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ABOUT COVER

Editorial board member of *World Journal of Clinical Oncology*, Dr. Takura is a Project Professor in the Department of Healthcare Economics and Health Policy, Graduate School of Medicine, University of Tokyo, Japan. He is part-time Research Fellow of the Cabinet Office and Chairman of the Specialized Agency of Cost-effectiveness Evaluation, Ministry of Health, Labour and Welfare. Dr. Takura also serves as a Guest Professor at Osaka University Graduate School of Medicine. Dr. Takura's research has focused on socioeconomic evaluation of cancer treatments and renal transplantation, and cost effectiveness analysis of revascularization for ischemic heart disease. His current approach to this work involves integrating big data from various sources. (L-Editor: Filipodia)

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EXPERT RECOMMENDATIONS

GOECP/SEOR clinical recommendations for lung cancer radiotherapy during the COVID-19 pandemic

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Abstract

The coronavirus disease 2019 crisis has had a major and highly complex impact on

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the clinical practice of radiation oncology worldwide. Spain is one of the countries hardest hit by the virus, with devastating consequences. There is an urgent need to share experiences and offer guidance on decision-making with regard to the indications and standards for radiation therapy in the treatment of lung cancer. In the present article, the Oncological Group for the Study of Lung Cancer of the Spanish Society of Radiation Oncology reviews the literature and establishes a series of consensus-based recommendations for the treatment of patients with lung cancer in different clinical scenarios during the present pandemic.

Key words: Lung cancer; COVID-19; Pandemic; Radiotherapy; Recommendations

Core Tip: The coronavirus disease 2019 crisis has had a major impact on the clinical practice of radiation oncology worldwide. Spain is one of the countries most affected by the devastating consequences of the pandemic. There is an urgent need to share experiences and offer guidance on the indications and standards of radiotherapy in the treatment of lung cancer. In this document, the Oncologic Group for the Study of Lung Cancer/ Spanish Society of Radiation Oncology establishes recommendations for triage, patient prioritization, and radiotherapy treatment regimens for the different clinical scenarios of lung cancer.

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INTRODUCTION

As of this writing, the global pandemic caused by the novel betacoronavirus, known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has affected more than 3 million people worldwide, with nearly 250000 deaths related directly or indirectly to the disease caused by this virus [coronavirus disease 2019 (COVID-19)], mainly the elderly and/or individuals with pre-existing conditions[1]. The COVID-19 pandemic has overwhelmed health care systems around the world, which has had a major impact on human lives as well as healthcare resources. This pandemic has affected not only the medical specialties directly involved in the fight against the coronavirus, but also other specialities, most notably those-such as radiation oncology-that treat cancer patients. One of the immediate consequences of the pandemic on the organization of health care systems is that most available hospital resources are dedicated to helping to control the disease, which necessarily imposes limitations on other areas of medicine and other important medical conditions. This situation is particularly serious for cancer patients, especially those with lung cancer.

Lung cancer is an aggressive biological entity, with a high proliferative and invasive capacity. Consequently, any delay in the initiation of oncological treatment can directly influence the clinical course of the disease, negatively impacting both disease control and mortality[2-4]. In the present health crisis, patients with lung cancer are especially vulnerable due to the convergence of a series of factors. First, the clinical manifestations of lung cancer often coincide with those of COVID-19 infection (cough, dyspnea, *etc*.), which could hinder an appropriate early diagnosis and alarm other cancer patients due to the fear of infection. In addition, the clinical characteristics of these patients-such as advanced age, the presence of an underlying lung disease, and other comorbidities (hypertension, heart disease, diabetes, among others)-place them at a higher risk of developing complications from COVID-19[2-4]. In this context, the diagnostic and therapeutic procedures necessary to manage lung cancer generally require multiple visits to the hospital and/or other health care centres, thereby increasing the risk of exposure to the virus. Moreover, lung cancer patients infected with the virus are much more likely to present a clinical course that is more serious

than in the general population[2-4].

Given the exceptional circumstances caused by this pandemic, which may limit our ability to offer standard radiotherapy treatment in patients with lung cancer, radiation oncologists must seriously consider alternative therapeutic strategies aimed at minimising the risk of infection in these patients while simultaneously ensuring that the alternative approach does not imply a delay in cancer treatment or a lower quality, less efficacious approach.

Due to the novelty of COVID-19, there is a lack of high-quality scientific evidence to guide clinical decision-making. Consequently, consensus-based expert recommendations are needed. Several prestigious international oncological scientific societies (ASTRO, ASCO, ESTRO) have recently published recommendations to guide the management of these patients during the pandemic[2-4]. The aim of the present document, developed jointly by the Spanish Society of Radiation Oncology (SEOR) and the Oncologic Group for the Study of Lung Cancer (GOECP), is to provide an updated review of the current scientific evidence to establish clinical recommendations regarding the therapeutic options for the optimal treatment of lung cancer during the COVID-19 pandemic.

RADIATION ONCOLOGY DURING THE COVID-19 PANDEMIC

Although lung cancer patients are assumed to be at increased risk of death from SARS-CoV-2 infection, the evidence to support this claim is limited and largely based on retrospective, single-centre studies with small sample sizes and significant methodological deficiencies[5-9]. To overcome these limitations, several professional organizations, including the European Society for Medical Oncology, the International Association for the Study of Lung Cancer, and the European Platform for Thoracic Oncology, have developed a global registry called TERAVOLT, whose purpose is to better understand the effect of COVID-19 on patients with thoracic cancers to help guide oncologists in the optimal management of these patients. An analysis of preliminary data from this registry indicates that patients with comorbidities present higher hospitalization and mortality rates, but the available data do not indicate that cancer treatment has negatively affected these patients[5].

Radiotherapy plays an essential role in the treatment of all stages of lung cancer, and it is also a safe and effective alternative to surgery in certain cases. Accordingly, any limitations in patient access to radiotherapy, or delays in the start of treatment, would negatively affect survival and quality of life outcomes in these patients. Due to the pandemic, radiation oncology departments face important challenges as they attempt to strike a balance between ensuring that patients receive the appropriate oncological care and minimizing the risk of COVID-19 infection in patients and health care professionals. In this context, there is a clear need for a multi-level communication policy based on rigorous, up-to-date, and convincing data to raise awareness among patients, their families, and health care professionals, of the importance of implementing appropriate preventive measures.

Since SARS-CoV-2 first began to spread widely, various official bodies, organizations, and experts have all urged radiation oncologists to implement standards and protocols designed to ensure the continued safety of oncological treatments[10-13]. These recommendations and protocols include posters and brochures, screening measures to detect infected individuals upon arrival at the department (*e.g.*, respiratory symptoms, fever, or contact with a possible or confirmed positive COVID case), limitations in the number of people allowed to accompany the patient, strict compliance with scheduled appointment times, as well as the routine use of masks and gloves, hand hygiene, and social distancing. In addition to these basic safety measures, the department must also develop and implement a comprehensive plan for cleaning and disinfecting physical spaces (controls, waiting rooms, booths, bunkers), contact surfaces (counters, screens, keyboards, telephones, handles), linear accelerators, the simulation computed tomography (CT) scanner, treatment tables, and immobilization devices.

The implementation of these measures in the radiotherapy department—aimed at guaranteeing the appropriate sanitary conditions-can potentially limit the department's capacity to offer treatment due to the risk of an outbreak among staff, reassignment of personnel other departments involved in the front-line fight against COVID-19, and/or reuse of physical and material resources. For this reason, a rational approach to planning cancer care is essential, with decision-making based on high scientific and ethical standards in which the indications for treatment-priority, delay,

interruption, or even refusal-are clearly defined. To achieve this in a clinical scenario with limited capacity, we must balance the risk of exposure to the coronavirus (and the associated morbidity) with the risk of failing to treat a potentially fatal cancer according to the usual standards. Multiple factors must be considered, including the patient's general condition, life expectancy, potential for cure, existence of alternative treatments with a similar therapeutic efficacy, and the presence of an active infection. The decision to delay or interrupt radiotherapy treatment in a COVID-19-positive lung cancer patient must be carefully evaluated to avoid infecting other patients and healthcare professionals. The best approach to managing this complexity is to include other specialists involved in the treatment of this pathology in the decision-making process, which implies that thoracic tumour boards should continue to meet, albeit in a virtual setting, to ensure quality of care.

From a practical point of view, it is essential to develop a contingency plan that considers all of the following: (1) The division of departmental staff into independent operating groups (*i.e.*, shift-based health care activity); (2) The possibility of working from home; (3) The reorganization of physical resources (waiting rooms, consultations, linear accelerator treatment room) to minimize the number of people in the same place, for example by converting a waiting room for a clinical consultation or CT scan into a waiting room for the linear accelerator; (4) The rational use of hypofractionated regimens; a simplified approach to treatment verification and administration; and (5) Measures to minimize follow-up consultations for toxicity. Whenever possible, follow-up consultations should be performed by telephone or videoconference.

If a patient undergoing radiotherapy develops COVID-19, an individualized assessment must be performed to select the appropriate therapeutic approach. In all cases, the main priority is the patient's health, which may involve treatment continuation or a temporary interruption until the infection resolves. Various factors must be considered, including those related to COVID-19 infection-severity, presence or absence of pneumonia, and the patient's respiratory status and general condition-which will determine whether the patient is able to receive radiotherapy. Other factors related to the neoplasm include the extent of the oncological disease, the presence or absence of tumour-related symptoms (*e.g.*, obstruction, hemoptysis, or pain), and the risk of tumour progression.

Interrupting or delaying treatment should only be considered in patients with COVID-19-related symptoms in whom a temporary interruption or delay in starting treatment is unlikely to lead to significant tumour progression (for example, prophylactic cranial irradiation, postoperative radiotherapy, *etc*.). Once the COVID-19 symptoms have resolved and the patient tests negative for the disease (PCR), treatment should be restarted or initiated as soon as possible. In the event that the decision is made to continue radiotherapy in a COVID-19-positive patient, this should be done according to recommendations of national and international organizations, which include the implementation of strict safety measures to protect health care personnel. The main safety measures include avoiding physical contact between infected and non-infected patients in the department (*i.e.*, different rooms for COVID-19 patients) and/or by treating these patients at different times, and strict cleaning and disinfection protocols[14,15].

STEREOTACTIC BODY RADIOTHERAPY FOR STAGE T1-T2 NON-SMALL CELL LUNG CANCER

The recommendations described here are based on an appropriate balance between the risk and benefits of the proposed radiotherapy regimens. The main risk associated with stereotactic body radiotherapy (SBRT) in patients with lung cancer is the potential for treatment-related toxicity, which is mainly a function of the location of the target lesion, the total dose and dose per fraction, and lesion size. Timmerman *et al*[16] found that the risk of treatment-related toxicity is higher in central versus peripheral lesions for the same dose fraction. For this reason, several groups[17,18] have proposed basing the fraction size on the tumour location (applicable to tumours with a maximum diameter < 5 cm). In addition to the established indication for SBRT in inoperable patients, in the context of this pandemic, if the surgery department is at full capacity, then SBRT should also be considered in operable patients in accordance with recommendations of both American and European experts[3].

Fractionation in central tumours

The appropriate treatment approach to central tumours is based mainly on the phase 1/2 RTOG 0813 trial (19)[19], a dose escalation trial performed to assess a five-fraction SBRT schedule ranging from 10 to 12 Gy/fraction (total dose, 50 to 60 Gy) delivered every other day. In that trial, the maximum tolerated dose was 12.0 Gy/fraction, with ≥ grade (G) 3 toxicity rate of 7.2%. These regimens can be compared to more conservative regimens, such as that described by Haasbeek *et al*[20], in which patients received 60 Gy delivered in 8 fractions, with a local control rate at 3 years of 92.6% and ≥ G3 toxicity rate of 7.9%.

Based on the available evidence for the risk of toxicity versus the probability of achieving local control in central tumours, a reasonable recommendation would be a total dose of 50-60 Gy delivered in 5 fractions, with dose adjustment (10 to 12 Gy/fraction) as appropriate to comply with dose limits to the organs at risk (OAR)[21]. Table 1 summarizes the recommended treatment regimens according to tumour location.

Lesions adjacent to the chest wall

In patients with lesions adjacent to (< 2 cm) or in contact with the chest wall, the European recommendations[22] allow for a dose of up to 48 Gy in 4 fractions. Another proposed fractionation schedule in this location is 60 Gy in 5 fractions[23]. Based on the published results of these two schedules in terms of local control and toxicity, we recommend 48 Gy in 4 fractions.

Peripheral lesions in the "safe" zone

Tumours located in a "safe" zone are understood as those located in an area that cannot be considered central, and at least 2 cm from the chest wall. In these cases, extreme hypofractionation consisting of a single fraction of 30-34 Gy-based on the findings of two prospective phase 2 trials[24,25] may be considered in well-selected patients. The classic fractionation regimen (60 Gy in 3 fractions) proposed by Timmerman *et al*[26] offers excellent 3-year local control rates (90.6%-94%) in peripheral tumours, with an acceptable toxicity rate (≥ G3), ranging from 10%-16.3%[27]. In short, administration of a single fraction of 30-34 Gy can be considered in lesions located in the safe zone provided that dosimetric restrictions to the OARs are met[28-32]; otherwise, the Timmerman scheme (54 Gy in 3 fractions), corrected for heterogeneity, should be administered.

RADIOTHERAPY IN LOCALLY-ADVANCED NON-SMALL CELL LUNG CANCER

Concomitant chemoradiotherapy (CRT) is considered the standard treatment for unresectable, stage III non-small cell lung cancer (NSCLC)[33]. However, the impact of the SARS-CoV-2 pandemic on surgery departments, including thoracic surgery, will likely increase the number of patients with potentially-resectable stage III NSCLC who receive non-surgical treatment-that is, some combination of chemotherapy and radiation therapy. Nevertheless, in patients in reasonably good physical condition [performance status (PS) 0-1, weight loss < 5 kg, good lung function, no significant comorbidities], if the available human and material resources allow, the treatment of choice is the standard external radiotherapy regimen: 60-66 Gy (30-33 fractions) administered concomitantly with platinum-based chemotherapy[34]. However, during a pandemic, the greater toxicity associated with concomitant CRT in these patients, especially lymphopenia, deserves special consideration as it could significantly increase the risk of complications in patients with COVID-19 (in whom lymphopenia is a common symptom), potentially leading to a worse prognosis[35-37].

In this context, sequential administration of systemic therapy followed by radiotherapy could be an option, for three main reasons. First, sequential treatment would reduce the potential immunosuppressive effects of concurrent CRT, which would, in turn, minimize the risk of complications caused by COVID-19. Second, a sequential therapeutic regimen would minimize exposure of the patient to the hospital environment, where the risk of infection is high. Third, it would optimize the available human and material resources in the radiation oncology department, which have been greatly depleted during the peak of the pandemic.

Therefore, it is reasonable to propose the use of hypofractionated thoracic radiotherapy in selected cases and in situations of severe shortage of radiotherapy

Table 1 Stereotactic body radiotherapy schemes for stage T1-T2 non-small cell lung cancer

Prescription	BED_{10}	LC (yr)	Distance	
			Thoracic wall	Mediastinum
30-34 Gy single fraction	149.6 Gy	94% (3 yr)	> 2 cm	> 2 cm
54 Gy (18 Gy × 3 fx)	151.2 Gy	94% (3 yr)	≥ 2 cm	> 2 cm
48 Gy (12 Gy × 4 fx)	105.6 Gy	85.4% (3 yr)	< 2 cm	NA
50-60 Gy (10-12 Gy × 5 fx)	100-132 Gy	75%-84% (3 yr)	> 2 cm	≤ 2 cm

LC: Local control; Fx: Fraction; BED: Biologically effective dose.

resources. Some schemes, such as 15-20 fractions administered at doses ranging from 2.75 to 4 Gy per fraction to the target volume, with or without integrated boost have been tested. These regimens would maintain an appropriate biologically-equivalent dose, which previous studies have shown to be both safe and effective[38,39].

The use of these hypofractionated regimens has long been routine in the United Kingdom[40]. In lung cancer patients, the use of hypofractionated radiotherapy administered sequentially after chemotherapy was widely used before the current pandemic, mainly due to its efficiency and favourable toxicity profile, and this approach should be considered as a possible strategy of choice in the current context[41]. Table 2 summarizes the most commonly used hypofractionated schedules. References in Table 2[41-43].

However, the current evidence to support hypofractionated radiotherapy combined with chemotherapy remains limited. Although a systematic review found that some studies reported high toxicity[44], many of those studies used older radiotherapy techniques, rather than the more modern-and more precise-techniques available today. Nevertheless, the findings of several studies suggest that concomitant CRT [with a risk-adjusted chemotherapy (cisplatin + vinorelbine) dose] can be considered in well-selected patients, although a greater risk of toxicity must be assumed.

In terms of quality standards for radiotherapy, it is advisable-regardless of whether a conventional (30-33 fractions) or hypofractionated regimen is prescribed-to perform a simulation CT (preferably 4D-CT) to assess respiratory motion. In addition, whenever possible, highly conformal techniques with dynamic modulation such as intensity-modulated radiotherapy or volumetric modulated arc therapy should be used. Appropriate image guidance and positioning verification (image-guided radiotherapy) using cone beam CT is essential-particularly for hypofractionated regimens-in order to continuously monitor the treatment volume to minimize the radiation dose to the OARs[45-47].

Once sequential CRT has been completed, depending on the patient's PD-L1 status, maintenance treatment with durvalumab for one year should be considered[48]. However, because the patients in the PACIFIC trial were treated with concomitant CRT, there is only limited evidence to support maintenance therapy with durvalumab in sequentially-treated patients. Moreover, the costs of the adjuvant treatment with durvalumab in sequentially treated patients may not be covered by the Spanish public health care system. Consequently, in patients with PD-L1 > 1%, the application of sequential schemes could negatively influence overall survival; nevertheless, consolidation treatment after sequential CRT is currently being investigated in the PACIFIC 6 trial (ClinicalTrials.gov Identifier: NCT03693300). An unplanned subgroup analysis of the PACIFIC trial data found that patients who started treatment with durvalumab within 14 d from the end of consolidation treatment had better outcomes; nonetheless, in the full patient cohort, durvalumab was initiated from 1 to 42 d post-CRT with good outcomes, findings that should be taken into account in the current situation.

To conclude, our recommendation is to prescribe, whenever possible, the standard external radiotherapy regimen-60-66Gy (30-33 fractions)-administered concomitantly with platinum-based chemotherapy followed by consolidation with Durvalumab accordingly with the patient´s PD-L1 status. However, if concomitant treatment is not possible, a valuable strategy could be the use of hypofractionated schemes in a sequential manner to chemotherapy.

Table 2 Hypofractionated radiotherapy for locally-advanced non-small cell lung cancer

Study	CT	Fx	Dose	Time	EQD-2_{10}	HDV	Comments
Soccar trial Randomized, Phase II[41]	Sequential; Concomitant	20 × 2.75 Gy	55 Gy	26 d	58.4 Gy	Lungs-GTV: V_{20} < 35%; MLD < 18 Gy; Spinal cord: Dmax 44 Gy; Esophagus: D1 cc < 55 Gy; Heart: V_{30} < 36%; Brachial plexus: Dmax 55 Gy	High concomitant toxicity; CT adjusted dose required; Not compared vs standard (60 Gy); Widely used in United Kingdom
Anderson, Retrospective[42]	Sequential	15 × 3 Gy	45 Gy	19 d	48,7 Gy	Not published	Retrospective comparison; Similar results to standard: 30 × 2 (60 Gy)
Toronto Retrospective[43]; Anderson Randomized, Phase III[45]	Sequential	15 × 4 Gy	60 Gy	19 d	70 Gy	Lungs-GTV: V_{20} < 30%; V_5 < 60%; MLD < 20 Gy; Spinal cord: Dmax 38 Gy; Esophagus: Dmax < 50 Gy; V_{45} < 10cc; Heart y MBV: Dmax < 63 Gy; V_{57} < 10 cc; Trachea y MB: Dmax < 63 Gy; V_{57} < 10 cc; Rib: Dmáx < 63 Gy; V30 < 30 cc; Brachial plexus: Dmax < 50 Gy; Skin < 50 Gy	Used for SBRT; Lack of data in stage III; Phase III RCT; 15 × 4 *vs* 30 × 2; Final results not published

CT: Computed tomography; Fx: Fraction.

NEOADJUVANT AND POSTOPERATIVE RADIOTHERAPY IN NON-SMALL CELL LUNG CANCER

The neoadjuvant approach (radiotherapy +/- chemotherapy followed by surgery) in stage III NSCLC (stage IIIA and, in some cases, potentially-resectable stage IIIB) is highly complex and should only be performed in centres with multidisciplinary experience. Although some phase 2 trials conducted at centres of excellence have reported excellent 5-year survival rates (up to 50%) with low morbidity and mortality, a recent meta-analysis found no significant survival benefit for neoadjuvant CRT plus surgery versus CRT alone[49]. For this reason, most international guidelines that have explored the optimization of radiotherapy resources during the COVID-19 pandemic have largely omitted any discussion of the neoadjuvant approach. Even the recommendations of the Memorial Sloan-Kettering Cancer Center, a well-known supporter of neoadjuvant chemotherapy in selected stage III patients, do not include this indication[50]. The Fox Chase Cancer Center guidelines comprehensively address the treatment of NSCLC during the COVID-19 pandemic stating "Patients with resectable disease can be treated with definitive non-operative management if surgical resources are limited or the risks of perioperative care are high..."[51]. Nevertheless, it is essential that the treatment approach be individualised within the parameters set by an interdisciplinary tumour board. For example, after induction therapy, the patient should undergo surgery within 4-12 wk, keeping in mind the high risks and high resource utilization in surgery departments. The final decision to perform surgery will ultimately depend on the phase of the pandemic and the impact of COVID-19 on surgery departments. Clearly, surgery is more feasible in the initial phases of the pandemic, whereas in advanced phases hospital resources are likely to be limited. If thoracic surgery is considered, all patients (even those who are asymptomatic) should

undergo a CT scan and be tested for COVID-19 to check for the presence of bilateral pulmonary infiltrates.

In patients in whom neoadjuvant CRT is indicated, the GOECP/SEOR recommends, if possible, the use of radical CRT alone, without surgery. The GOECP/SEOR also recommends against performing induction chemotherapy as this is a suboptimal cancer treatment unless subsequent radical local treatment (surgery or radiotherapy) can be guaranteed.

Postoperative radiotherapy (PORT) has several evidence-based clinical indications[52]. PORT is recommended in patients with NSCLC with involvement of multiple nodal stations (pN2) and/or capsular rupture, according to the explicit indication of the ESTRO-ASTRO guidelines[3]. These same guidelines note there is a strong consensus (82% of experts) that PORT could be delayed for 4-6 wk, with a recommended dose of 54 Gy at standard fractionation (2 Gy per fraction). Those guidelines do not recommend hypofractionated regimens in these patients due to the increased risk of morbidity and mortality.

Various international guidelines, such as those published by the MSKCC, emphasize the clinical utility of PORT in patients with involved surgical margins[53,54], recommending a total dose of 54-60 Gy (1.8-2 Gy/fraction) to the high risk surgical bed. Other guidelines advise a shorter fractionation schedule (50 Gy in 25 fractions) to limit exposure to the hospital setting. However, some guidelines do not even specifically include PORT among the recommendations[55].

The GOECP/SEOR believes that PORT, as an adjuvant treatment, should be deferred, whenever possible, until the current pandemic is under control. In our view, PORT is indicated in patients with involved margins (including massive capsular rupture) and in patients with multi-station mediastinal node involvement, with a recommended dose of 60 Gy in the former group and 50-54 Gy in the latter, in both cases using standard fractionation schedules (2 Gy/fraction), assuming that patients have good PS (0-1) and appropriate functional tests. Indications for other clinical scenarios, such as involvement of the parietal pleura and chest wall without clearly affected margins, are more ambiguous due to the scant published data; these cases should be presented to the weekly tumour board and discussed with pathologists and thoracic surgeons. As a general rule, we do not recommend hypofractionation in these patients due to the risk of toxicity. In any case, as with any adjuvant therapy, each case must be evaluated individually to assess potential benefits and risks. If the likely benefit in local control and/or survival is modest, then treatment should be delayed. Finally, in patients who test positive for COVID-19 during treatment, radiotherapy (both neoadjuvant and PORT) should be interrupted.

STEREOTACTIC BODY RADIOTHERAPY IN PATIENTS WITH OLIGOMETASTIC NON-SMALL CELL LUNG CANCER

It is estimated that 60%-70% of patients with NSCLC are diagnosed with stage IV disease and, of these, approximately 20% meet criteria for oligometastatic disease[56], although this percentage may be even higher, as staging techniques such as positron-emission tomography become more widely used. Oligometastatic disease has two main forms of presentation: "*de novo*" oligometastasis (≤ 3-5 lesions at diagnosis) or "induced" oligometastasis (persistence of ≤ 3 lesions after treatment).

The concept of induced oligometastatic disease was recently described by Guckenberger *et al*[57], who subclassified this into three clinical presentations: (1) Oligopersistence: < 5 lesions persisting after systemic treatment; (2) Oligoprogression: Progression in ≤ 3 sites after systemic treatment; and (3) Oligoresistance: Response to systemic treatment, with evidence of disease in ≤ 3 sites.

Studies have shown that the use of local therapy to treat patients with oligometastatic disease can improve overall survival[58]. Three prospective trials evaluated subgroups of patients with oligometastatic disease at diagnosis[59-61], finding that metastatic patients who respond to-or who do not develop disease progression-after systemic treatment are more likely to benefit from local therapies such as radiotherapy. Although immunotherapy was not considered within the treatment arms in those trials, data from more recent trials that did assess the role of immunotherapy have changed the treatment paradigm for patients with metastatic disease[62], with numerous clinical trials currently underway to assess the immunotherapy combined with local radiation therapy.

In the current pandemic, our recommendation is to identify the "true" oligometastatic patient, defined as the patient who, after systemic treatment, shows a

response or at least no evidence of progression. If the event that the conditions related to the pandemic did not allow for systemic treatment, then patients with the best prognosis should be selected for locally ablative therapy. In retrospective series of patients with oligometastatic disease, the factors most consistently associated with better prognosis were as follows: Gender (male *vs* female); histology (adenocarcinoma *vs* other histologies); presentation (metachronous *vs* synchronous); performance status (PS 0-1 *vs* the rest); the number of metastatic lesions (1 *vs* 2-3 *vs* the rest); size (< 3 cm *vs* the rest); and location (lung and bone *vs* adrenal glands and lymph nodes *vs* other sites)[63-69].

The most appropriate SABR/SBRT treatment regimen will depend on the characteristics of each patient, although the objective should always be to offer the treatment approach that most limits the patient's exposure to the hospital environment and-depending on the metastatic location-the treatment with the lowest risk of toxicity. It is important to stress that, depending on the tumour localisation a more or less aggressive regimen can be selected. The most common sites for metastases in patients with stage IV NSCLC are the brain, lung, liver, bones, and adrenal glands. Table 3 shows the currently accepted doses and fractions for the treatment of these different metastatic locations[70-82].

RADIOTHERAPY IN PATIENTS WITH SMALL-CELL LUNG CANCER

Small-cell lung cancer (SCLC) accounts for 13% of all lung cancers. The standard treatment in limited stage SCLC (LS-SCLC) is concomitant radiotherapy initiated in the first or the second cycle of chemotherapy. Consolidation thoracic radiotherapy has become widely used in clinical practice in patients with extensive stage SCLC (ES-SCLC) who show a good response to systemic treatment. In both of the aforementioned clinical scenarios, prophylactic cranial irradiation (PCI) is recommended in responders. New treatments such as immunotherapy are being progressively incorporated into the therapeutic armamentarium for SCLC, although more slowly than in NSCLC[83].

Limited-stage SCLC

A key lesson that has been learned in recent years with regard to the management of SCLC is that time is crucial factor in treatment outcomes due to the aggressive nature of this histological subtype, which has a rapid doubling time, a tendency towards early dissemination, and-in some cases-rapid symptom onset. For this reason, curative-intent treatment should not be delayed by more than 4-6 wk; however, in patients with COVID-19, radiotherapy should be deferred, when possible, until the patient is asymptomatic and tests negative for the disease. If the diagnosis of COVID-19 is made when the patient has already started radiotherapy, the decision to interrupt treatment should consider all relevant factors, including the presence of symptoms (due to the tumour or virus), and the phase of treatment (*i.e.*, closer to the start or finalisation of radiotherapy).

The standard recommendation for treatment sequencing in terms of early concomitant therapy in the first and second cycles of chemotherapy should be reviewed in these circumstances. Studies have shown that overall survival outcomes are better when radiotherapy is administered in the first eight weeks after chemotherapy[84] and in patients in which the start and completion of radiotherapy is less than 30 d[85]. Recent studies suggest that radiotherapy has a similar therapeutic efficacy when administered with the third cycle of chemotherapy, a finding that implies that a slight delay is acceptable if the start of radiotherapy coincides with the onset of clinical manifestations of COVID-19[86,87].

Fractionation plays a key role in the treatment of lung cancer. In general, we do not recommend modifying the standard CRT regimens: 45 Gy in 30 fractions for 3 wk (2 fractions of 1.5 Gy/day)[88] or 60-66 Gy in 30-33 fractions for 6 to 6.5 wk[89]. However, the individual radiation oncology department must decide whether they prefer to extend the duration of treatment (one fraction/day) or shorten it (two fractions/day), with the latter implying an increased exposure to the hospital environment. The choice will depend on factors such as the effectiveness of prevention measures against COVID-19 or the logistical capacity of the department to administer two daily sessions.

Although only scant evidence is available to support hypofractionated regimens in LS-SCLC, this approach could merit consideration based on the extrapolation of results obtained in patients with NSCLC using either 45 Gy or 40-42 Gy delivered in 15

Table 3 Stereotactic body radiotherapy schemes for oligometastatic non-small cell lung cancer

Localisation	Fractions/Total dose
Brain	1 fx: 18-24 Gy; 3 fx: 24-27 Gy; 5 fx: 25-35 Gy
Lung	3 fx: 54 Gy; 4 fx: 48 Gy; 5 fx: 50-60 Gy
Adrenal gland	3 fx: 36-45 Gy; 5 fx: 40-50 Gy
Liver	1 fx: 24-26 Gy; 3 fx: 45-60 Gy; 5 fx: 40-50 Gy; 8 fx: 60 Gy
Bone (spinal column included)	1 fx: 16-24 Gy; 3 fx: 27-30 Gy; 5 fx: 30-40 Gy

Fx: Fraction.

daily fractions, Table 4. The administration of concomitant chemotherapy should be carefully evaluated, including the possibility of administering systemic treatment sequentially to reduce the risk of significant toxicity. Given that SCLC often presents as a centrally-located bulky mass, these regimens should only be considered in well-selected patients in the context of resource restrictions related to the pandemic in which standard treatment is not possible[50,90,91]. In patients with early stage LS-SCLC (T1-T2 N0M0), SBRT at doses ranging from 50-60 Gy in 5 fractions is always an option[92].

Prophylactic cranial irradiation at a dose of 25 Gy in 10 fractions has been shown to reduce the risk of symptomatic brain metastases and to improve survival[93,94], and therefore this fractionation schedule should not be modified, even in the present pandemic[95]. Although PCI for LS-SCLC must remain the standard recommendation during the pandemic, PCI could be delayed if brain MRI is used to closely monitor the patient, an approach that has been used in patients with ES-SCLC[96]. If we postpone PCI (due to the pandemic) until CRT has finalized, we would have two months to administer PCI after completing CRT. However, if instead of administering PCI, we decide to monitor the patient with brain MRI, then the minimum duration of MRI-based follow-up should be two years, performed every three months the first year and every six months the second year, as indicated in the protocol of the Japanese trial[96]. In COVID-19 positive patients, PCI should be delayed or interrupted until the patient is asymptomatic and tests negative for the disease.

Extensive-stage SCLC

Consolidative thoracic radiotherapy (CTRT) has been shown to improve survival in patients with ES-SCLC who show a significant response to chemotherapy[97]. PCI has also been shown to reduce symptomatic metastases and may also improve survival in this subgroup[98]. Indeed, in recent years, CTRT and PCI have both been added to the therapeutic armamentarium of radiation oncology departments. However, during the present pandemic, omitting CTRT could reasonably be considered in patients with a complete pulmonary response to chemotherapy; in selected cases with tumour persistence, the traditional regimen (30 Gy in 10 fractions) could be considered. In general, our recommendation is to avoid PCI, preferring instead brain MRI to monitor brain lesions[96].

PALLIATIVE RADIOTHERAPY IN LUNG CANCER

In the context of the current pandemic, it is essential to consider the following points regarding palliative-intent radiotherapy: (1) A detailed risk/benefit analysis should be performed; (2) Consider possible repercussions of delaying radiotherapy if the patient's clinical condition allows; (3) Consider therapeutic alternatives; (4) Palliative radiotherapy is not indicated in patients with life expectancy < 3 mo; and (5) When indicated, the maximum hypofractionated dose should be applied to reduce hospital visits.

For palliative thoracic radiotherapy (to treat hemoptysis, severe cough, dyspnea secondary to bronchial obstruction, or atelectasis), it is preferable to offer the most hypofractionated regimen possible, such as 20 Gy in 5 fractions , 17 Gy in 2 fractions , or a single fraction of 10 Gy[99-102], Table 5.

In patients with vena cava syndrome, alternatives to radiotherapy (*e.g.*, endovascular stent, thrombolysis, *etc*.) should be considered. If these alternatives are

Table 4 Hypofractionated radiotherapy for limited stage small-cell lung cancer

Ref.	Type of study	Total dose (Gy)	Dose per fraction (Gy)	No. fractions	Radiotherapy technique, Dose tolerance
Giuliani *et al*[90], 2015	Retrospective	40	2.67	15	3DCRT/IMRT; Lung: V_{20} ≤ 30%, D_{media} 20 Gy, Esophagus: D_{max} < 105%
Grønberg *et al*[91], 2016	Prospective, phase 2 trial	42	2.8		

IMRT: Intensity-modulated radiotherapy; CRT: Chemoradiotherapy.

Table 5 Palliative radiotherapy in lung cancer

Clinical indication	Fractionation
Palliative thoracic treatments (VCS, hemoptysis, dyspnea…)	20 Gy/5 fx; 17 Gy/2 fx; 10 Gy/1 fx;
Bone metastases / Spinal cord compression	8 Gy/1 fx
Multiple brain metastases	20 Gy/5 fractions; Omit in patients with poor ECOG

VCS: Vena cava syndrome.

not possible, then one of the hypofractionated radiotherapy regimens described above can be administered.

To manage bone metastases, Table 5, the first step is to compare radiotherapy to other treatment options (*e.g.*, modification of analgesic therapy, bisphosphonates, *etc.*) to ensure that radiotherapy is the best option. If so, the available evidence indicates that a single fraction of 8 Gy is as effective as more fractionated regimens[103]. Indeed, a recent trial also evaluated this regimen to treat metastatic spinal cord compression[104] and some recommendations[105] support this regimen. For pathological fractures, PORT is not recommended during the pandemic.

The use of whole brain radiotherapy to treat multiple metastases is controversial, in part because a study performed by Mulvenna and colleagues demonstrated that a similar quality of life can be achieved in many cases with corticosteroids treatment alone[106]. However, those authors also showed that whole brain radiotherapy appears to improve survival in a well-defined patient subgroup (age < 60 years, good PS, controlled primary tumour). Consequently, a hypofractionated regimen consisting of 20 Gy in 5 fractions would be an appropriate recommendation in this patient subgroup, Table 5.

In patients with COVID-19 scheduled to undergo palliative radiotherapy, it is better to delay or suspend treatment when possible until the patient has clinically recovered (negative PCR test). However, radiation therapy may be necessary in COVID-19+ patients who present potentially life-threatening symptoms, such as spinal cord compression or grade 4 vena cava syndrome, where the risk of delay is too great.

CONCLUSION

All recommendations of the GOECP should be considered in accordance with the resources available at the treating centre. A summary of the international guidelines, expert consensus, and the recommendations of the present manuscript are shown in Table 6.

Table 6 Summary of recommendations of the main clinical guidelines and of the GOECP/SEOR for lung cancer radiotherapy during the coronavirus disease 2019 pandemic

	ESTRO-ASTRO	MSKCC	Yale radiation oncology	GOECP/SEOR
Stage I NSCLC	SBRT: 45-54 Gy in 3 fx, 48 Gy in 4 fx; Maximum hypofractionation supported, 30-34 Gy 1 fx	SBRT; Peripheral lesions: 34 Gy, 1 fx; Central tumours: 10 Gy × 5 fx; Ultracentral tumours: 7.5 Gy × 8 fx	SBRT; Peripheral lesions: 30-34 Gy, 1 fx (first option). -45 Gy in 3 fx; Central tumours: 45 Gy in 3 fx (first option); -50 Gy/5 fx; Ultracentral or very large tumours: 60-72 Gy in 15-18 fx *vs* 60 Gy in 8 fx	SBRT; Safe Zone: -30-34Gy, 1 fx (first option). -54 Gy in 3 fx; Peripheral Lesions: 48 Gy in 4 fx (first option); Central Tumour: 50-60 Gy in 5 fx *vs* 60 Gy in 8 fx
Stage III NSCLC	CRT 60-66 Gy in 30-33 fx	CRT 55 Gy in 20 fx	CRT 60 Gy in 30 fx	CRT 60-66 Gy in 30-33 fx
Stage III NSCLC; Radiotherapy Alone/sequential	60 Gy in 15 fx (33%); 60 Gy in 20 fx (27%); 60-66 Gy in 24-30 fx (2.2-2.75 Gy/d) (23%) 24; 55 Gy in 20 fx (13%)	45 Gy in 15 fx (or more hypofractionated)	52.5-60 Gy in 15 fx	55 Gy in 20 fx[1] (first option); 45 Gy in 15 fx
PORT NSCLC	50-60 Gy over 5-6 wk	50 Gy in 25 fx	Delay treatment	Delay treatment
LS-SCLC	CRT 60-66 Gy in 30-33 fx over 6-6.5 wk, or 45 Gy in 30 fx over 3 wk using BID fractions of 1.5 Gy	-45 Gy in twice daily 1.5 Gy (first option); -66-70 Gy in 33-35 daily fx; -45 Gy in 15 daily fx	40-42 Gy in 15 daily fx	CRT 60-66 Gy in 30-33 fx over 6-6.5 wk, or 45 Gy in 30 fx over 3 wk using BID fractions of 1.5 Gy[1]
PCI; SCLC	LS-SCLC: 25 Gy in 10 fx over 2 wk	LS-SCLC: 25 Gy in 10 fx; ES-SCLC: 20 Gy in 5 fractions or MRI surveillance	Delay treatment	LS-SCLC: 25Gy in 10 fx; ES-SCLC: MRI surveillance (if available)
Palliative	Preferred fractionation schedule: 20 Gy in 5 fx (30%); 17 Gy in 2 fx (37%); 8-10 Gy in 1fx (33%)	-20 Gy in 5 fx; -17 Gy in 2 fx; -10 Gy in 1fx	Pain or bony lesion: 8 Gy × 1 fx; Bleeding: 10 Gy × 1 fx; If single fraction not possible, hypofractionate dose to extent possible; Brain metastases can be deferred per algorithm, and treated with single fraction radiosurgery; Endobronchial obstruction: Consider 8 Gy × 1 or 17 Gy in 2 weekly fractions	Pain or bony lesion: 8 Gy × 1 fx; Bleeding: 10 Gy x 1 fx, 20 Gy × 5fx; If single fraction not possible, hypofractionate dose to extent possible; Multiple brain metastases: 20 Gy × 5 fx (in favourable subgroup); MSCC: 8 Gy × 1fx

[1]Selected cases, if there are resource limitations in the radiotherapy department, consider hypofractionated radiotherapy administered sequentially after chemotherapy. CRT: Chemoradiotherapy; Fx: Fraction; NSCLC: Non-small cell lung cancer; SCLC: Non-small cell lung cancer; LS: Limited stage; ES: Extensive stage; MSCC: Malignant spinal cord compression; MSKCC: Memorial sloan-kettering cancer center; PCI: Prophylactic cranial irradiation; PORT: Postoperative radiotherapy; GOECP: Oncologic Group for the Study of Lung Cancer; SEOR: Spanish Society of Radiation Oncology; SBRT: Stereotactic body radiotherapy.

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REVIEW

B-cell lymphoma-2 inhibition and resistance in acute myeloid leukemia

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Abstract

Spurred by better understanding of disease biology, improvements in molecular diagnostics, and the development of targeted therapies, the treatment of acute myeloid leukemia (AML) has undergone significant evolution in recent years. Arguably, the most exciting shift has come from the success of treatment with the B-cell lymphoma-2 inhibitor venetoclax. When given in combination with a hypomethylating agent or low dose cytarabine, venetoclax demonstrates high response rates, some of which are durable. In spite of this, relapses after venetoclax treatment are common, and much interest exists in elucidating the mechanisms of resistance to the drug. Alterations in leukemic stem cell metabolism have been identified as a possible escape route, and clinical trials focusing on targeting metabolism in AML are ongoing. This review article highlights current research regarding venetoclax treatment and resistance in AML with a focus on cellular metabolism.

Key words: Acute myeloid leukemia; B-cell lymphoma-2; Venetoclax; Metabolism; Leukemic stem cell; Resistance

Core tip: The B-cell lymphoma-2 inhibitor venetoclax has drastically changed the treatment paradigm for acute myeloid leukemia; however, much is unknown about mechanisms of relapse after treatment with this agent. Alterations in cellular metabolism have been identified as a potential resistance mechanism and may be able to be targeted with novel treatments.

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of aggressive hematologic malignancies characterized by the uncontrolled proliferation of genetically altered immature myeloid cells. Accumulated clonal leukemic stem cells (LSC) are inherently nonfunctional and arrested in differentiation causing rapid bone marrow failure and, if untreated, eventual death[1].

An estimated 21500 patients are diagnosed with AML yearly in the United States[2]. Despite advances in molecular prognostication and therapeutic targeting, AML remains a significant cause of morbidity and mortality. The current 5-year survival rate remains < 30%[3]. Patients above age 65 and those with poor performance status, pre-existing comorbidities, or biologically aggressive disease have especially poor prognoses, as do patients who relapse after hematopoietic stem cell transplant[4].

Since the first publication by Yates *et al*[5] in 1973, the standard therapeutic approach for treating AML has relied upon intensive induction chemotherapy with the 7 + 3 protocol, a cytarabine and anthracycline based regimen. Individuals who were unable to tolerate intensive chemotherapy had few options[5]. It has only been in the last decade that a myriad of new drugs have changed this paradigm and gained approval for the treatment of AML.

Despite improvements in the success of up-front AML therapy, treatment for relapsed disease remains a significant challenge. Relapse occurs due to the emergence of chemotherapy resistant leukemic stem cells[6]. Over the past decade, much has been learned about the complexity of the metabolic and molecular transformations that LSCs undergo. Interestingly, some of the same metabolic dysregulations are seen in other malignancies including colon, breast, and prostate cancer[7]. Whole-genome mapping and targeted sequencing of serial samples of leukemia cells from individual patients has led to the discovery of distinct metabolic aberrations that play a role in relapse and, in some cases, are targets for drug development[8].

Novel therapies such as venetoclax, a specific B-cell lymphoma-2 (Bcl-2) inhibitor, have triggered a paradigm shift in the approach to AML and reinvigorated discussions about the link between metabolism and cancer. Though the majority of patients respond to venetoclax-based treatment, the depth and duration of response remain inadequate[9]. Thus, understanding the metabolic rewiring that allows treatment resistance to develop is crucial. This review summarizes Bcl-2 inhibition in AML with a focus on mechanisms of resistance to venetoclax, in particular those related to leukemic cell metabolism.

LEUKEMIC STEM CELL METABOLISM

During evolution from normal hematopoietic progenitors to LSCs, cells undergo significant alterations in metabolic pathways including glycolysis, amino acid metabolism, and fatty acid metabolism. Similar to normal progenitors, primitive LSCs retain the ability to self-renew and remain in the G0 phase, allowing them to escape eradication by cytotoxic chemotherapy, which targets actively dividing blasts[10].

Glucose metabolism

Leukemogenic cells exist in a stressful hypoxic microenvironment and, in response, upregulate certain energy producing conduits to meet proliferative demand. Enhanced glycolysis plays a prime role in LSC proliferation. Increased glucose flux is directed by activated oncogenes, particularly expression of BCR-ABL and MLL-AF9, along with overexpression of hypoxia inducible factor 1[11]. These genes upregulate glucose transporter 1 receptor expression, thereby promoting glucose entry and subsequent phosphorylation by hexokinase. Increased levels of hypoxia inducible factor 1, hexokinase, and genes upregulate glucose transporter 1 are described in patients with relapsed AML with poor response to chemotherapy[12]. *In vivo* studies of aggressive leukemia cells have demonstrated a correlation between high glycolysis flux and decreased levels of autophagy, an evolutionary intracellular degradation process that

is bypassed by LSCs[13].

Historically, it has been thought that malignant cells preferentially use cytoplasmic anaerobic glycolysis as a major carbon source (the so-called Warburg effect) over mitochondrial oxidative phosphorylation (OX-PHOS)[14]. However, metabolomic studies have shown that mitochondrial OX-PHOS may be upregulated in LSCs as an adaptive mechanism[15]. Excess oxidative stress has been described in various hematologic malignancies as a critical factor in initiation and progression of disease. There is growing evidence showing that AML LSCs generate increased levels of reactive oxygen species (ROS) primarily driven by mitochondrial NADPH oxidase and other pro-oxidant mechanisms. Sallmyr *et al*[16] suggest that acquired genetic changes in myeloid malignancies lead to DNA damage and defective repair by directly increasing ROS production. Certain genetic abnormalities in AML such as RAS, IDH1/IDH2 and fms-like tyrosine kinase 3 (FLT3)/ITD mutations can directly disturb ROS metabolism causing an eventual shift to amplified ROS production[16].

Interestingly, the majority of LSCs preferentially maintain a low ROS state due to their quiescent nature. These low ROS LSCs were isolated *ex vivo* and subject to gene expression studies using RNA sequencing methods. Remarkably, they displayed a uniform overexpression of the Bcl-2 protein without upregulation of other anti-apoptotic members[17,18].

Glutamine metabolism

The non-essential acid glutamine can be metabolized by glutaminases to glutamate and then α-ketoglutarate, which can go on to fuel the tricarboxylic acid cycle in the mitochondria[19]. To sustain high proliferative advantage, LSCs may adapt a metabolic preference for glutamine to drive biomass. This so-called glutamine addiction has been demonstrated in multiple studies and represents a potential target for anti-leukemic therapy[20-23].

A number of oncogenes and pathways work to potentiate glutamine addiction in AML, including FLT3. In fact, metabolomic studies reveal that FLT3 inhibited LSCs are impaired in their glycolytic function and fittingly switch to utilize glutamine as primary fuel. Therefore, this metabolic dependency on glutamine metabolism poses a potential therapeutic vulnerability when targeted with FLT3 inhibition[24]. Concurrent reduction of glutamine and Bcl-2 inhibition are being studied to compromise mitochondrial energy production and induce apoptosis, respectively[23].

The mammalian target of rapamycin 1 (mTORC1) signaling pathway is involved in numerous cellular processes including metabolism, cell growth, and apoptosis. Moreover, it has been shown to play an integral role in LSC development and proliferation[25-27]. Glutamine availability is a rate-limiting step for mTORC1; therefore, removal of glutamine accordingly inhibits mTORC1 signaling and may be another metabolic mechanism for the treatment of AML[28].

B-CELL LYMPHOMA-2 MEDIATED MITOCHONDRIAL APOPTOSIS

Control of cellular proliferation and apoptosis is deregulated in cancer cells. Mitochondria play an intrinsic role in programmed cell death through release of soluble proteins from the intermembrane space, a process called mitochondrial outer membrane permeabilization (MOMP). A group of over 20 specialized proteins, known as the Bcl-2 family, are the prime mediators of this process[29] (Figure 1).

Apoptosis is tightly regulated by an intricate balance between pro-apoptotic Bax-like proteins (*e.g.*, BAX, BAK and BAD) and anti-apoptotic Bcl-2 like proteins (*e.g.*, Bcl-2, Bcl-XL, Bcl-W and MCL-1) which are predominantly localized in the mitochondria. Bcl-2 prevents apoptosis by inactivating BAX and BAK. Bcl-XL blocks apoptosis by rendering mitochondrial pores impermeable thus inhibiting cytochrome C release. BAX and BAK proteins promote apoptosis by simply opposing Bcl-2 and forming oligomeric pores essential in MOMP[30].

Each of these apoptotic proteins are structurally distinguished by four groups of Bcl-2 homology (BH) domain (1-4). Functionally these BH domains, specifically the "BH3-only proteins" (*e.g.*, BID, BIM, BAD, PUMA, NOXA and BIK/NBK), sense cellular stress, activate pro-death signals, and coordinate the activity of other Bcl-2 proteins[31]. The binding of apoptotic proteins is highly selective: BAD binds exclusively to Bcl-2, Bcl-XL, and Bcl-W, NOXA to MCL-1 and A1, and BIM can bind to all anti-apoptotic members[32]. Upstream of the intrinsic Bcl-2 pathway, PUMA serves as a critical mediator of cell death *via* p53-dependent and independent activation of BAX, BAK and dismissing inhibition of Bcl-2 family proteins. Most BH3-only proteins

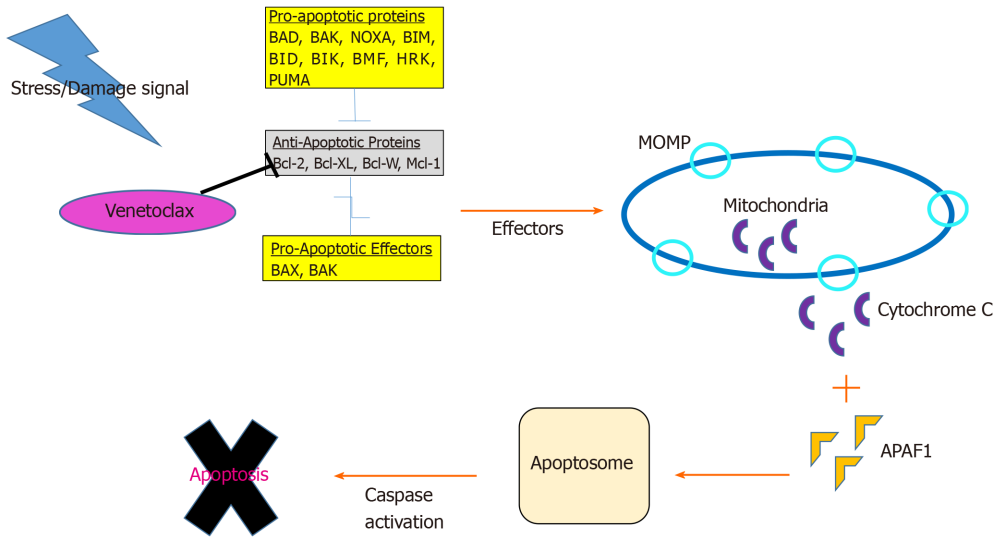

Figure 1 Diagram of the intrinsic apoptotic pathway. When a cell stress or damage signal is received, pro-apoptotic proteins inhibit the anti-apoptotic proteins leading to the subsequent release of effector proteins, BAX and BAK. This induces mitochondrial outer membrane permealization and allows for the release of cytochrome C. Cytochrome C binds to Apoptotic protease activating factor 1, which leads to the formation of the apoptosome, release of caspases, and ultimately, cell death. Venetoclax inhibits B-cell lymphoma-2. Bcl-2: B-cell lymphoma-2; MOMP: Mitochondrial outer membrane permealization; APAF1: Apoptotic protease activating factor 1.

exist in an ambiguous conformation and at relatively low levels. Chemotherapeutic agents induce activation of BH3 only proteins to overcome the anti-apoptotic threshold resulting in cell death[33].

In response to cellular derangement, BH3-only proteins concurrently inhibit anti-apoptotic members and activate pro-apoptotic members, BAX and BAK. Intracytoplasmic signaling leads to transformation of BAX into homo-oligomers and translocation of the proteins into the mitochondrial membrane forming pores to induce MOMP. As a result, voltage dependent anion channels are unlocked facilitating release of cytochrome C into cytosol, binding to apoptotic protease-activating factor 1, apoptosome formation, caspase activation, DNA fragmentation, and ultimately cell death[30].

Mitochondrial response to pro-apoptotic members, a process known as "priming", has been studied as a measure of sensitivity to chemotherapy. Artificial priming of myeloblast mitochondria with BH3-only proteins (BIM or BAD BH3-peptide) supported the hypothesis that Bcl-2 inhibition may be a powerful strategy in targeting AML cells. Analysis of poorly primed, chemo-refractory AML cells showed increased sensitivity to BAD BH3-peptide mediated killing with potential for BH3 mimetic benefit even in low-primed AML[34]. Knowing the level and specificity of priming prior to treatment may help in predicting the synergistic action of chemotherapeutic agents and Bcl-2 inhibitors. This functional approach to predicting mitochondrial response to BH3 peptides, termed BH3 profiling, could distinguish alterations between AML myeloblasts and HSCs. Certain BH3 peptides used for profiling inhibit selective Bcl-2 family proteins (*e.g.*, BAD BH3 peptide indicates dependence on Bcl-2, Bcl-XL, or Bcl-w)[35]. MOMP induced by targeting such peptides hints at specific dependence on certain anti-apoptotic proteins through which they inhibit cell death[36].

Human LSCs were first discovered to modify expression of death receptors (*e.g.*, FAS and TRAIL receptors) to evade apoptosis. LSCs with very immature phenotype of CD34+/CD38- were able to confer both chemotherapy resistance and decreased capacity to induce Fas-induced apoptosis[37]. Prominently, alteration of the Bcl-2 mediated pro-survival pathway and variant expression of effector proteins (BAX and BAK) are potent methods employed by LSCs to inactivate death signals. Bcl-2 is normally expressed in early myeloid progenitors but downregulated during myeloid differentiation. However, transgenic studies have shown that overexpression of Bcl-2 protects LSCs from various apoptosis-inducing stimuli[38]. Bcl-2 overexpression leads to increased LSC numbers in the bone marrow and enhanced colony formation *in vitro* and *in vivo*[39]. Remarkably, the bone marrow stromal microenvironment may facilitate this mechanism. Leukemic blasts thrive by exhibiting a higher degree of Bcl-2 when co-cultured with stromal cells. It is therefore possible that eliminating Bcl-2 protein function can eradicate early LSCs[40].

TARGETING B-CELL LYMPHOMA-2 IN ACUTE MYELOID LEUKEMIA

In 2005, ABT-737, a high-affinity small molecule Bcl-2/Bcl-XL/Bcl-W inhibitor, demonstrated single agent mechanistic killing of lymphoma and various solid tumor cell lines. Later studies demonstrated effective killing of primitive CD34+/CD38- populations with independent and synergistic action of conventional chemotherapeutics. Remarkably, this disruption was specific to LSCs without apparent damage to normal HSCs[41]. Certain LSCs with increased MCL-1 and phosphorylated Bcl-2 were unaltered by ABT-737, proposing a potential co-target to bypass resistance in AML[42].

Progenitor blasts and chemo-resistant LSCs are heterogenous and possess a certain degree of metabolic plasticity. As discussed, LSCs adapt to rely on OX-PHOS as their predominant source of carbon as suggested by high mitochondrial mass and increased oxygen consumption[43]. Chemically blocking Bcl-2 causes prompt and severe impairment of OX-PHOS with the potential to cut off a major power source for LSCs[17].

Bcl-2 dependence has been described as a hallmark of multiple hematologic malignancies including AML. This led to the study of venetoclax (ABT-199), an oral Bcl-2 inhibitor, as a single agent and in combination with hypomethylating agents for the treatment of AML. Venetoclax is highly specific for Bcl-2 but also inhibits several other members of the Bcl family, including Bcl-W[17,44]. Strong preclinical data was evidenced by a median IC50 of approximately 10 nmol/L, and mitochondrial apoptosis occurring within 2 h of exposure[45].

Venetoclax monotherapy was first studied in high-risk relapsed/refractory AML patients and was found to have an underwhelming overall response rate of 19%[46]. Given these results, the success of the combination of venetoclax with a hypomethylating agent (HMA) (either 7 d of azacitidine or 5 d of decitabine) or low-dose cytarabine in newly diagnosed, elderly AML patients was somewhat unexpected. Studies have demonstrated 50%-70% response rates for combination therapy in this high-risk population[47,48]. In addition, in the HMA + venetoclax study, median overall survival was increased by 17.5 mo (double that of an HMA alone)[47]. These pivotal results led, in November 2018, to the FDA approval of the combination of venetoclax plus an HMA or low dose cytarabine combo for adults > 75 years who are not candidates for intensive induction chemotherapy[44]. Patients with mutations in FLT3, IDH1/2, or mutations in the nucleophosmin gene were noted to have the most favorable responses[47].

Interim results from a Phase II study of ten-day decitabine plus venetoclax were recently presented and build upon the results of these initial studies. In this heterogeneous cohort of patients, those with newly diagnosed de novo AML had a CR/CRi rate of 95%. Furthermore, 80% of these patients became MRD negative and 90% were alive at 6 mo[49].

Unfortunately, retrospective results for venetoclax combination therapy in the relapsed/refractory setting have not been as promising, however, prospective studies are ongoing. Overall response rates in these patients, some of whom have been heavily pre-treated, range from 21%-64%[50,51]. Patients with secondary AML and those whose AML harbors a TP53 mutation have the poorest responses[52]. Identifying the reasons for the disparity between response rates in newly diagnosed and relapsed disease has been the focus of much investigation, and has centered on a discussion of leukemic cell metabolism.

MECHANISMS OF VENETOCLAX RESISTANCE

There is increasing interest in understanding the mechanisms underlying venetoclax resistance. Genomic and protein analyses of expanding clones of LSCs after venetoclax treatment have identified a variety of potential adaptive mechanisms, including alterations in leukemic cell metabolism.

Initial hypotheses about resistance mechanisms focused on alterations in BH3-family protein expression. Reductions in Bcl-2 expression have been shown to promote primary and acquired resistance to venetoclax by alternate pathway activation and upregulated expression of other anti-apoptotic proteins such as MCL-1 and Bcl-xL[53]. A study of AML cell lines *in vitro* showed a definite and inverse correlation with the ratios of Bcl-2/MCL-1 transcripts and venetoclax sensitivity suggesting the importance of MCL-1 effect on sensitivity[54]. Similarly, Niu *et al*[55] demonstrated that Bcl-2/MCL-1 transcript ratio may represent a potential biomarker in predicting response[55]. As such,

methodical targeting of MCL-1 during venetoclax therapy may delay the acquisition of venetoclax resistance[56]. However, MCL-1 upregulation is only part of the venetoclax resistance story.

To try and better understand the basis of resistance, Chen *et al*[57] performed a genome-wide CRISPR/Cas9 loss of function screen in venetoclax-sensitive and venetoclax resistant clones (VRCs). The analysis demonstrated that specific genes involved in mitochondrial physiology, namely CLPB with HAX1, contribute to development of VRCs. CLPB, also known as chaperonine, is a protein-coding gene thought to maintain mitochondrial integrity by preventing the release of cytochrome C following death stimulus. Loss of CLPB impairs mitochondrial structure thereby triggering defective OXPHOS and glycolysis. CLPB was notably upregulated in VRCs suggesting a potential dependency and an amenable target. Correspondingly, analysis of CLPB-deficient AML cells showed that they were more sensitive to venetoclax treatment[57].

Similarly, Sharon *et al*[58], performed a genome-wide CRISPR knockout screen to look for potential genes that could be inactivated to reestablish venetoclax sensitivity[58]. Interestingly, a glycine-to-valine mutation at amino acid position 101 was not identified in the Bcl-2 gene of VRCs. This mutation was previously proposed as an acquired venetoclax resistance mechanism in chronic lymphocytic leukemia[59]. Instead, multiple genes-DAP3, MRPL54, MRPL17, and RBFA- encoding key parts of the mitochondrial translation apparatus were identified. LSCs exposed to the bacterial mitochondrial ribosome inhibitors tedizolid and doxycycline, both alone and in combination with venetoclax, showed a depleted CD34+ fraction with combination therapy, but not with venetoclax alone, suggesting that pharmacologic inhibition of mitochondrial translation may overcome resistance[58].

Findings of a study by Pollyea *et al*[60] demonstrated that deeper and more durable responses to treatment with venetoclax and azacitadine were due to effective eradication of OXPHOS dependence. Direct *in vitro* measurement of ETC complex II activity and SDHA glutathionylation in primary AML cells upon venetoclax and azacitidine exposure confirmed decreased glutathione levels and correlating reduction in ETC activity[60]. However, Jones *et al*[61] showed that OXPHOS levels in the LSCs of patients with relapsed AML are not reduced after HMA and venetoclax exposure suggesting that altered metabolism is an escape route for LSCs[61]. Further evaluation of these LSCs identified an increased reliance on fatty acid metabolism, which may be targetable.

OVERCOMING RESISTANCE WITH VENETOCLAX COMBOS

Clinically, combining venetoclax with one or more other agents may be the key to overcoming resistance; many studies of this kind are underway[62]. A comprehensive list of is found in Table 1.

VENETOCLAX + METABOLIC INHIBITION

Exploiting dependency on OXPHOS concurrently with Bcl-2 inhibition is a potential therapeutic strategy. Preclinical combination of OPB-111077, an OXPHOS inhibitor, with decitabine synergistically hindered the proliferation LSCs with a tolerable side effect profile. Triplet therapy with OPB-111077 + HMA and venetoclax in AML cells increased apoptosis rates to a greater degree than exposure to single agent OPB-111077 or venetoclax[63]. A Phase I study of the triplet is ongoing.

The OXPHOS inhibitor IACS-010759 is another small molecule with promising *in vivo* and *in vitro* activity in LSCs in AML cell lines. This agent binds and inhibits complex I of the electron transport chain (NADH ubiquinone oxidoreductase) and is being studied in a phase I study of patients with relapsed/refractory AML. Safety is yet to be established with dose escalation, but mechanistically this is a sensible combination strategy with venetoclax[64].

Metformin, a biguanide used in diabetes management, has shown potential for anti-leukemic activity by directly targeting electron transport chain complex I activity and inhibition of constitutive mTOR activation. This in turn induces AMPK-independent apoptosis. Promising combination strategies with chemotherapy or other targeted therapies have been described with all-trans retinoic acid, ABT-737 (Bcl-2 inhibitor) and sorafenib in acute promyelocytic leukemia, T-cell acute lymphoblastic leukemia, and FLT3-ITD positive AML[65]. Given its mechanism of action, the combination of

Table 1 Clinical trials investigating venetoclax combination therapy

ClinicalTrials.gov identifier	Treatment combination	Phase	Population
NCT03709758	Venetoclax + daunorubicin + cytarabine	Ib	Untreated
NCT03214562	Venetoclax + fludarabine, cytarabine, filgrastim, idarubicin	Ib/II	Untreated Relapsed/refractory
NCT03471260	Venetoclax + ivosidenib ± azacitidine	I/II	Relapsed/refractory
NCT02993523	Venetoclax + placebo or azacitidine	III	Untreated
NCT03069352	Venetoclax + placebo or low dose cytarabine	III	Untreated
NCT03466294	Venetoclax + azacitidine	II	Untreated-elderly
NCT03404193	Venetoclax + decitabine 10 d	II	Untreated
NCT03586609	Venetoclax + low dose cytarabine+ cladribine + azacitidine	II	Untreated
NCT03236857	Venetoclax ± chemotherapy (various)	I	Relapsed/refractory malignancies (including AML)
NCT03455504	Venetoclax + fludarabine + cytarabine + idarubicin	II	Untreated
NCT03629171	Venetoclax + liposomal daunorubicin -cytarabine	II	Untreated Relapsed/refractory
NCT03862157	Venetoclax + azacitidine + pevonedistat	I/II	Untreated
NCT03390296	Venetoclax + azacitidine + avelumab	I/II	Untreated
NCT03390296	Venetoclax + azacitidine + gemtuzumab ozogamicin + anti-OX40 antibody	I/II	Relapsed/refractory
NCT03867682	Venetoclax + lintuzumab-Ac225	I/II	Relapsed/refractory
NCT03932318	Venetoclax + azacitidine + lintuzumab-Ac225	I/II	Relapsed/refractory
NCT03672695	Venetoclax + S64315	I	Relapsed/refractory
NCT03797261	Venetoclax + AMG-176	Ib	Relapsed/refractory
NCT03063944	Venetoclax + decitabine + OPB-111077	Ib/II	Relapsed/refractory
NCT03484520	Venetoclax + dinaciclib	Ib	Relapsed/refractory
NCT03441555	Venetoclax + alvocidib	Ib	Relapsed/refractory
NCT02670044	Venetoclax + cobimetinib; Venetoclax + idasanutlin	I/II	Relapsed/refractory
NCT03940352	Venetoclax + HDM201	I	Relapsed/refractory
NCT03874052	Venetoclax + ruxolitinib	I	Relapsed/refractory
NCT03471260	Venetoclax + ivosidenib	Ib/II	Relapsed/refractory
NCT04092179	Venetoclax + enasidenib	Ib/II	Relapsed/refractory
NCT03735875	Venetoclax + quizartinib	Ib/II	Relapsed/refractory
NCT03625505	Venetoclax + gilteritinib	I	Relapsed/refractory

AML: Acute myeloid leukemia.

metformin with venetoclax may be effective.

Finally, as discussed earlier, CLPB targeting can compromise mitochondrial matrix adding to Bcl-2 inhibition. Interestingly, a bacterial CLPB inhibitor has been developed and proposed as an antimicrobial agent with possible use in this setting[57].

VENETOCLAX+ DAUNORUBICIN/CYTARABINE

In vitro studies conducted in AML cell lines and patient-derived AML samples have shown that venetoclax in combination with daunorubicin or cytarabine reduced MCL-1 protein levels resulting in increased DNA damage[66]. Preclinical synergy translated to the clinical setting in an open label, multicenter trial study with 82 patients in which CR rate was 54% with a median OS of 10.1 mo. Lower response rates were observed

for patients with prior hypomethylating agents[67]. Investigations for Venetoclax with daunorubicin/cytarabine (7 + 3) and consolidation therapy are currently underway.

VENETOCLAX + MCL1 INHIBITOR/ CYCLIN-DEPENDENT KINASE 9 INHIBITION

MCL-1 inhibitors are under development to target VRCs. Direct MCL-1 inhibition with S63845 and A-1210477 plus venetoclax leads to synergistic cell killing of VRCs *in vivo* and *in vitro*. Additionally, several studies demonstrate preclinical synergy of A-1210477 and venetoclax where successful neutralization of MCL-1-dependent AML cells have been demonstrated[68]. Dual inhibition of Bcl-2 and MCL-1 (with S55746 and S63845, respectively) has also shown strong activity against LSCs with relative sparing of normal progenitors. Researchers observed prolonged survival of xenograft models of AML with this combination[69].

More recent studies suggest synergy between venetoclax and inhibitors of Cyclin-dependent kinase 9 (CDK9), a transcriptional regulator of MCL-1, *via* indirect targeting of MCL-1. Drivers of LSC survival like MCL-1 and MYC have very short half-lives making them expeditious targets to CDK9 inhibition. Alvocidib, aka flavopiridol, was the first of the CDK9 agents tested in combination with conventional chemotherapy[70]. A newer agent voruciclib that inhibits CDK9, 4, and 6 kinase diminishes transcription of MCL-1 downstream with better toxicity profile in comparison[71].

VENETOCLAX + MITOGEN ACTIVATED PROTEIN KINASE INHIBITION

Based on preclinical data, mitogen activated protein kinase pathway inhibitors such as cobimetinib (also a MEK1/2 inhibitor) have been studied with concomitant targeting of Bcl-2 in relapsed or refractory AML. Padua *et al*[72] demonstrated disruption of the RAS/Bcl-2 complex in AML patient derived samples suggesting potential efficacy of the combination[72]. Likewise, Han *et al*[73] studied co-targeting of Bcl-2 and mitogen activated protein kinase in Bcl-2 protein enriched leukemic cells and synergistic killing was appreciated with over 60% growth inhibition in AML samples, including VRCs[73]. Preliminary phase 1B clinical trial results, however, revealed increased gastrointestinal toxicity, mainly diarrhea, associated with cobimetinib[74]. Newer MAP kinase inhibitors with better safety profiles are currently under development.

VENETOCLAX + PHOSPHATIDYLINOSITOL-3 KINASE/ MAMMALIAN TARGET OF RAPAMYCIN 1 INHIBITION

Dual Bcl-2 and phosphatidylinositol-3-kinase (PI3K/AKT) inhibition may help overcome both acquired and intrinsic venetoclax resistance requiring and is being evaluated in AML[75]. Co-administration of venetoclax and apitolisib (GDC-0980:PI3K/mTOR inhibitor) or taselisib (GDC-0032: p110β-sparing PI3K inhibitor) induced profound cytochrome C release and apoptosis in various AML cell lines. AKT/mTOR inactivation and MCL-1 downregulation were also noted, with BAX and BAK mediated apoptosis of a CD34+/38-/123+ population while sparing the normal HSCs.

VENETOCLAX + MOUSE DOUBLE MINUTE 2 ANTAGONIST

Small molecule mouse double minute 2 homolog (MDM2) antagonists reactivate the tumor suppressor function of wildtype-p53 leading to downstream stimulation of pro-apoptotic BAX and NOXA. Further apoptotic pathways are promoted, like PUMA and BAD, to stabilize and degrade MCL-1. Studies with a combination of Nutlin-3a, a first-generation MDM2 inhibitor, and ABT-737, a Bcl-2 inhibitor, published a decade ago displayed durable induction of mitochondrial apoptosis of AML cells by the combination[76]. Given preclinical rationale, researchers tested the combination of Bcl-2 and MDM2 inhibition (by idasanutlin) in wildtype-AML to boost activity of venetoclax and prevent upfront resistance[77]. Safety and efficacy of venetoclax and idasanutlin has been studied in 39 patients with relapsed refractory elderly AML patients. Overall

response rate was 46% with superior responses in IDH1/2, RUNX1, JAK2, MPL, and CALR mutations. TP53 and FLT3 mutations were associated with primary or secondary refractoriness[78]. Additionally, updated data in both safety and efficacy appears to show reasonable tolerance to MDM2 and Bcl-2 inhibition.

VENETOCLAX + JAK2 INHIBITION

JAK inhibitors may combine with venetoclax to counteract bone marrow stroma-mediated resistance in AML. Cytokines activated by JAK/STAT signaling like GM-CSF support AML cell proliferation and switch dependency of Bcl-2 to Bcl-XL[79]. Correspondingly, *ex vivo* studies of isolated AML blasts expressed sensitivity to venetoclax + ruxolinitib combination as an effective method of killing[80].

VENETOCLAX + IDH INHIBITION

The small molecule IDH inhibitors enasidenib (IDH2) and ivosidenib (IDH1) are FDA approved for the treatment of AML. Inhibition of altered IDH1 and IDH2 enzymes along with hypomethylated genes can allow differentiation of LSCs[81]. Studies investigating safety and tolerability of IDH1 and Bcl-2 inhibition are currently ongoing with ivosidenib and venetoclax, respectively[82].

VENETOCLAX + FLT3 INHIBITION

Sequencing studies were performed to assess the combination of venetoclax and the small molecule FLT3 inhibitor quizartinib in specific FLT3 ITD mutated xenograft models. The combination induced durable tumor regression for up to 3 mo after cessation of treatment[83]. However, Chyla *et al*[84] noted that FLT3-ITD or PTPN11 mutations may confer intrinsic and acquired resistance to venetoclax[84]. Clinical trials evaluating venetoclax and FLT3 inhibitor combination therapy are ongoing[9].

CONCLUSION

Up-front AML treatment with venetoclax in combination with a hypomethylating agent has shown impressive responses in multiple trials. Unfortunately, response durations are variable and patients still inevitably relapse. Attempts at identifying the cellular and molecular changes that occur after exposure to venetoclax have provided insight into mechanisms of resistance, namely alterations in LSC metabolism. Improved techniques to understand mitochondrial adaptations and the stromal microenvironment may aid in designing new therapeutic strategies. With more potent BH3 mimetics in development and rational combination therapies under investigation, the right strategy for building on the success of venetoclax treatment in AML is within reach.

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REVIEW

Combination drug regimens for metastatic clear cell renal cell carcinoma

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Abstract

Renal cell carcinomas (RCC) make up about 90% of kidney cancers, of which 80% are of the clear cell subtype. About 20% of patients are already metastatic at the time of diagnosis. Initial treatment is often cytoreductive nephrectomy, but systemic therapy is required for advanced RCC. Single agent targeted therapies are moderately toxic and only somewhat effective, leading to development of immunotherapies and combination therapies. This review identifies limitations of monotherapies for metastatic renal cell carcinoma, discusses recent advances in combination therapies, and highlights therapeutic options under development. The goal behind combining various modalities of systemic therapy is to potentiate a synergistic antitumor effect. However, combining targeted therapies may cause increased toxicity. The initial attempts to create therapeutic combinations based on inhibition of the vascular endothelial growth factor or mammalian target of rapamycin pathways were largely unsuccessful in achieving a profile of increased synergy without increased toxicity. To date, five combination therapies have been approved by the U.S. Food and Drug Administration, with the most recently approved therapies being a combination of checkpoint inhibition plus targeted therapy. Several other combination therapies are under development, including some in the phase 3 stage. The new wave of combination therapies for metastatic RCC has the potential to increase response rates and improve survival outcomes while maintaining tolerable side effect profiles.

Key words: Renal cell carcinoma; Immunotherapy; Targeted therapy; Vascular endothelial growth factor; Programmed-death receptor 1; Programmed-death receptor ligand-1; Tyrosine kinase inhibitors

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Core tip: The treatment of metastatic clear cell renal cell carcinoma (ccRCC) remains a challenge given the broad spectrum of disease presentations and outcomes, variety of treatment options without clear optimal sequencing, and the low rate of complete response to systemic monotherapy. The core of this work reviews the current status of systemic combination drug options in the treatment of metastatic ccRCC, encompassing the novel combinations of tyrosine kinase inhibitors and immune checkpoint inhibitors, with a focus on rationale for use, efficacy, and side effect profiles. We also discuss the role of biomarkers in the development of future therapeutic options.

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INTRODUCTION

Kidney cancer is one of the top 10 most common cancers in men and women. In the United States, there are expected to be about 65340 new cases of kidney cancer in 2019 with about 14970 deaths. Renal cell carcinomas (RCC) account for approximately 3.8% of all new cancers and make up about 90% of renal cancers[1]. According to the American Cancer Society, the risk for developing kidney cancer in men is 1 in 47 and in women is 1 in 82[2].

Most renal masses are incidentally found and small (≤ 4 cm). Even patients with advanced disease are often asymptomatic at the time of diagnosis with about 20% of patients having metastatic disease at the time of presentation. The 5-year survival rate for metastatic RCC is 12.0%[3]. Approximately 80% of renal cell carcinomas are clear cell renal cell carcinomas (ccRCC). The other 15%-20% are non-clear cell renal cell carcinomas (nccRCC) which comprise a diverse group of histologic subtypes, each with varying molecular profiles. Histologies include clear cell-papillary, papillary type I or II, chromophobe, collecting duct, and other rare forms[4]. The focus of this paper will be management of clear cell histology.

Patients with metastatic RCC (mRCC) are categorized into risk groups by combining independent prognostic factors for survival. In addition to the Tumor, Nodes, Metastasis (TNM) staging system[5], the two most widely used RCC prognostic models are the Memorial Sloan Kettering Cancer Center (MSKCC)[6] and the International Metastatic RCC Database Consortium (IMDC)[7]. Table 1 summarizes the three prognostic models. The heterogeneous clinical behavior and variable response to therapy seen in RCC pose a challenge in developing therapeutic drug trials.

Surgery is considered the first line of treatment for Stage I to III disease while cytoreductive nephrectomy (CN) followed by systemic therapy is often used to treat metastatic disease[8]. However, the role of CN in advanced RCC is has been challenged in recent years given the efficacy of newer systemic therapies[9]. RCC is not highly responsive to cytotoxic chemotherapy or radiotherapy[10], making systemic targeted therapies and immune checkpoint inhibitors (ICI) critically important, which will be the focus of this review.

MECHANISMS OF ACTION

The state-of-the-art therapy for RCC has undergone rapid transformation over the past fifteen years. Prior to 2005, cytokine therapy with interferon alpha (IFN-α) and then high dose interleukin 2 (HDIL-2) were considered the standard of care for the treatment of metastatic renal cell carcinoma[11,12]. The antitumor mechanism of HDIL-2 and IFN-αare mediated *via* activation of cytotoxic T lymphocytes and other cytokines. Since response rates were modest with these agents, high doses were administered, which resulted in substantial toxicity[13,14]. HDIL-2 side effects often need to be managed in an intensive care unit and were associated with a mortality rate of 1% to 5%[15]. The overall response rates (ORR) of IL-2 and IFN-α range between 5% to 20% with a median overall survival (OS) of about 10 to 15 mo[13]. Though the use of HDIL-2 has mostly fallen out of favor, some centers continue its use, often in clinical trials in

Table 1 Renal cell carcinoma prognostic models

Model	Prognostic factors	Prognostic risk groups
Memorial Sloan Kettering Cancer Center[6]	(1) Interval from diagnosis to treatment of less than 1 year; (2) Karnofsky performance status less than 80%; (3) Serum lactate dehydrogenase greater than 1.5 times the upper limit of normal (ULN); (3) Corrected serum calcium greater than the ULN; and (4) Serum hemoglobin less than the lower limit of normal	(1) Low-risk group: No prognostic factors; (2) Intermediate-risk group: One or two prognostic factors; and (3) Poor-risk group: Three or more prognostic factors
International Metastatic RCC Database Consortium[7]	(1) Less than one year from time of diagnosis to systemic therapy; (2) Performance status < 80% (Karnofsky); (3) Hemoglobin < lower limit of normal; (4) Calcium > upper limit of normal; (5) Neutrophil > upper limit of normal; and (6) Platelets > upper limit of normal	(1) Favorable-risk group: No prognostic factors; (2) Intermediate-risk group: One or two prognostic factors; and (3) Poor-risk group: Three to six prognostic factors
Tumor, Nodes, Metastasis Staging System for Kidney Cancer[5]	(A) Primary tumor (T): (1) Primary tumor cannot be assessed (TX); (2) No evidence of primary tumor (T0); (3) Tumor ≤ 7 cm in greatest dimension, limited to the kidney (T1); (4) Tumor > 7 cm in greatest dimension, limited to the kidney (T2); (5) Tumor extends into major veins or perinephric tissues, but not into the ipsilateral adrenal gland and not beyond Gerota's Fascia (T3); and (6) Tumor invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland) (T4); (B) Regional Lymph Nodes (N): (1) Regional lymph nodes cannot be assessed (NX); (2) No regional lymph node metastasis (N0); and (3) Metastasis in regional lymph node(s) (N1); and (C) Distant Metastasis (M): (1) No distant metastasis (M0); and (2) Distant metastasis (M1)	Stage I: T: T1; N: N0; M: M0; Stage II: T: T2; N: N0; M: M0; Stage III: T: T1-T2; N: N1; M: M0; and T: T3; N: NX,N0-N1; M: M0; Stage IV: T: T4; N: Any N; M: M0; and T: Any T; N: Any N; M: M1

RCC: Renal cell carcinoma.

combination with immune checkpoint inhibitors[16].

Targeted therapies

Advances in genomics and molecular biology have led to the development of targeted therapies for RCC[17,18]. The turning point has been the identification of mutation or loss of von-Hippel Landau (VHL) tumor suppressor gene in 60% to 90% of sporadic cases of RCC either through somatic mutation or promoter methylation[19]. Inactivation of VHL leads to overexpression of hypoxia-inducible factors (HIF) and transcription of genes such as vascular endothelial growth factor (VEGF)[20,21]. HIF-1α is an important stimulus to angiogenesis. VEGF binds to VEGF receptor (VEGFR) on endothelial cells and is a potent mediator of angiogenesis. It leads to increased vascular permeability, endothelial cell proliferation, migration, and cancer progression[22]. This has led to the development of various strategies to inhibit VEGF signal transduction such as humanized neutralizing anti-VEGF monoclonal antibodies and VEGFR inhibitors.

Bevacizumab is the only monoclonal antibody targeting VEGF that has been approved for RCC by the U.S. Food and Drug Administration (FDA). Currently, six small molecule oral tyrosine kinase inhibitors (TKIs) with potent activity against VEGF receptors have been approved for use in RCC (axitinib, cabozantinib, lenvatinib, pazopanib, sorafenib, and sunitinib).

Another critical regulating factor in RCC is mammalian target of rapamycin (mTOR), a serine/threonine kinase, an important component of the phosphoinositide 3-kinase/AKT signaling pathway, which is often dysregulated in RCC. Hyperactivity of mTOR signaling promotes cell growth and proliferation leading to growth and invasiveness of tumor cells. The mTOR component 1 (mTORC1) increases cellular levels of HIF-α and TNF-α, which in turn can cause overproduction of VEGF, PDGF-α and TNF-α in tumor cells resulting in further increase in mTOR signaling. Inhibition of mTOR would result in decreased cell growth, proliferation, cellular metabolism and angiogenesis[23-25]. FDA approved mTOR inhibitors for treatment of RCC are everolimus and temsirolimus.

Immune checkpoint inhibitors

While targeted therapies have changed the course of RCC by improving outcomes, the duration of response is limited by the development of drug resistance and complete responses are rare[26]. This spurred a search for novel therapeutic strategies—specifically in the realm of immuno-oncology. The ICIs are the latest class of immunotherapy (IO) under development. These include programmed death receptor 1/programmed death receptor ligand 1 (PD-1/PD-L1) and cytotoxic T lymphocytes antigen 4 (CTLA-4) inhibitors. PD-1 is a transmembrane protein present on activated effector T cells and has two known ligands (PD-L1 and PD-L2) found on other cells including tumor cells. When bound to its ligand, PD-1 normally acts as an

"off switch" preventing an effective T-cell response. Most RCC tumor cells express PD-L1 on the cell membrane which helps them evade an immune attack. The immune checkpoint inhibitors, by providing PD-1 inhibition or PD-L1 inhibition block this pathway, releasing the "off switch" on the immune system, increasing the ability of T-cells to kill tumor cells[27,28]. CTLA-4 inhibition stops autoreactive T cells during the immune priming phase, thereby supporting the activation and proliferation of effector T cells[29]. FDA has approved two PD-1 inhibitors (nivolumab and pembrolizumab), and one CTLA-4 inhibitor (ipilimumab) for use in RCC. PD-L1 inhibitors under development for use in RCC include atezolizumab, and durvalumab. A CTLA-4 inhibitor under development for RCC is tremelimumab. The precise and detailed mechanism of action of different drugs, their molecular pathways, and the pathophysiologic effects on tumor cells and their microenvironments are beyond the scope of this article. Table 2 summarizes the FDA approved monotherapies for the treatment of clear cell RCC.

COMBINATION THERAPY

Rationale for combination therapy

The goal of combining various modalities of systemic therapy is to potentiate a synergistic antitumor effect. However, combining various targeted therapies may cause increased toxicity. As of now there are five FDA approved combination treatments for metastatic ccRCC: Bevacizumab plus IFN-α, lenvatinib plus everolimus, ipilimumab plus nivolumab, and most recently, pembrolizumab plus axitinib, and axitinib plus avelumab. The more recently approved combinations of immunotherapy and TKIs also allow for combinations of very different therapeutic mechanisms of actions with the aim of improved and potentially rapid response rates as well as potential durable responses.

Unsuccessful combination therapies

Table 3 summarizes initial attempts of combination therapies with unexpectedly high toxicity or lack of anticipated antitumor synergy. Patients with mRCC treated on a phase I study of the combination of bevacizumab and sunitinib were found to have a high degree of hypertension, vascular, and hematologic toxicities at the maximum tolerated dose level (sunitinib 50 mg plus bevacizumab 10 mg/kg). Discontinuation of treatment was observed in 48% of patients due to adverse events[30].

In a phase II combination study of bevacizumab and everolimus, the median progression-free survival (PFS) and OS in previously untreated mRCC patients was longer than inpatients previously treated with sunitinib and sorafenib (PFS: 9.1 mo *vs* 7.1 mo; OS: 21.3 mo *vs* 14.5 mo, $P = 0.11$). Median PFS for all patients was 8.1 mo (95%CI: 6.3 to 10.8 mo). However, 14% of patients discontinued treatment due to serious adverse events (SAEs) such as proteinuria, pulmonary embolism, stomatitis, and anorexia[31].

Similarly, in a phase I combination study of everolimus and sorafenib in mRCC patients, a partial response (PR) rate of 25% was observed. However, due to gastrointestinal toxicities and dose reductions, study discontinuation was necessary. Moreover, there was a higher than expected incidence of rash typically seen with either drug as a single agent[32].

In a phase III trial, the combination of temsirolimus plus interferon-α was compared with temsirolimus or interferon-α alone with the primary end point of OS. OS in the combination-therapy group did not differ significantly compared with the interferon group [Hazard ratio (HR), 0.96; 95%CI: 0.76 to 1.20; $P = 0.70$]. Median OS in the interferon group, the temsirolimus group, and the combination-therapy group was 7.3, 10.9, and 8.4 mo, respectively. Ultimately, the addition of temsirolimus to interferon did not improve survival[33].

In a phase I dose escalation combination trial of tremelimumab plus sunitinib in mRCC patients, 9 of 21 (43%) evaluable patients achieved partial response. All patients developed treatment - related AEs, ten patients (36%) had serious AEs, and seventeen patients (61%) had grade 3 or 4 AEs. DLTs were reported in 2/5 patients receiving sunitinib 50 mg/d plus tremelimumab 6 mg/kg resulting in further exploration done with lowered sunitinib dose at 37.5 mg/d. Of these 4/14 (29%) and 3/6 (50%) developed DLTs with tremelimumab at 10 mg/kg and 15 mg/kg, respectively. Acute renal failure was the most common DLT reported in 4 patients (14%)[34] though it is not a common toxicity with either drug used alone. Acute renal failure did not appear to be related to tremelimumab concentration as deduced from the limited

Table 2 Food and Drug Administration approval monotherapies for the treatment of renal cell carcinoma

Drug	Mechanism of action	Line of therapy	Study	PFS	OS	ORR	Associated toxicities	Ref.
Pazopanib	TKI	First	Pazopanib *vs* placebo	9.2 mo *vs* 4.2 mo HR 0.46; 95%CI: 0.34 to 0.62; $P < 0.0001$	22.9 mo (95%CI: 19.9 to 25.4) *vs* 20.5 (95%CI: 15.6 to 27.6) mo; HR 0.91; 95%CI: 0.71-1.16; one sided stratified log rank $P = 0.224$	30% (95%CI: 25.1 to 35.6) *vs* 3% (95%CI: 0.5 to 6.4), median duration of response 58.7 wk by independent review[1]	Diarrhea, hypertension, hair color changes, nausea, anorexia, vomiting. Grade 3 toxicities included elevated ALT (30%) and AST (28%)	[99,100]; Comment: Lack of correlation between OS and PFS was attributed to extensive crossover of placebo-treated patients to pazopanib group
Pazopanib	TKI	Second	Pazopinibvs placebo after prior progression on sunitinib or bevacizumab	7.5 mo (95%CI: 5.4 to 9.4) *vs* 7.5 mo (95%CI: 5.5 to 14.1) *vs* 6.7 mo (95%CI: 3.6 to 9.3)	14.8 mo (95%CI: 12 to 28.8) *vs* 24.2 mo (95%CI: 14.7 to not reached) *vs* 10.9 (95%CI: 8.2 to 12)	27% (95%CI: 17% to 40%) *vs* 26% (95%CI: 15% to 41) *vs* 31% (95%CI: 14 to 55%)	Grade 1 and 2 toxicities were common. Grade 3 and 4 occurring in ≥ 10% included fatigue (185), proteinuria (13%), hypertension (13%), and diarrhea (11%)	[101]
Sunitinib	TKI	First	Sunitinib *vs* interferon	11 mo (95%CI: 11 to 13 mo *vs* 5 mo (95%CI: 4 to 6); HR 0.42 (95%CI: 0.451 to 0.643); $P < 0.001$	26.4 mo (95%CI: 23 to 32.9) *vs* 21.8 (95%CI: 17.9 to 26.9); HR, 0.821; 95%CI: 0.673 to 1.001; $P = 0.051$	31% (95%CI: 26 to 36) *vs* 6% (95%CI: 4 to 9; $P < 0.001$)	Grade 3 events included hypertension (12%), fatigue (11%), diarrhea (9%), and hand-foot syndrome (9%)	[102,103]
Axitinib	TKI	First	Axitinib *vs* sorafenib	10.1 mo (95%CI: 7.2 to 12.1) *vs* 6.5 mo (95%CI: 4.7 to 8.3); Stratified HR; 0.77 (95%CI: 0.56 to 1.05)[1]	Median OS (95%CI: 21.7 mo (18.0-31.7) with axitinib *vs* 23.3 mo (18.1-33.2) with sorafenib (stratified HR, 0.995; 95%CI: 0.731-1.356; 1-sided $P = 0.4883$)	32% *vs* 15%; risk ratio 2.21; (95%CI: 1.31 to 3.75; stratified one-sided $P = 0.0006$)	Diarrhea (50%), hypertension (49%), weight decrease (40%), decreased appetite (29%), dysphonia (23%). Any grade events were more common n axitinib *vs* sorafenib ≥ 10%	[104,105]
Axitinib	TKI	Second	AXIS: Axitinibvssorafenib after 1 prior systemic therapy	8.3 mo (95%CI: 6.7 to 9.2) *vs* 4.7 mo (95%CI: 4.7 to 6.5); HR 0.656, 95%CI: 0.552 to 0.779; one sided $P < 0.001$	20.1 mo (95%CI: 16.7 to 23.4) *vs* 19.2 (95%CI: 17.5 to 22.3)	19% (95%CI: 15.4 to 23.9) *vs* 34% (95%CI: 6.6 to 12.9), $P = 0.0001$	Adverse events of all grades were more frequent with axitinib were hypertension, fatigue, dysphonia, and hypothyroidism. Adverse events more frequent with sorafenib with hand-foot syndrome, rash, alopecia, and anemia	[57,106]
Sorafenib	TKI	Second line	TARGET: Sorafenib *vs* placebo for patients who progressed on prior therapy	5.5 mo *vs* 2.8 mo	17.8 mo *vs* 14.3 mo, HR= 0.88; $P = 0.146$		Skin rash/ desquamation, hand foot skin reaction, fatigue. Hypertension and cardiac ischemia were rare but SAEs.	[107]
Cabozantinib	Inhibitor of multiple TKReceptors including MET, VEGFRs, and AXL	First	The Alliance A031203 CABOSUN Trial: Cabozantinib *vs* sunitinib	8.2 mo (95%CI: 6.2 to 8.8 mo) *vs* 5.6 mo (95%CI: 3.4 to 8.1 mo); Adjusted HR, 0.66; 95%CI: 0.46 to 0.95; one-sided $P = 0.012$	30.3 mo (95%CI: 14.6 to 35.0 mo) *vs* 21.8 mo (95%CI: 16.3 to 27.0 mo); Adjusted HR, 0.80; 95%CI: 0.50 to 1.26	33% (95%CI: 23% to 44%) *vs* 12% (95%CI: 5.4% to 21%)	Fatigue, hypertension, diarrhea, AST/ALT elevation	[62]
Cabozantinib		Second	METEOR: Cabozatinib *vs* everolimus for those that progressed on anti VEGF therapy	7.4 mo (95%CI: 5.6 to 9.1) *vs* 3.8 mo (95%CI: 3.7 to 5.4); HR 0.51 (95%CI: 0.41 to 0.62); $P < 0.001$	21.4 mo (95%CI: 18.7-not estimable) *vs* 16.5 mo (95%CI: 14.7 to 18.8); HR 0.66 (95%CI: 0.53 to 0.83; $P = 0.00026$)	17% (95%CI: 13 to 22) *vs* 3% (95%CI: 2 to 6), $P < 0.0001$	Grade 3 or 4 events were hypertension (15%), diarrhea (13%), fatigue (11%), palmar-plantar erythrodysaesthesia syndrome (8%)	[63,108]

Everolimus	mTOR Inhibitor	Third	RECORD-1: Patients who progressed on sunitinib, sorafenib, or both were given everolimus *vs* placebo	4.9 mo (95%CI: 3.7 to 5.5) *vs* 1.9 (95%CI: 1.8 to 1.9); HR 0.33, 95%CI: 0.25 to 0.43; $P < 0.001$	14.8 mo *vs* 14.4 mo; HR 0.87, 95%CI: 0.65 to 1.15; $P = 0.162$	1% *vs* 0%	Stomatitis (40% *vs* 8%), rash (25% *vs* 4%), fatigue (20% *vs* 16%), pneumonitis (8%)	[109,110]
Temsirolimus	mTOR Inhibitor	First	IFN-α-alone *vs* temosirolimus alone *vs* IFN-α+ temosirolimus[1], poor risk patients with ≥ 3 of 6 unfavorable prognostic factors.	3.1 mo (95%CI: 2.2 to 3.8) *vs* 5.5 (95%CI: 3.9 to 7) *vs* 4.7 (95%CI: 3.9 to 5.8); ($P < 0.001$)	7.3 mo (95%CI: 6.1 to 8.8) *vs* 10.9 mo (95%CI: 8.6 to 12.7) *vs* 8.4 mo (6.6 to 10.3); HR for death, 0.73; 95%CI: 0.58 to 0.92; $P = 0.008$	4.8% (95%CI: 1.9 to 7.8) *vs* 8.6% (95%CI: 4.8 to 12.4) *vs* 8.1% (95%CI: 4.4 to 11.8); HR, 0.96; 95%CI: 0.76 to 1.20; $P = 0.70$)	Rash, peripheral edema, hyperglycemia, and hyperlipidemia were more common in the temsirolimusgroup, asthenia was more common in the interferon group (26% *vs* 11%)	[33]
Temsirolimus	mTOR Inhibitor	Second	INTORSECT: Temsirolimus *vs* sorafenib as second line after treatment with sunitinib[1] with response duration < 180 d	4.3 mo (95%CI: 4 to 5.4) *vs* 3.9 mo (95%CI: 2.8 to 4.2); Stratified HR = 0.87; 95%CI: 0.71 to 1.07; two-sided $P = 0.19$	12.3 mo (95%CI: 10.1 to 14.8) *vs* 16.6 mo (95%CI: 13.6 to 18.7); Stratified HR, 1.31; 95%CI: 1.05 to 1.63, $P = 0.01$ (two sided log-rank)	8% *vs* 8%	Rash and fatigue more commonly associated with temsirolimus and PPE + diarrhea higher in sorafenib group	[111]
Nivolumab	ICI- Anti PD-1 Inhibitor	Second	Checkmate 025: Nivolumab *vs* everolimus	4.6 mo (95%CI: 3.7 to 5.4) *vs* 4.4 mo (95%CI: 3.7 to 5.5); HR, 0.88; 95%CI: 0.75 to 1.03; $P = 0.11$	25.0 mo (95%CI: 21.8– NR for nivolumab) *vs* 19.6 mo (95%CI: 17.6–23.1)	25% *vs* 5%; odds ratio, 5.98 (95%CI: 3.68 to 9.72); $P < 0.001$	Fatigue	[66,67]

[1]Not statistically significant. HR: Hazards Ratio; NR: Not reached; mAb: Monoclonal antibody; DFS: Disease-free survival; ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival.

pharmacokinetic data available. The relationship with sunitinib could not be determined. Fever was noted to accompany all acute renal failure events postulating the possibility of an immune-related mechanism when the two drugs are used in combination. Given the high incidence of renal failure, further evaluation of doses more than 6 mg/kg tremelimumab plus sunitinib 37.5 mg daily was not recommended.

Approved combination therapies

Currently, there are five FDA approved combination therapies for mRCC (Table 4).

Bevacizumab plus IFN-α: A multicenter, randomized, double-blind phase III trial compared OS, PFS, and safety in 649 patients who either received bevacizumab plus IFN-α or placebo plus IFN-α. A total of 641 patients were treated with 325 in the combination group and 316 in the placebo plus IFN-α group. The combination group of bevacizumab plus IFN-α had a significantly longer PFS (10.2 mo *vs* 5.4 mo) and ORR (30.6% *vs* 12.4%). There were significantly more grade 3 or higher adverse events for the bevacizumab group than the control group in terms of fatigue (12% *vs* 8%) and asthenia (10% *vs* 7%)[35], but toxicity was felt to be acceptable.

In a similar trial, patients with previously untreated mRCC ($n = 732$) were randomized to receive either IFN-α monotherapy or the combination of bevacizumab

Table 3 Unsuccessful combination therapy trials

Combination therapy	TrialPhase	Comparator	Side-effect profile	Comments	Ref.
Bevacizumab + sunitinib	I	3 cohorts of escalating doses of Sunitinib	High degree of hypertension, vascular and hematologic toxicities, leading to discontinuation in 48%		[30]
Bevacizumab + everolimus	II		Increased proteinuria, pulmonary embolism, stomatitis and anorexia leading to discontinuation in 14%		[31]
Everolimus + sorafenib	I		Discontinuation due to high gastrointestinal toxicity and grade 3 rash		[32]
Temsirolimus + IFN-α	III	IFN-α		Failed to improve overall survival	[33]
Tremelimumb + sunitinib	I		Rapid onset renal failure		[34]

plus IFN-α. The PFS of the combination group was higher than the control group (8.5 mo *vs* 5.2 mo) but the OS was not significant (18.3 mo *vs* 17.4 mo). The combination group had a higher objective response rate (25.5% *vs* 13.1%). There was significantly greater toxicity in the bevacizumab plus IFN-α group than the control group in the form on grade 3 to 4 hypertension, fatigue, anorexia, and proteinuria[36]. In July 2009, the FDA granted approval for the use of bevacizumab in combination with IFN-αfor the treatment of patients with metastatic RCC. Despite bevacizumab being approved as combination therapy with IFN, many practitioners have used bevacizumab as monotherapy rather than combination as the added benefit of IFN was unclear[37].

Everolimus plus lenvatinib: Resistance to targeted monotherapy in RCC is believed to be due to feedback mechanisms that are mediated *via* biological changes permitting tumor growth and perfusion independent of VEGF or mTOR pathways. This can offset targeted inhibition and permit tumor growth[38,39]. Hence, sequential treatments with a single anti-VEGF agent followed by a mTOR inhibitor often results in the development of resistance. Consequently, combination therapy with both VEGF and mTOR inhibitors was thought to potentially surmount monotherapy resistance[40]. Lenvatinib is a tyrosine kinase inhibitor ofVEGFR1, VEGFR2, VEGFR3 and everolimus is an mTOR inhibitor.

Motzer *et al*[41] conducted a phase II, randomized, open-label efficacy and safety study with lenvatinib or everolimus alone, or lenvatinib plus everolimus in patients with metastatic or unresectable, locally advanced, clear cell RCC who had received prior treatment with a VEGF-targeted therapy and progressed within 9 mo of drug discontinuation. The primary objective was PFS using investigator-assessed objective responses. Lenvatinib plus everolimus significantly prolonged PFS compared with everolimus alone (14.6 mo *vs* 5.5 mo, $P = 0.0005$), but not compared with lenvatinib alone (7.4 mo, $P = 0.12$). Single agent lenvatinib significantly prolonged PFS compared with everolimus alone ($P = 0.048$). But retrospective independent radiological review of the study did not show any significant difference in PFS between lenvatinib alone *vs* everolimus alone groups ($P = 0.12$). This was attributed to small sample size.

Lenvatinib plus everolimus showed significantly increased median OS of 25.5 mo (95%CI: 20.8-25.5), compared with 18.4 mo (13.3–NE) for single-agent lenvatinib, and 17.5 mo (11.8–NE) for single-agent everolimus. In the post-hoc updated analysis, median OS between patients assigned lenvatinib plus everolimus was significantly improved at 25.5 mo (95%CI: 16.4–NE) compared with single-agent everolimus 15.4 mo (11.8-19.6); HR 0.51, 95%CI: 0.30-0.88; $P = 0.024$. However, OS did not differ between patients who received lenvatinib plus everolimus (HR 0.75, 0.43-1.30; $P = 0.32$), and single-agent lenvatinib [median OS 19.1 mo (95%CI: 13.6-26.2)] or single-agent everolimus (HR 0.68, 95%CI: 0.41-1.14; $P = 0.12$)[41].

The safety profile for lenvatinib plus everolimus was similar to the known toxic effects of each individual agent. Grade 3-4 treatment-emergent adverse event (TEAE), occurred in fewer patients allocated single-agent everolimus (50%) compared with those assigned lenvatinib alone (79%) or lenvatinib plus everolimus (71%). The most common grade 3 or 4 TEAE in patients allocated lenvatinib plus everolimus was diarrhea (20%),in those assigned single-agent lenvatinib it was proteinuria (19%), and in those assigned single-agent everolimus it was anemia (12%). One case of fatal drug-related AE (cerebral hemorrhage) was reported in the lenvatinib plus everolimus group.

Table 4 Approved combination therapies

Combination therapy	FDA approval date	Line of therapy	Trial	Comparator	Efficacy outcomes						Side-effect profile	Comments	Ref.
					OS (exp) (Mo)	OS (contr) (Mo)	PFS (exp) (Mo)	PFS (contr) (Mo)	RR (exp) (%)	RR (contr) (%)			
Bevacizumab + IFN-α	2009	1st	AVOREN	IFN-α	23.3	21.3	10.2	5.4	30.6	12.4		No significant increase in SEs in combination *vs* IFN; OS difference not significant	[35]
Bevacizumab + IFN-α	2009	1st	CALGB	IFN-α	18.3	17.4	8.5	5.2	25.5	13.1		Increased toxicity in combination; No significant increase in OS	[36]
Lenvatinib + Everlimus	2016	2nd		Everolimus	25.5	15.4	14.6	5.5			Fatigue, mucosal inflammation, proteinuria, diarrhea (20%), vomiting, hypertension, and nausea, Grade 3-4 SEs occurred in 71% compared with 50% in everlimus group	Median OS for lenvatinib alone was 18.4 mo	[41]
Nivolumab + Ipilimumab	2018		CheckMate 214	Sunitinib	Not reached	26			42	27	Similar SE profile but discontinuation in 22% *vs* 12% in comparison group		[44]
Pembrolizumab + axitinib	2019	1st	KEYNOTE-426	Sunitinib			15.1	11.1	59.3	35.7	Gr3 or higher adverse event of any cause occurred in 75.8% of patients in the pembrolizumab-axitinib group and in 70.6% in sunitinib group		[45]
Avelumab + axitinib	2019	1st	JAVELIN Renal 101	Sunitinib	ongoing	ongoing	13.8	8.4	51.4	25.7	Grade 3 or higher treatment-elated AEs in the overall population groups, were reported in 71.2% of patients in combination arm *vs* 71.5% in sunitinb arm with discontinuation in 7.6% and 13.4% respectively	Similar responses were observed for PFS and ORR in the PD-L1positive patients	[46]

DFS: Disease-free survival; ORR: Objective response rate; OS: Overall survival; PD-L1: Programmed cell death-ligand 1; PFS: Progression-free survival.

These efficacy results were promising and in May 2016 led to the FDA approval for the treatment of advanced RCC after failure of prior antiangiogenic (TKI) therapy at the lenvatinib dose of 18 mg/daily in combination with everolimus 5 mg/daily.

Nivolumab plus ipilimumab: In April 2018, the FDA approved the combination therapy of ipilimumab and nivolumab for the treatment of intermediate or poor risk advanced RCC. Both of these drugs work to prevent the inactivation of T-cells but *via* different mechanisms, which is why they are effective in combination.

The CheckMate 016 study was an open-label, parallel-cohort, phase 1 study that evaluated the safety and efficacy of nivolumab plus ipilimumab. The study included patients with poor (n = 6), intermediate (n = 47), and favorable risk (n = 47) disease according to the MSKCC risk categorization. Patients in the expansion cohort (intermediate and favorable risk patients) were treatment naïve with the exception of

either prior adjuvant or neoadjuvant therapy or cytokine treatment. Patients were separated into three treatment arms: 3 mg/kg nivolumab plus 1 mg/kg ipilimumab (N3l1), 1 mg/kg nivolumab plus 3 mg/kg ipilimumab (N1l3), 3 mg/kg nivolumab plus 3 mg/kg ipilimumab (N3l3). All the patients in the N3l3 group were censored out of the study because of dose-related toxicities. The N3l1 and N1l3 combination groups had similarly efficacious results (2-year OS was 67.3% and 69.6% respectively) but the N3l1 group had significantly less treatment related adverse events (38.3% *vs* 61.7%)[42].

The CheckMate 214 trial was an open-label phase III study evaluating OS and PFS for the combination of nivolumab plus ipilimumab *vs* sunitinib monotherapy, in previously untreated patients with advanced ccRCC. Patients (n = 1096) were assigned to either the combination group of 3 mg/kg nivolumab with 1 mg/kg ipilimumab or the control group of 50 mg sunitinib. The co-primary end points were OS, PFS, and ORR in the intermediate or poor-risk patients (n = 425/550 patients in combination arm and n = 422/546 patients in sunitinib arm). The median OSwas not reached for the combination group *vs* 26 mo (HR for death, 0.63; P < 0.001) for the sunitinib group. The ORRs were significantly higher with combination therapy than with sunitinib monotherapy (42% *vs* 27%, P < 0.001), and the complete response rate (CRR) was 9% *vs* 1% (P < 0.001). This is the best CR rate any RCC treatment has shown to date, and an updated 30 mo follow-up analysis reported slightly higher CR rate 11%[43].

The 18-mo OS rate in the intermediate or poor-risk patients was 75% (95%CI: 70-78) with combination therapy and 60% with sunitinib (95%CI: 55-65). The median PFS (11.6 mo *vs* 8.4 mo, HR, 0.82, P = 0.03) was not statistically significant. Similar numbers of treatment-related AEs occurred in both the combination and sunitinib groups (93% *vs* 97%) however these AEs led to discontinuation of 22% of the nivolumab plus ipilimumab group *vs* 12% of the sunitinib group. Grade 3 or 4 events occurred in 46% and 63% patients, respectively. The most common types of AEs were fatigue, rash, diarrhea, pyrexia, and arthralgia[44].

The intent-to-treat population in the CheckMate 214 study also included favorable-risk patients (n = 125 in combination arm and n = 124 in sunitinib group). The 18-mo OS in the overall intent-to-treat population favored nivolumab plus iplimumab *vs* sunitinb (78% *vs* 68%), but exploratory analyses of just favorable-risk patients favored sunitinib (88% *vs* 93). The ORR (29% and 52%; P < 0.001) and median PFS (14.3 and 25.1 mo; HR, 2.18; 99.1%CI: 1.29-3.68; P < 0.001) were also lower in the favorable group patients taking nivolimumab plus ipilimumab *vs* sunitinib.

Pembrolizumab plus axitinib: In April 2019, the FDA approved the combination therapy of pembrolizumab plus axitinib for first-line treatment of patients with advanced RCC irrespective of risk category. In the phase 3 KEYNOTE-426 trial, 861 patients with previously untreated ccRCC were randomly assigned to receive axitinib plus pembrolizumab (n = 432) or sunitinib (n = 429) in the first line setting. The IMDC risk factors were favorable for 31.2%, intermediate for 56.2%, and poor for 12.5% patients.. The dual primary end points were PFS and OS and the secondary end point was ORR, both as decided by blinded independent central review. Median PFS was 15.1 (95%CI: 12.6 to 17.7) mo in the axitinib plus pembrolizumab group and 11.1 mo (95%CI: 8.7 to 12.5) (HR = 0.69; 95%CI: 0.57 to 0.84, P < 0.0001) in the sunitinib group. ORR in the axitinib plus pembrolizumab group was 59.3% (95%CI: 54.5 to 63.9) and 35.7% (95%CI: 31.1 to 40.4) in the sunitinib group (P < 0.001). In the axitinib plus pembrolizumab group, the complete response rate was 5.8% (n = 25) *vs* 1.9% (n = 8) in the sunitinib group. After 1 year, 90% of patients were alive in the combination group *vs* 78% in the sunitinib group (HR for death, 0.53; 95%CI: 0.38 to 0.74; P < 0.0001). Grade 3 or higher adverse event of any cause occurred in 75.8% of patients in the pembrolizumab plus axitinib group and in 70.6% in sunitinib group with the most common adverse event of any cause being diarrhea and hypertension[45]. Medication discontinuation due to AEs of any cause and deaths attributed to treatment-related AEs occurred in 30.5% and 4/11 patients, respectively in pembrolizumab plus axitinib group. The corresponding data was 13.9% and 7/15 patients in sunitinib group.

Avelumab plus axitinib: In May 2019, the FDA approved avelumab in combination with axitinib for first-line treatment of patients with advanced RCC irrespective of risk category. In the phase III JAVELIN Renal 101 trial, patients with advanced untreated ccRCC (n = 886) were randomized in a one to one fashion to receive either avelumab plus axitinib (n = 442) or sunitinib (n = 444) as first line therapy. Patients across all MSKCC and IMDC prognostic risk groups were included. The dual primary end points were PFS and OS among patients with PD-L1 positive (> 1%) tumors. Secondary end points were PFS and OS among all patients regardless of PD-L1 expression. These determinations were made by blinded independent central review.

Among patients with PD-L1-positive tumors, median PFS (13.8 mo *vs* 7.2 mo), confirmed ORR (55.2% *vs* 25.5%) and CRR (4.4% *vs* 2.1%) were approximately twice as robust with the avelumab plus axitinib *vs* sunitinib groups, respectively.

Similar responses were observed in the overall population, with PFS (13.8 mo *vs* 8.4 mo), confirmed ORR (51.4% *vs* 25.7%) and CRR (3.4% *vs* 1.8%) in the avelumab plus axitinib *vs* sunitinib groups, respectively.

Grade 3 or higher treatment-related AEs in the overall population were reported in comparable percentage of patients (71.2% *vs* 71.5%) inavelumab plus axitinib *vs* sunitinib groups, respectively. However, discontinuation was higher in the sunitinib group compared to the avelumab plus axitinib group (13.4% *vs* 7.6%, respectively). The most common adverse reactions were diarrhea, fatigue, hypertension, musculoskeletal pain, nausea, mucositis, palmar-plantar erythrodysesthesia, dysphonia, decreased appetite, hypothyroidism, rash, hepatotoxicity, cough, dyspnea, abdominal pain and headache. Of patients treated with combination arm, 38.2% experienced immune-related AEs of which 9% had severity grade 3 or higher, and the most common immune-related AE was hypothyroidism. Serious adverse reactions occurred in 35% of patients receiving combination regimen and the incidence of major adverse cardiovascular events was also higher compared with sunitinib[46].

ONGOING TRIALS AND FUTURE DIRECTIONS

Table 5 summarizes ongoing phase 3 trials of combination therapy in RCC, studies designed in part to further optimize optimal first line regimens. New agents under investigation in the treatment of RCC include NKTR-214 and abexinostat. NKTR-214 is a novel IL2 pathway agonist, designed to provide sustained signaling through heterodimeric IL2 receptor βγ to drive increased proliferation and activation of $CD8^+$T and natural killer cells without unwanted expansion of T regulatory cells in the tumor microenvironment[47]. Abexinostat is a novel histone deacetylase (HDAC) inhibitor. HDAC inhibitors target HDAC enzymes leading to highly acetylated histones and chromatin reshaping. In addition to altering histone acetylation, HDAC inhibitors can also influence the degree of acetylation on non-histone proteins, increasing or repressing their activity. HDAC inhibition thus inhibits the proliferation of cancer cells and induce cancer cell death, or apoptosis. Through the epigenetic modulation of vascular endothelial growth factor expression, it is thought that abexinostat can prolong the therapeutic effect of pazopanib and prevent resistance[48]. In a recently completed Phase Ib/II trial of pembrolizumab with bevacizumab, the combination regimen was found to be safe and effective in the treatment of mRCC[49]. It may be potentially helpful in patients who cannot tolerate TKIs.

Atezolizumab plus bevacizumab

In the phase III IMmotion151 trial, patients (n = 915) were stratified by PD-L1 status (n = 362 PD-L1+), MSKCC risk score[6] and presence of liver metastases. In PD-L1+ patients, PFS was 11.2 mo (95%CI: 8.9 to 15.0) in atezolizumab plus bevacizumab *vs* 7.7 mo (95%CI: 6.8 to 9.7) in sunitinib (P = 0.0217); ORR was 43% (95%CI: 35 to 50) in atezolizumab plus bevacizumab *vs* 35% (95%CI: 28 to 42) in sunitinib. Duration of response was not reached for atezolizumab plus bevacizumab *vs* 12.9 mo for sunitinib treated patients. The combination arm was well tolerated. Treatment-related grade 3-4 AEs were noted in 40% of atezolizumab plus bevacizumab and 54% of sunitinib treated patients; 12% and 8% of treatment-related all-Grade AEs led to discontinuation, respectively[50]. Five treatment–related deaths were recorded in combination group *vs* 1 with sunitinib. Although the PFS benefit was met in the PD-L1+patients as well as in other subgroups and in the intent to treat (ITT) population, the Independent Radiological Review-assessed PFS in PD-L1 patients did not show a statistically significant benefit. Because of this variance, the fate of this combination is uncertain.

TREATMENT SELECTION

Treatment dilemma

There is no single established sequence of systemic therapies in metastatic ccRCC. Treatment choices are based on evidence-based efficacy data, individual patient

Table 5 Ongoing phase 3 trials of combination therapy in renal cell carcinoma

Treatment	Trial name	ClinicalTrials.gov No.	Enrollment	Primary endpoint	Status
Nivolumab-cabozantinib *vs* sunitinib	CheckMate 9ER	NCT03141177	630	PFS	Estimated primary completion date: January 2020
Lenvatinib-everolimus or lenvatinib-pembrolizumab *vs* sunitinib	CLEAR	NCT02811861	1050	PFS	Estimated primary completion date: April 2020
Nivolumab-ipilimumab followed by nivolumab *vs* nivolumab-cabozantinib	NCI-2018-03694	NCT03793166	1046	OS	Estimated primary completion date: September 2021
NKTR-214-nivolumab *vs* sunitinib or cabozantinib	CA045002	NCT03729245	600	ORR	Estimated primary completion date: December 2021
Pazopanib-abexinostat *vs* pazopanib	XYN-602	NCT03592472	413	PFS	Estimated primary completion date: January 2022
Nivolumab-ipilimumab *vs* placebo	CheckMate 914	NCT03138512	800	DFS	Estimated primary completion date: September 2022
Nivolumab-ipilimumab *vs* nivolumab	CA209-8Y8	NCT03873402	418	PFS	Estimated primary completion date: December 2022

DFS: Disease-free survival; ORR: Objective response rate; OS: Overall survival; PD-L1: Programmed cell death-ligand 1; PFS: Progression-free survival.

factors, co-morbidities, and the toxicity profiles of the potential agents.

The National Comprehensive Cancer Network (NCCN) Kidney Cancer Panel has categorized all systemic kidney cancer regimens as "preferred", "other recommended", or "useful under certain circumstances"[51].

The first line therapies are further categorized according to the IMDC[7,52] prognostic model which provides the primary selection criteria. Patients are largely stratified into low- or favorable-risk and intermediate-or poor-risk groups, based on clinical and laboratory risk factors.

First-line therapies: (1) Low-or favorable-risk patients: The NCCN preferred category 1option for low-risk patients is the combination of pembrolizumab plus axitinib, which was recently approved (April 2019) across all risk groups. KEYNOTE 426[45] demonstrated a 47% lower risk of death and a 31% lower risk of disease progression or death on treatment with pembrolizumab plus axitinib compared with sunitinib. The ORR was 23% higher in the combination group than in sunitinib group. The benefits of improved PFS and OS were observed in all subgroups of patients, including across all IMDC risk groups and regardless of PD-L1 expression. The significant improvement in OS is of utmost importance because this has not been achieved before with any single or combination therapy. A head to head trial is needed to compare the combination of pembrolizumab plus axitinib with a newer TKI monotherapy, such as cabozantinib, *vs* other combinations to make further progress in selecting preferred category 1 option in low-risk patients.

The alternative category 1 options for low-risk patients are pazopanib and sunitinib. A phase III non-inferiority direct comparison of pazopanib *vs* sunitinib (COMPARZ study)[53,54] in treatment naïve mRCC patients showed a comparable efficacy profile. The PFS with pazopanib was non-inferior (median 8.4 mo) to sunitinib (median 9.5 mo). The median OS was 28.4 and 29.3 mo respectively. Certain adverse events were more frequent with sunitinib, namely fatigue 63% *vs* 55%, hand-foot syndrome 50% *vs* 29%, and thrombocytopenia 78% *vs* 41%. Although liver function abnormalities (60% with pazopanib *vs* 43% with sunitinib), weight loss and alopecia were noted more with pazopanib, several quality-of-life indicators favored pazopanib[51,53]. This is further supported by phase III crossover study (Pisces study) where significantly more patients preferred pazopanib (70%) over sunitinib (22%) while only 8% had no preference[55]. In a subgroup analysis of COMPARZ trial, safety profile of the two drugs was studied in Asian *vs* non-Asian populations[56]. In general, Asian patients experienced higher incidences of hypertension, hematologic toxicity, hand-foot syndrome, liver chemistry abnormalities with either drug compared to non-Asian patients. On the other hand, non-Asian patients experienced higher incidences of gastrointestinal AEs, mucosal inflammation, and headache. This may reflect ethnic differences in absorption, metabolism, and tolerance of the drugs. Effects of other translational factors related to genetic and non-genetic factors may also be into play

and will require further research.

The other options approved for low-risk group are cabozantinib, nivolumab plus ipilimumab, and axitinb plus avelumab. Cabozantinib use as category 2B is extrapolated from its response in intermediate to poor risk patients. The nivolumab plus ipilumumab combination was FDA approved (CheckMate 214 trial)[44] for intermediate to poor-risk patients. However, it may be used in low-risk patients who cannot receive a TKI, as in severe hepatic impairment, uncontrolled hypertension, or significant cardiovascular disease or in patients with high PD-L1 expression in the tumor cells.

In May 2019 the FDA approved avelumab plus axitinib as part of a combination regimen, regardless of tumor PD-L1 expression. In the JAVELIN Renal 101 study, patients with advanced RCC across IMDC prognostic risk groups (21% favorable, 62% intermediate and 16% poor) demonstrated significantly improved median PFS (13.8 mo *vs* 8.4 mo) and ORR (51.4% *vs* 25.7%) with the combination of avelumab plus axitinib compared with sunitinib. The study is continuing for OS and further data are expected. The grade 3 or higher AEs were similar in the two groups. Hypertension, diarrhea, fatigue, nausea, and palmar-plantar erythrodysesthesia were the most frequent AEs and not significantly different in safety profiles of these drugs used individually or in combination. Axitinib was selected as VEGFR inhibitor in preference to sunitinib, because it has demonstrated longer PFS than sorafenib among patients treated previously with sunitinib, though the benefit was relatively small[57]. Secondly, it reduces the risk of potential hepato-toxicity observed with sunitinib and pazopanib combined with nivolumab, an immune checkpoint inhibitor[58].

Active surveillance may be considered an initial option in patients with slowly progressive, asymptomatic disease given the toxicity and non-curative nature of systemic therapy. In a prospective phase 2 trial, 52 patients with treatment-naive, asymptomatic, mRCC were enrolled and observed until start of systemic therapy, with specific radiologic assessments timed per protocol. Therapy was initiated at the discretion of the treating physician. Median time on surveillance until initiation of systemic therapy was 14.9 mo in the 48 patients analyzed. Higher numbers of IMDC adverse risk factors and metastatic disease sites were associated with a shorter surveillance period, as per multivariate analysis. Twenty-two (46%) patients died during the study period, all from mRCC. However, selection criteria, risk/benefit, and end-point criteria have not been validated[59].

And (2) Intermediate-or poor-risk patients: Nivolumab plus ipilimumab is a preferred category 1 option for patients with intermediate or poor-risk disease, particularly given its significant complete response rate[60]. At 30 mo of follow up of intermediate and poor-risk previously untreated ccRCC patients from the CheckMate 214 trial, OS was 60% *vs* 47%, ORR were 42% *vs* 29%, CRR was 11% *vs* 1%, respectively, between immunotherapy combination and sunitinib groups. The number of deaths were least in younger age group (< 65 year) compared with elderly (75 years), but this was also noted in sunitinib group. The overall safety profile was similar to prior trials of nivolumab plus ipilimumab. The relatively higher discontinuation rate of treatment due to AEs (22% in nivolumab plus ipilimumab *vs* 12% in the sunitinib groups) may be due to inability for dose reduction of the combination *vs* sunitinib. The most common grade 3/4 AEs in the combination group were fatigue (4%) and diarrhea (4%). In the sunitinib arm, the most common grade 3/4 AEs were hypertension (16%), fatigue (9%), and palmar-plantar erythrodysesthesia syndrome (9%). The combination is however, contraindicated in patients with autoimmune or neuromuscular disorders, or receiving immunosuppressive therapies.

The combination of pembrolizumab plus axitinib was recently approved (April 2019) as a preferred category 1 option as well, though indicated across all risk groups.

Cabozantinib is a recommended category 2A preferred first-line treatment for intermediate to poor risk patients based on the CABOSUN study. This study demonstrated a significantly improved investigator assessed median PFS (8.2 mo *vs* 5.6 mo), which was consistent with an independent post-hoc retrospective radiology review committee (IRC) assessment. The ORR per IRC was 20% for cabozantinib *vs* 9% for sunitinib. All responses were partial. The disease control rate (complete responses + partial responses +stable disease) was 75% with cabozantinib and 47% with sunitinib. These results are further significant given the disease burden and poor prognostic features in addition to 81% classified as intermediate risk and 19% as poor risk as per the IMDC criteria. Notably, 25% had no prior nephrectomy and 36% had bone metastases[61].

Further, subgroup analysis of PFS per IRC assessment based on stratification factors and MET expression level were consistent with overall results. The observed improvement in PFS with cabozatinib compared with sunitinib may be due, in part, to

inhibition of MET and AXL by cabozantinib in addition to VEGF receptors. Subgroup analyses of PFS based on MET expression level favored cabozantinib over sunitinib (HR < 1) regardless of MET status. Although the HR more strongly favored cabozantinib for MET-positive *vs* MET-negative tumors, subgroup sizes were small. Grade 3 or 4AEs occurred for 68% cabozantinib-treated patients and 65% sunitinib-treated patients. The most common grade 3 or 4 adverse events in the cabozantinib and sunitinib treatment groups were hypertension (28% *vs* 21%), diarrhea (10% *vs* 11%), fatigue (6% *vs* 17%), palmar-plantar erythrodysesthesia (8% *vs* 4%), and thrombocytopenia (1% *vs* 11%)[62].

Additionally, pazopanib, sunitinib, and avelumab plus axitinib are listed as other recommended option for the first-line treatment of patients with intermediate –poor-risk features.

High dose IL-2 (category 2A), given its significant toxicity profile, is approved as first-line treatment only in a highly selected subgroup of patients for all risk groups. The selection is based largely on assessment of safety *vs* risk factors. Axitinib (category 2B) is used as single agent generally only as a highly advanced line of therapy across all risk groups. Temsirolimus is still included as category 1 first line treatment option in poor-risk patients but must be used only if TKIs and immunotherapy are contraindicated. Sorafenib is excluded given better treatment options.

Subsequent-line therapies: The need for subsequent therapy is currently based on intolerable AEs or progression of disease on first-line therapy. There is uncertainty yet, regarding the optimal duration of first-line therapy for patients who respond to treatment, particularly IOs, and do not experience significant adverse events. Induction of resistance remains a concern and indices for optimizing therapy duration will need to be ascertained as more prospective data becomes available.

In patients with progression after previous TKI or immunotherapy, cabozanitinb is the current preferred NCCN category 1 choice[51,63,64]. As demonstrated in the METEOR trial, cabozanitib was found superior to everolimus in patients who progressed on anti VEGFR therapy, with a significantly improved median OS (21.4 mo *vs* 16.5 mo) and ORR (17% *vs* 3%). The most common treatment-related grade 3 or 4 AEs with cabozantinib were hypertension, diarrhea, and fatigue and those with everolimus were anemia, fatigue, and hyperglycemia. The rate of treatment discontinuation due to AEs was similar in both arms. Cabozantinib is particularly recommended in patients with bone metastasis. In a subgroup of patients with bone metastases in the METEOR trial, median PFS (7.4 mo *vs* 2.7 mo), OS (20.1 mo *vs* 12.1 mo), and ORR (17% *vs* 0%) were all improved for patients treated with cabozantinib *vs* everolimus[61]. In a meta-analysis comparing cabozanitinb with everolimus, nivolumab, axitinib, sorafenib, or best supportive care, cabozanitinb appeared to show a longer PFS as a second line treatment choice[65].

Nivolumab is another preferred category 1 option. It was found to be superior to everolimus in patients who progressed on prior antiangiogenic therapy (excluding mTOR) in a phase III trial (CheckMate 025) with a median OS 5.4 mo longer in comparison.The ORR was also 5 times greater with nivolumab compared to everolimus. Treatment related AEs of any grade were reported in 79% with nivolumab, in 88% with everolimus and grade 3-4 AEs were noted in 19% and 37% respectively. Treatment discontinuation from toxicities was seen in 8% with no treatment-related deaths in nivolumab patients. Corrresponding numbers were 13% and 2 deaths respectively in everolimus patients[66,67]. The effect of nivolumab continuation was evaluated after first Response Evaluation Criteria in Solid Tumors (RECIST) disease progression in CheckMate 025 trial patientswho showed clinical benefit and tolerated the therapy. A reduction in tumor burden was seen in approximately50% patients of which 13% of patients had a ≥ 30% tumor burden reduction[51]. AEs of any grade were reported less frequently after progression (59%) than before progression (71%)[68].

Lenvatinib, a multi-targeted TKI plus everolimus, an mTOR inhibitor, is another category 1 combination approved for subsequent therapy. In a phase II trial, patients with advanced RCC, previously treated with antiangiogenic therapy were randomized to receive the combination of lenvatinib plus everolimus *vs* everolimus alone *vs* lenvatinib alone. The median PFS (14.6 mo *vs* 5.5 mo; HR 0.40; 95%CI: 0.24-0.68) and OS (25.5 mo *vs* 15.4 mo; HR 0.67; 95%CI: 0.42-1.08) were significantly improved for the combination compared to everolimus alone[41,69].

Nivolumab plus ipilimumab is preferred as category 2A in patients who have progressed on one prior systemic therapy. Several other regimes may be recommended in appropriate settings as indicated in NCCN guidelines[51]. Although, single agent everolimus is not used as first or second line therapy, it may be worth

considering it in patients with mutation in mTOR pathway but future studies directed at this strategy are required[70,71]. However, determining the ideal combination of therapies and the sequence in which they can be used remains an area for exploration.

Biomarkers

Development of a sensitive biomarker would help to formulate an efficacious therapeutic course, and to prognosticate outcomes[72]. While prognostic biomarkers play a role in forecasting patient outcomes, predictive biomarkers identify the best treatment options with the fewest adverse effects and toxicities. Given that many ccRCC cases are diagnosed in the advanced or metastatic stage, development of validated and reliable biomarkers is a crucial goal. To date, perhaps, the IMDC model remains the single most validated clinical prognostic model in mRCC. It is used for patient counseling, risk stratification in clinical trials, and treatment selection. Although several biomarkers have been the focus of recent research, no single other biomarker has been validated for use in ccRCC[73]. Therefore, several biomarkers are used in combination to generate a patient tailored approach.

PD-L1 expression continues to be a potential biomarker of clinical interest[74]. However, in the CheckMate 025, a phase II trial, a positive response was observed with nivolumab irrespective of PD-L1 expression. This was postulated to be related to variation in histologic subclasses. In CheckMate 214, phase II study of nivolumab plus ipilimumab *vs* sunitinib, a longer median PFS was observed in nivolumab plus ipilimumab treated subjects with 1% or greater PD-L1 expression (22.8 mo *vs* 5.9 mo) but not in those with less than 1% PD-L1 expression (11 mo *vs* 10.4 mo). Similar result was observed among patients with ≥ 5% or < 5% PD-L1 expression. A higher ORR was observed with nivolumab plus ipilimumab across all patient groups *vs* sunitinib but the response was more robust in patients with 1% or greater PD-L1 expression (58% with nivolumab plus ipilimumab *vs* 22% with sunitinib) compared with those with less than 1% PD-L1 expression (37% *vs* 28%)[66,74]. In IMmotion 151, phase III study, with atezolizumab (PD-L1 inhibitor) plus bevacizumab (VEGF inhibitor) *vs* sunitinib, patients were stratified by their PD-L1 expression (< than 1% *vs* ≥ 1% expression). Patients with clear cell as well as sarcomatoid histology were included. The two treatment arms were PD-L1 ≥ 1% and the entire ITT population. A higher PFS was noted in both groups compared with sunitinib. The response was higher in PD-L1 positive patients (but the difference was small). Higher PFS was observed in patients with sarcomatoid histology. The role of PD-L1 expression, although limited as a prognostic biomarker, continues to be explored as a predictive biomarker[75,76].

In addition to the varying levels of PD-L1 expression in tumor cells, recent advances in genetic and genomic studies have shown significant inter-tumor and intra-tumor genomic heterogeneity of ccRCC. Of these, mutation in the *VHL* gene, located on 3p25, is the fundamental event and most researched but it is not the single driver gene. Several other tumor suppressor genes are now identified, importantly *PBRM1* (40%), *SETD2* (15%), and *BAP1* (10%) *KDM5C* (7%), and *TP53* (5%) and the oncogene *MTOR* (5%-6%)[77]. *PBRM1*, *SETD2*, and *BAP1* are all located on 3p21 and encode for tumor suppressor chromatin-and histone-modifying proteins and their mutations are associated with more aggressive clinical features for all stages of ccRCC[78,79].

SETD2 mutations are associated with advanced stage, grade and worse cancer specific survival. An overall metastatic rate of 36% is reported in *SETD2* mutated ccRCC tumors, suggesting a link between *SETD2* and cancer metastasis[80,81]. However, *SETD2* loss is not yet correlated with poor targeted treatment outcomes[82,83]. This needs further validation and additional studies evaluating response of targeted therapies.

BAP1 mutations are prevalent in about 10% of human ccRCC cases, and loss of *BAP1* function is associated with tumors of high grade, worse cancer specific survival[80] as well as overall poor clinical response despite targeted therapy[83,84]. As such, *BAP1* regulated pathways are an appropriate future therapeutic target. The relatively inferior OS noted with *BAP1* mutations in comparisons with *SETD2* and *KDM5C* mutations by Tennenbaum *et al*[83] needs further research and confirmation. *BAP1* and *PBRM1* mutations are usually mutually exclusive. Their simultaneous occurrence, which is observed rarely are associated with more aggressive disease.

Two distinct subtypes and prognostic features (ccA/ccB) are defined by molecular stratification of ccRCC using consensus clustering[85]. The ccB classified tumors demonstrated increased tumor size, grade and rate of metastasis as well as decreased recurrence free survival and OS[86]. ClearCode 34 is a genetic signature developed from this classification to predict recurrence[87]. This tool is validated despite limitation from tumor heterogeneity, making it a potentially valuable prognostic biomarker.

Another prognostic multigene signature has been proposed using a 16-gene assay to predict recurrence after nephrectomy in localized RCC[88]. The recurrence score was

validated as a predictor of outcome in patients with stage I-III ccRCC. A signature of four specific genomic aberrations using FISH) was developed which can identify tumors with a high metastatic potential, and may be a better predictor of OS, CSS CSM and PFS, compared with clinico-pathologic variables[89].

Although c-Met overexpression has been observed and correlated with significantly worse pathological features in RCC, its clinicopathological impacts remain uncertain[90]. OS, PFS, and ORR were improved with cabozantinib *vs* sunitinib in patients with advanced RCC[62], but the benefit was noted regardless of tumor expression levels of MET in the METEOR study[63]. Thus its role as a biomarker appears to have limitations.

The role of pathogenic variants in genes associated with DNA damage repair (DDR), frequently encountered in mRCC patients, was evaluated. Presence of a deleterious DDR gene alteration was associated with improved survival in patients treated with IO (HR 0.29, $P = 0.04$) but not in those treated with TKI. However, DDR alterations were not associated with improved PFS in either group. Despite limitations of the study, it requires validation and can provide another path forward in treatment selection[91].

Given the relation between hypercalcemia and poor prognosis in ccRCC patients (IMDC), investigators have recently studied the prognostic role of calcium-sensing genes on plasma membrane. In one study, higher levels of DYSF (Dysferlin) were found in ccRCC cells compared with normal kidney cells and this, within ccRCC patients, was a predictor of improved prognosis[92]. It is postulated that DYSF may act as a metastasis suppressive gene and perhaps be a promising prognostic tool in ccRCC patients, but replication of data is required by future studies.

Recently, use of plasma and urine nucleic acids as biomarkers in ccRCC also have been a focus of investigations and need reproduction and validation[93].

DISCUSSION

The landscape of first-line therapy for advanced RCC is evolving very rapidly with recent FDA approvals of ICI in combination with another ICI or with an anti-VEGFR TKI. Combination therapies, as outlined below, are the current standard of care in the management of RCC.

For patients with favorable risk disease, the preferred current choice of treatment is combination of pembrolizumab (ICI) plus axitinib (TKI). KEYNOTE-426 trial[45] showed improved OS, ORR and PFS with combination of pembrolizumab plus axitinib compared to sunitinib (TKI). Although this benefit was noted across all risk groups and independent of PD-L1 expression, the choice of therapy is less clear in the intermediate to poor risk patients, where another effective option is available.

For patients with intermediate to poor risk disease, the preferred current choice is nivolumab plus ipilimumab (ICI + ICI). In the CheckMate 214 trial[44], CRR was 9% with nivolumab plus ipilimumab, the best so far, compared to that of 1% in control arm with sunitinib. The updated 30 mo follow-up analysis reported an even higher CR rate of 11%. The OS and ORR were also significantly better in the combination arm. Comparing the combinations of pembrolizumab plus axitinib with that of nivolumab plus ipilimumab, both tested against sunitinib, although the ORR was higher (59% *vs* 42%) in the former combination, it was the CRR of 9% (and 11% on 30 mo follow-up) in the nivolumab plus ipilimumab combination compared to 5.8% in pembrolizumab plus axitinib, that makes it one of the preferred choice in patients with intermediate to poor risk RCC. In terms of AEs also, nivolumab plus ipilimumab combination appeared to be tolerated better with Grade 3 or 4 AEs encountered in 46% of patients compared to grade 3 or higher AEs in 76% patients who received pembrolizumab plus axitinib. In contrast, combination of nivolumab plus ipilimumab did not hold up in favorable risk patients, in whom ORR and PFS favored sunitinib over combination of nivolumab plus ipilimumab. However, OS data from long term follow up are still awaited.

Two VEGFR agents (pazopanib and sunitinib) are also recommended options as first-line therapy in favorable-risk patients with advanced RCC who cannot receive ICIs (pembrolizumab plus axitinib). Pazopanib was non-inferior to sunitinib in the COMPARZ study[53] with several quality of life indicators and AEs profile favoring pazopanib. The VEGFR alternative to ICI therapy in patients with intermediate to poor risk is cabozantinib. Cabozantinb (CABOSUN study)[62] had significantly improved ORR and PFS in comparison with sunitinib but all responses were partial. Grade 3 or 4 AEs occurred in a comparable percentage of patients in the two groups. Cabozantinib

is particularly recommended in patients with bone metastasis. Of new interest is the combination of avelumab (ICI) plus axitinib (TKI), approved by the FDA in May 2019 (JAVELIN Renal 101 trial) for first-line treatment of patients in advanced RCC across all risk groups. PFS and OR benefit were observed irrespective of PD-L1 expression but results of OS are awaited.

The consensus on second-line treatment is still controversial. The general understanding to date is that for patients who progress on immunotherapy, VEGFR targeted therapy is recommended. For patients who progress on initial VEGFR targeted therapy, either single agent nivolumab or the combination ICI regimen nivolumab plus ipilimumab is recommended. If ICI therapy is unavailable or not advisable, other VEGFR agents can be tried. For patients who progress on VEGFR-agent plus ICI, the choices are combination of nivolumab plus ipilimumab or a different VEGFR-agent. Large retrospective and prospective studies are mandated to further analyze the differential benefit/risk ratios of the different available options. Although the choice of a specific therapeutic agent remains controversial, the current trend is discussed below.

For patients treated previously with IO, cabozantinib may be the preferred agent for subsequent therapy. Cabozantinib showed significantly improved PFS and OS compared to everolimus in the METEOR trial. It is particularly beneficial in patients with bone metastasis. In a retrospective analysis of 69 patients with progression on IO alone, or in combination with VEGFR agent or others, the one-year OS was 53% with cabozantinib as a subsequent agent[94]. The appropriate drug holiday before starting a TKI after progression on ICI is undecided. Although an overlap may potentially improve efficacy, it must be remembered that ICIs have long half-lives and can contribute to both, continued response as well as AEs long after discontinuation[68,95]. There is also very limited data evaluating the safety and efficacy of VEGFR agents following progression on IOs[96,97] and larger future studies are awaited. It is imperative to note here that AEs from IO agents can be severe, can affect any organ system, and can be life-threatening. Grade 2 toxicities can be managed by treatment interruption and supportive care but grade 3 or higher toxicities may require high-dose glucocorticosteroids over a prolonged time period. Close monitoring is therefore required for all patients on IO agents.

For patients who progressed on prior VEGFR-agent (but not mTOR inhibitor), nivolumab was found superior to everolimus (CheckMate 025 trial) in ORR and OS benefit. Additionally, nivolumab treatment continuation beyond first progression was noted to have benefit in a subset of patients[68]. Based on the initial success of nivolumab plus ipilimumab (CheckMate 016 trial)[98] which was later confirmed in further study (CheckMate 214 trial), this is a leading alternative.

Other recommended regimens and potential drug choices under specific conditions are as listed in the NCCN guidelines[51]. Ultimately the choice of therapy is a multifactorial decision, depending not only on patient clinical factors but also on other external variables such as cost and availability, practice setting and treatment experience of health care provider. Many questions still remain challenging and unanswered. Several promising drug trials are underway and we expect slow but steady evolutions to treatment regimes. Ultimately, the discovery of sensitive predictive and prognostic biomarkers, or more likely a combination of biomarkers, will define the therapeutic success in treating patients with RCC.

CONCLUSION

The emergence of ICIs and combination therapies has revolutionized the treatment of ccRCC. Significant improvement in efficacy profiles have been appreciated. The best preferred combination regimen and sequencing of treatments will continue to evolve as newer therapeutic agents get FDA approved. These will have to be tried and judged in the balance of AE profiles. Would there be place for a triple drug combination instead of dual drug combination treatment without further adding to the burden of adverse events? In the face of this changing horizon, need for a reliable and validated biomarker(s) is both an increasingly pressing need and a challenge.

Biomarkers can guide in initial treatment selection as well as in sequencing treatments and follow-ups. The IMDC risk model is currently the only validated biomarker based on clinical data and laboratory tests, which classifies metastatic ccRCC patients as having favorable, intermediate/poor prognostic status and accordingly defines their treatment options as first-, second-, or third- line therapies. However, risk stratification based initially on the TNM staging system[5] and later

modified by IMDC classification[7] does not address the critical factor of genetic heterogeneity, differential metastatic potentials, or aggressive subtypes. In view of the high intertumoral and intratumoral heterogeneity, multiple genetic and molecular biomarkers may be required to identify specific responsible genes and the genetic/molecular pathways that are activated in aggressive tumors. The future generation of preferred therapeutic options for ccRCC should molecularly target the most common and aggressive pathways affected by different mutations. Further, prospective clinical trials are required to evaluate the clinical utility of suggested genetic and molecular signatures. Ultimately, the biomarkers may allow treatment to be personally tailored to the needs of each patient, enabling patients to get maximal potential benefit while minimizing unnecessary risks by avoiding regimens with limited efficacy.

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REVIEW

Circular RNA and its potential as prostate cancer biomarkers

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Abstract

Advancing knowledge of the transcriptome has revealed that circular RNAs (circRNAs) are widely expressed and evolutionarily conserved molecules that may serve relevant biological roles. More interesting is the accumulating evidence which demonstrates the implication of circRNAs in diseases, especially cancers. This revelation has helped to form the rationale for many studies exploring their utility as clinical biomarkers. CircRNAs are highly stable due to their unique structures, exhibit some tissue specificity, and are enriched in exosomes, which facilitate their detection in a range of body fluids. These properties make circRNAs ideal candidates for biomarker development in many diseases. This review will outline the discovery, biogenesis, and proposed functions of circRNAs.

Key words: Circular RNA; RNA splicing; Prostate cancer; Biomarker; Cell-free RNA

Core tip: Circular RNAs are unusually stable RNA molecules that are tissue- and cell lineage-specific, abundantly expressed in cells, and enriched in exosomes. These properties facilitate their detection in different body fluids and probable utility as biomarkers. Herein, we review the literature that investigates their potential as biomarkers for prostate cancer.

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INTRODUCTION

Whilst they are amongst the last addition to the RNA family, circular RNAs (circRNAs) are not new discoveries[1]. Circular transcripts were originally found to naturally exist in plant viroids in 1976[2] and in the hepatitis delta virus in 1986[3]. They were noted as endogenous molecules in eukaryotes by a study investigating splicing in the *DCC* gene[4]. In this study, splicing was observed to occur in a non-sequential fashion by means of "exon scrambling"; upstream exons moved downstream to bind exons and yielded circular transcripts[4]. Because their exons are inverted compared to the exonic arrangement on the genomic open reading frame, circRNAs were initially labeled as by-products of splicing error[5]. This narrative began to change upon discovery that the *Syr* gene in adult mice was only expressed as 1.23-kb circular transcripts[6]. Given the importance of this gene in sex determination during embryogenesis, it inferred possible pre-determined biological of circRNAs, albeit being grouped as non-coding RNAs (ncRNAs) at this time[7]. However, renewed interest in circRNAs occurred when Salzman *et al*[8] identified a myriad of circRNAs in a variety of normal, and malignant cell types. Additionally, the functional exploration of CDR1as revealed its ability to sponge miR-7 in neuronal tissue, inferring that miRNA sponging may be a function of other circRNAs as well[9]. Consequently, interest in the mechanistic machinery that drives the genesis of circRNAs, as well as their function has intensified over the last few years.

CIRCULAR RNA BIOGENESIS

The combinatorial model best explains the alternative splicing (AS) mechanism that facilitates exon skipping. In this model, splicing regulatory factors coordinate the splicing order to determine which exons are included in the final mRNA transcript[10]. The outcome is multiple isoforms of a protein with different functions[11]. AS not only coordinates diversity amongst the linear transcriptome, it also facilitates a diverse group of circRNAs formed *via* backsplicing[12]. In the backsplicing process, circular transcripts are generated through covalently fusing the 5′ site of an upstream exon (acceptor) with the 3′ end of the same, or a downstream exon (donor)[5,13,14] (Figure 1A). The diversity amongst circRNAs was evidenced with multiple genes in a recent study- a salient example was the *BIRC6* gene which was shown to generate over 500 circular isoforms[15]. Unsurprisingly, the study also highlighted that diversity amongst circular isoforms was directly proportional to exon counts in the gene[15].

Interestingly, backsplicing is flanked by the canonical splicing motif, AG-GT[15] and the circular RNAs and their relative linear RNAs share canonical splice sites suggesting that they are both generated by the same spliceosome machinery[16]. One study demonstrated that introducing mutations into the canonical splice sites significantly decreased circRNA production[16]. This study, as well as others[17] have also projected that circular and linear RNAs are competitively generated by the same spliceosome.

Liang *et al*[18] indicate that circRNAs are seldomly formed from the first or last exons as these exons lack splicing binding sites. Moreover, the number of exons in a single circRNA usually ranges between one and five exons, with several sources reporting that circRNAs with two to three exons are most prevalent[4,5,8,12]. Nonetheless, exons are not exclusive components of circRNAs; circularization of introns, long non-coding RNAs (lncRNAs), antisense transcripts, and intergenic regions is also possible[8,19]. Fascinatingly, there are multiple pieces of evidence of circRNAs consisting of both exonic and intronic regions[5,8,20,21], but exonic circRNAs are still most prevalent and studied[12,20]. Interestingly, Vo *et al*[15], mentioned a new subset of circRNAs generated from exons provided by adjacent genes on the same strand called read-through circRNAs (rt-circRNAs). The specific mechanisms of backsplicing are intricate and are still being investigated as bioinformatics of circRNA mapping improves. However, the following models are recurrently proposed to facilitate backsplicing: Exon skipping model (Lariat model), Intron-pairing, and the RNA-binding protein (RBP) models.

Exon skipping model (Lariat model)

In the exon skipping model, canonical splicing occurs first, producing the mRNA transcript, and an intermediate lariat consisting of introns and skipped exons[1,5] (Figure 1B). The intermediate lariat is unstable and undergoes further splicing (intra-lariat splicing) in which circRNA(s) are produced *via* backsplicing, and the intron lariat forms a separate RNA strand[1,5,20]. However, backsplicing *via* exon skipping can also

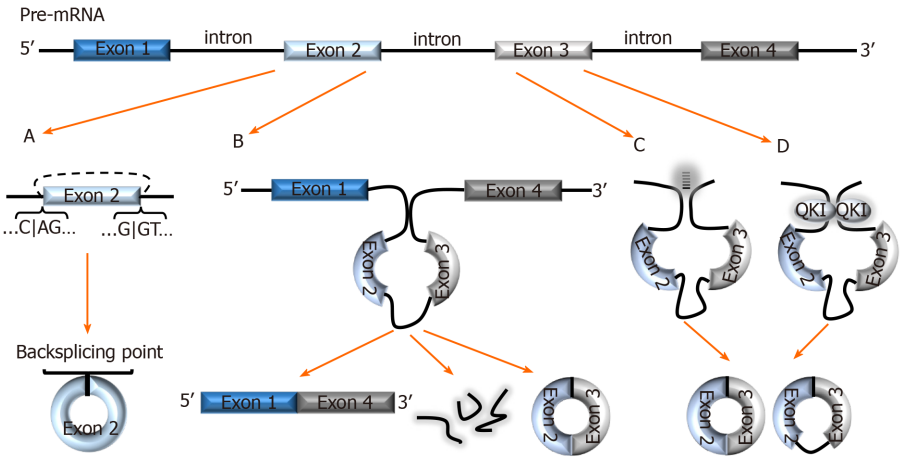

Figure 1 Biogenesis of circRNAs. A: In backsplicing, circRNAs are usually flanked by the canonical splicing motifs, AG-GT, and covalently fuse the 5' site of an upstream exon (acceptor) with the 3' end of a downstream exon (donor); B: In the exon skipping model, an unstable intermediate lariat consisting of introns and skipped exons are generated after splicing. The intermediate lariat is then spliced to produce circRNA; C: Flanking introns containing complementary sequences (Alu repeats) bind and increase the possibility of backsplicing; D: RNA-binding proteins, such as Quaking can bind to flanking introns and dimerize to create a closed RNA loop which facilitates backsplicing. QKI: Quaking.

occur independent of lariat formation by means of direct backsplicing[5].

Intron-pairing

A common feature amongst circularized exons is the presence of long flanking introns containing complementary sequences (Alu repeats)[20] (Figure 1C). This characteristic makes it possible to predict the backsplicing sites of circularization using bioinformatics. Hybridization of these complementary sequences increases the proximity of exonic backsplicing sites and facilitates backsplicing of said sites[18,20]. In this model, the circRNA generation is prioritized over linear transcripts, unlike in the exon skipping model[5,20]. Thus further suggesting that circRNAs are purposely produced, and according to Eger *et al*[5], explain the higher expression of certain circRNAs for some genes over linear transcripts. Interestingly, multiple studies propose that flanking intronic sequences represented in this model can be considered modulators in circularization efficiency[16,20,22]. Zhang *et al*[21] calls this model of backsplicing "alternative circularization", and adds that alternative circularization in concert with alternative splicing, also enhances exonic circularization diversity from a single gene.

RBPs-mediated backsplicing

Multiple studies have demonstrated RBPs-mediated exon circularization with RBPs such as Quaking (QKI) and Muscleblind protein (MBL)[16,23]. In this model, RBPs bind to flanking introns (near to splicing sites) and dimerize to create a closed RNA loop that facilitates backsplicing[23,24] (Figure 1D). Conn *et al*[23] showed that inserting synthetic QKI into intron sites significantly induced circRNA formation and confirmed QKI-directed biosynthesis of circRNA. Similarly, in a prior study, circMbl formation was significantly increased after cells were transfected with MBL variants. This finding was accompanied by a reduction in linear Mbl generation[16]. Altogether, these results not only demonstrated RBP-regulated circRNA generation but also demonstrated the role of RBPs in competitive splicing to generate circular versus linear mRNAs.

CIRCULAR RNA FUNCTIONS

Though there are several pieces of evidence supporting functions such as miRNA sponging in molecules like CDR1as, substantial investigation of general functionality have only been demonstrated in a handful of circRNAs. Herein, we highlight three proposed functions of circRNAs that have been investigated: MiRNA sponging, protein binding, and cap-independent translation. However, whether these functions are generally exhibited by all or most circRNAs is not known.

CircRNAs are miRNA sponges and intermediate miRNA reservoirs

Perhaps the most examined function of circRNAs is their ability to sponge miRNAs. Some circRNAs harbor microRNA response elements (MREs) which facilitate the competitive binding of miRNAs[25,26]. The sequestration of miRNAs by circRNAs modifies their activity in regards to mRNA target gene expression[1,25]. In essence, circRNAs are indirectly involved in mRNA gene expression through miRNA sponging. For example, CDR1as contains over 70 conserved binding sites for miR-7[9,25,27], and the binding capacity is 10 times higher than that of any other transcript or mRNA target[27]. Hansen and colleagues further add that the competition between miR-7 targets and CDR1as creates a buffer effect that prevents transient fluctuations in miR-7 expression[28]. Furthermore, cleavage of CDR1as-miR-7 by argonaute 2 (AGO2) results in the release of miR-7 and the subsequent inhibition of miR-7 targets[25,28,29]. As such, CDR1as functions not only as a miRNA sponge but also as an intermediate reservoir for miR-7[29].

Protein binding

Some circRNAs can competitively bind RBPs as well as store, sort, and sequester proteins in the cytoplasm to limit nuclear entry, regulate their function, and act as scaffolds for protein-protein interactions[30,31]. For example, CircFOXO3 binds and prevents the interaction of p21 and CKD1 to suppress cell cycle progression at the G1 stage in a non-tumor cell line[32], and scaffolds p53 and Mdm2 in breast cancer cell lines to promote Mdm2-induced p53 degradation[33]. The interaction between circMbl and MBL is interesting as MBL can prioritize the generation of circMbl over linear forms, which in turn regulates MBL levels by sponging[16].

CircRNAs mediated protein translation in a cap-independent manner

The predominant opinion on circRNAs is that they are ncRNAs that do not translate proteins. However, the advent of engineered circRNAs that translate protein[20] fostered questions as to whether protein-coding endogenous eukaryotic circRNAs exist. Whilst the predominant stance still aligns with the former view, it has since come to light that there is a minute proportion (< 1%) of circRNAs that contain the start AUG codon, and are able to associate with ribosomes. Amongst them is circZNF609, which consists of a start and stop codon similar to those in the linear transcript. In their study, Legnini *et al*[34] were able to identify circ-ZNF609 as eukaryotic circRNAs that associate with polysomes, and are protein-coding. In circular transcripts like circ-ZNF609, the 5'untranslated regions (5'UTR) are included in the circular sequence during circularization. The 5'UTRs undergo folding to form internal ribosomal entry sites (IRES) which facilitate ribosomal association[34]. Some circRNAs such as circ-FBXW7 are also able to translate protein by other mechanisms such as N6-adenosine methylation[12,29]. Considering that most circRNAs are less abundant than their linear counterparts, it is unsurprising that the aforementioned examples of protein-coding circRNAs are less efficient in this activity than linear transcripts. Accumulating evidence also suggests that cap-independent translation is a cellular stress response to generate immediate and selective changes in protein levels[34].

THE POTENTIAL OF CIRCULAR RNA AS BIOMARKERS

Abundance

CircRNAs represent approximately 10% of the total RNA content in cells[35], with some being more abundantly expressed than their linear isoforms[8,36]. Their global expression and abundance can be stage-or-age dependent[37] as evidenced by several studies demonstrating variation in circRNA expressions at different developmental stages. Two studies reported the induction of circRNA expression during embryonic development in humans and flies across a range of tissues[38,39]. For example, the circular RNA generated from the *NCX1* gene (primarily expressed in cardiomyocytes) was most highly expressed during fetal development according to Szabo *et al*[38]. In the mouse brain, one study demonstrated that certain circRNAs were more expressed in aged mice versus mice half their age[40] suggesting a function in neuronal maturity; another study described circRNA abundance at different stages of hippocampus development in the brain[41]. Interestingly, circRNA abundance can be independent of linear RNA expression[42] indicating splicing preference for generating certain circRNAs at different biological stages and suggesting an overall function in development.

Tissue- and cell lineage-specificity

The expression of some circRNAs is cell and tissue-dependent[17,42,43] which suggests they can be used as molecular markers for different diseases. For example, the expression levels of circular isoforms of the *DCC* gene varied across human tissues and did not correlate with their linear counterparts[4]. Similarly, certain circRNAs are concentrated in different parts of mammalian brains, and also had varying ratios of circRNAs versus linear RNAs[17]. In mice, the circular forms of Rmst and Khl12 were highly expressed in the brains versus the liver and lungs[41]. These studies suggest that circRNA generation and subsequent expression is a widely regulated process. Furthermore, this regulation appears to be evolutionarily conserved across mammals, having had several studies document the conservation between mouse, pigs, flies, and humans in brain tissues[1,17,20,42].

Stability

Unlike linear transcripts, circRNAs are covalently closed loops that lack polyadenylated tails[8,20]. Hence, circRNAs are relatively more stable, and have increased protection from exonuclease degradation[8,20]. Considering that exonucleases, and not endonucleases are the predominant nucleases in host RNA cells[44], it is inferred that the accumulation and detection of circRNAs is favored over the linear transcripts. Though RNA circularization generally increases stability of RNA molecules, hepatitis delta virus (HDV) circular RNAs become more susceptible to degradation by nucleases as they increase in molecular size. However, there is evidence suggesting that these larger HDV circles can be stabilized by their interactions with RBPs such as Ag-S[45].

Unsurprisingly, most circRNAs also have a half-life that is approximately 2.5 times longer than their linear counterparts in mammalian cells[20,25]. Due to their relative stability, circRNAs can also be detected at higher levels (approximately 6.3 folds higher) in exosomes than in cells[46]. This is an important property which contributes to their detection in body fluids.

Exosome enrichment and detection in body fluids

CircRNAs are more enriched in exosomes compared to intracellular levels[30,46]. Exosomes are vesicles that facilitate cell-to-cell communication between parent and recipient cells[27]. CircRNAs are sorted into exosomes potentially as a response to stimuli or physiological needs[27]. Though the precise mechanism is largely unclear, the sorting of circRNAs into exosomes is considered to be a regulated and selective process and can be guided by different factors such as RBPs and miRNA abundance[30,46]. Because of their enrichment and stability in exosomes, circRNAs are detectable in a range of body fluids including saliva[47], plasma[48], urine[49], gastric fluid[50], and supports their consideration as minimally-invasive biomarkers. One study shows that a group of exosomal-circRNAs (exo-circRNAs) in serum could distinguish between colon cancer patients and healthy controls[46]. Another study demonstrated that circRNA-IARS in exosomes could be a potential early diagnostic and prognostic predictor of pancreatic ductal adenocarcinoma (PDAC)[51]. These two studies demonstrate the translational potential of exo-circRNAs as circulating clinical biomarkers.

Genomic information

Unlike protein biomarkers, circRNAs are transcriptomic molecules that entail nucleic acid sequences. These sequences could potentially convey genomic information pertaining to germline mutations, as well as therapy-related somatic mutations which may inform disease prognosis and facilitate therapy decision[52]. Although cell-free tumor DNA can also provide similar information, it reflects the tumor cell genome and is passively released from dead tumor cells. In contrast, circRNAs are gene transcripts and can be both passively and selectively released from tumor cells in exosomes. Therefore, circRNAs could be more effective early indicators of disease.

CIRCULAR RNA IN PROSTATE CANCER

Current biomarkers in prostate cancer

Prostate cancer (PCa) is one of the most common cancers amongst men worldwide[53,54]. Like many other cancers, PCa management is plagued with the possibility of metastasis, therapy resistance, and poor diagnostic and prognostic

biomarkers for screening[54]. Despite the emergence of a plethora of potential prostate cancer biomarkers, the prostate-specific antigen (PSA) still remains the best tool to general screening, and monitoring post-treatment[54]. Still, PSA testing is not without its shortcomings and controversies. Whilst it is prostate-specific, the PSA is not PCa specific, and its level in the blood can be affected by other factors such as age, trauma, inflammation, benign prostatic hypertrophy (BPH), *etc*[55]. Moreover, the established normal range of PSA (< 4.0 ng/mL) insufficiently captures PCa cases and often lead to under-diagnoses and false-positives[56,57]. Reports show that only 25%-30% of elevated PSA within the grey zone (4.0-9.9 ng/mL) cases are confirmed with PCa when biopsied[57,58]. From their study, Thompson *et al*[57] showed that normal PSA is also possible in men with PCa and high Gleason grade- this was observed in 15% of their study participants with normal PSAs.

The limitation of PSA also lies in deciding which cases move forward with biopsy for pathological diagnosis of PCa, which has been the blame for hundreds of thousands of unnecessary prostate biopsies in the United States yearly[59]. Serum levels of other PSA isoforms (*e.g.* p2PSA) show improved specificity to the PSA blood test[55]. Other potential biomarkers such as the prostate cancer antigen 3 (PCA3) score has shown utility in PCa diagnosis and monitoring[60]. PCA3 is a long non-coding RNA that is highly expressed in PCa (primary and metastatic cases)[60]. Whilst possessing a higher specificity than serum PSA, PCA3 score has variable sensitivity and requires a digital rectal examination to collect the specimen, which limits its clinical usage[61]. As evidenced by one study, using PCa-specific circRNAs (circ_0057558 and circ_0062019) from tissues and PSA levels together could offer a diagnostic advantage over just the PSA test[62]. In this study, the combination increased the AUC, specificity, and sensitivity for distinguishing between BPH and PCa[62]. However, reliable, and minimally-invasive PCa clinical biomarkers that can provide diagnostic and prognostic information solely, or in supplementation to the PSA test is still lacking.

CircRNAs as potential biomarkers of prostate cancer

The advancement of transcriptomic profiling has revealed a plethora of circRNAs worthy of further investigations for PCa biomarker development[15,36,63,64]. Chen and colleagues identified a group of circRNAs that are able to distinguish between localized PCa and normal prostate[36]. This study also proposed that circRNA abundance may not only be tissue-dependent but also based on functional roles in the tumor such as cell proliferation[36]. The functional analyses conducted in this study have strengthened the consideration of circRNAs as PCa biomarkers.

Along with establishing the MiOncoCirc catalog of circRNAs, Vo and colleagues identified a subset of circRNAs able to distinguish between PCa subtypes using tissue biopsies[36]. From this subset, circAMACR was upregulated and associated with androgen receptor (AR) amplification in castration-resistant prostate cancer. Additionally, circAURKA was upregulated in the suggestion of neuroendocrine prostate cancer (NEPC)[36]. These are promising markers for therapy-resistant PCa progression and warrant further investigations in clinical settings in different patient cohorts.

In collaboration with Yan Dong's Lab, we reported and validated that multiple circRNAs are encoded by the *AR* gene, and are widespread in PCa cells and xenograft models[65]. We have further demonstrated that one of the AR circRNAs, namely circAR3, is abundantly expressed in prostate tissues and detectable in patient plasma in prostate- and prostate cancer-specific manners[52]. It is worth to be noticed that the levels of intratumoral circAR3 reduced in high Gleason tumors, while plasma circAR3 is positively associated with high Gleason scores and positive lymph node metastasis, making it suitable for biomarker development in PCa[52]. This disproportional expression of circRNAs in tissue and blood may likely be explained by the release rates of circRNAs from tissue to bloodstream that can be affected by multiple factors (Figure 2): (1) CircRNAs can be selectively packaged into exosomes and actively released from the tumor into the circulatory system where they are detectable in plasma; (2) With PCa development, the prostate architecture is disrupted leading to faster release of circRNAs from the tissues into the stromal space. They can circumvent the endothelial cells of the blood vessels and enter the bloodstream. Similar to PSA, the plasma concentration of PCa-specific circRNAs can be increased in this way; (3) Cell death induced by stresses such as hypoxia, inflammation, and anti-tumor therapies can increase the release of circRNAs into the bloodstream; and (4) As tumor invasion and metastasis occur, microparticles containing circRNAs are shed from tumor cells, subsequently increasing the circRNA concentration in plasma. As indicated with circAR3, plasma levels were higher in lymph node metastasis than without[52]. Altogether, these form a complex network that constitutes the disproportion between

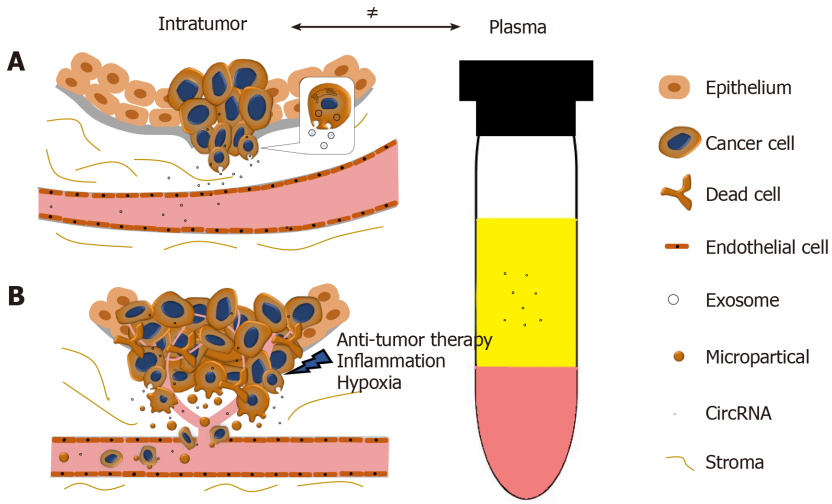

Figure 2 The disproportion of circRNAs between tumor and plasma. A: CircRNAs can be selectively enriched in exosomes and actively released into plasma as exosomes. During PCa progression, the integrity of normal prostatic tissues will be interrupted; this facilitates the release of circRNAs into the bloodstream; B: Stresses such as hypoxia, inflammation, and anti-tumor therapies will cause cell death and increase the release of circRNAs. Microparticles containing circRNAs shed from the metastasizing tumor will subsequently increase the circRNA concentration in plasma.

circRNA levels in tumors versus plasma.

The functional characterization of circRNAs in PCa cells further advocates that certain circRNAs could be developed into PCa biomarkers. CircRNA-miRNA mapping has revealed that studying the interaction between circRNAs and miRNAs may further help to characterize the role of certain circRNAs in PCa development. In vitro investigations of interactions such as CDR1as-miR-7[66], circRNA-MYLK- miR-29a[64], and circBAGE2-miR-103a[66] have implicated tumor suppressive and oncogenic roles of circRNAs, which could imply their utility as biomarkers as well as therapeutic targets[64]. Other studies have shown that some circRNAs may play roles in contributing to therapy-resistance PCa. For example, downregulated circFOXO3 promotes PCa progression to be resistance to docetaxel[67], while hsa_circ_0004870 downregulation is correlated with enzalutamide resistance[11].

CONCLUSION

The surmounting evidence linking circRNA expression to the development of PCa is promising. Their presence and stability in body fluids such as plasma and urine allow their expressions to be analyzed in regards to a range of urologic diseases. Moreover, their detectability in said body fluids is a key pro in regards to convenient, minimally invasive sample collection which is an important feature for ideal biomarkers. Most exciting is the validation of a circRNA that is prostate and prostate-cancer specific, and detectable in the plasma of patients. Overall, further investigations are needed to truly label circRNAs as biomarkers. Firstly, it might be useful to focus on functionally characterizing specific circRNAs in pathogenesis and or tumorigenesis.

Molecular pathological epidemiology (MPE) research focuses on the etiology and pathogenesis of diseases. The inclusion of MPE studies in the future could provide clearer correlations between circRNAs, tumor characteristics/molecular changes, risk factors (environmental, lifestyle, microbiome, genetic mutations, *etc*.), and disease outcome (including tumor subtypes) in PCa patients. It would also be interesting to see whether the findings of such studies could expand on the potential clinical applications of circRNAs in cancer management; specifically as it relates to constructing predictive models that could improve screening and personalized medicine. But, the success of MPE research is hindered by challenges such as the need for trans-disciplinary experts, and poorer success rates with funding applications[68]. Nonetheless, MPE research generally have strong impact[68], thus it is a promising direction for elevating prostate cancer research with circRNAs.

Furthermore, considering the wide expression of circRNAs, perhaps closer attention should be on defining disease-specific circRNA panels which could be used in addition to traditional diagnostic markers. Additionally, for clinical validation, sample

processing, detection method, and interpretation (cut-off) values need to be standardized across studies prior to truly establishing their clinical capacity as biomarkers. Nonetheless, with the growing capacity of next-generation sequencing and bioinformatics, the knowledge of circRNAs and their biomarker potential will undoubtedly continue to expand.

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REVIEW

Statins in risk-reduction and treatment of cancer

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Abstract

Statins, which are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, reduce cholesterol blood levels and the risk of developing cardiovascular diseases and their related complications. In addition to this main activity, statins show pleiotropic effects such as antioxidant, anti-inflammatory and antiproliferative properties, with applications in many pathologies. Based on their antiproliferative properties, *in vitro* and *in vivo* studies have investigated their effects on various types of cancer (*i.e.*, breast cancer, prostate cancer, colorectal cancer, ovarian cancer, lung cancer) with different genetic and molecular characteristics. Many positive results were obtained, but they were highly dependent on the physiochemical properties of the statins, their dose and treatment period. Combined therapies of statins and cytotoxic drugs have also been tested, and synergistic or additive effects were observed. Moreover, observational studies performed on patients who used statins for different pathologies, revealed that statins reduced the risk of developing various cancers, and improved the outcomes for cancer patients. Currently, there are many ongoing clinical trials aimed at exploring the potential of statins to lower the mortality and the disease-recurrence risk. All these results are the foundation of new treatment directions in cancer therapy.

Key words: Statins; Cancer; Pleiotropic effects; Risk reduction; Clinical trials; 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; Mevalonate pathway

Core tip: In the last few years, statins have been increasingly studied for their anticancer properties. This review presents the application of statins in cancer management by outlining the latest *in vitro* and *in vivo* studies. The results represent the foundation of the latest clinical trials in order to search for new treatment directions in cancer therapy.

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INTRODUCTION

Statistics published in September 2018 by the World Health Organization revealed that cancer is responsible for one in six deaths worldwide. The most diagnosed and deadly types of cancer are lung cancer (LC), breast cancer (BC), colorectal cancer (CRC) and prostate cancer (PC). The most common risk factors responsible for cancer occurrence include smoking, obesity, unhealthy diet, alcohol consumption and viral infections[1]. Cancer, which is represented by a large number of conditions, is defined as an uncontrolled proliferation of cells that possess metastatic properties. These cells are characterized by changes in their activity, such as the suppression of apoptotic mechanisms, the disruption of cell adhesion and signaling, and changes that occur as a result of genetic mutations[2].

Lately, high cholesterol levels have been associated with the development of some types of cancer, *i.e.*, CRC, PC and BC[3]. The literature describes two main paths through which cholesterol contributes to cancer onset. The first one involves the fundamental role of cholesterol in processes such as cell adhesion and signaling, necessary for normal cell functioning, while the second one refers to its function as a precursor in the synthesis of sex hormones and other isoprenoid intermediates, responsible for the development of particular types of cancer[4,5]. The latest treatment directions suggest that this field should be further explored due to the benefits that cholesterol-lowering drugs can bring in cancer treatment[4].

Statins are the first cholesterol-lowering agents discovered. Due to their significant ability to reduce cholesterol blood levels, international guidelines acknowledge statins as a first-line treatment for hypercholesterolemia[6]. By inhibiting the synthesis of cholesterol and its metabolites[7,8], statins have shown antiproliferative effects in various types of cancer[3]. A number of observational studies reported a risk reduction in the onset of cancer, or improvements in the outcomes of cancer, in statin users. The variable efficacy of different statins is related to their distinct physiochemical properties and the length of treatment[9]. Many *in vitro* and *in vivo* studies performed on different types of cancers underlined the molecular mechanisms through which statins inhibit cancer cell proliferation and metastasis[10]. These mechanisms were considered the basis for introducing statins in cancer treatment and prevention[8,11]. The antiproliferative effects of statins are a result of both inhibition of the mevalonate pathway and their pleiotropic effects, *i.e.*, antioxidant, anti-inflammatory and immune modulatory properties, with a major impact on patient survival and cancer recurrence[10,12].

The purpose of this review was to present the latest studies regarding the antiproliferative effects of statins. The paper is divided into two parts. The first section is dedicated to reviewing the latest published preclinical studies, highlighting the main mechanisms through which statins exert their anticancer properties. In the second part, several observational studies and clinical trials on statins, as single therapy or in combination with anticancer therapies, are summarized as future lines of research in cancer prevention/treatment.

MECHANISM OF ACTION OF STATINS

Cholesterol along with isoprenoid intermediates are synthesized through the mevalonate pathway. In this process, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) is converted into mevalonate *via* HMG-CoA reductase. Statins, due to their structural similarity to HMG-CoA, are competitive inhibitors of HMG-CoA reductase, and thereby have the ability to suppress cholesterol synthesis[13-15]. The affinity of statins for HMG-CoA reductase is in the nanomolar range, compared to the natural substrate whose concentration needs to be in the micromolar range[15]. Statins mainly act in the liver, where they induce an overexpression of low density lipoprotein (LDL) receptors at the surface of hepatocytes, thereby increasing the uptake of circulating LDL[15,16]. Through this mechanism, statins reduce lipoprotein blood levels,

and consequently decrease the risk of developing cardiovascular diseases and their related complications[14].

The potency of these drugs is highly influenced by their physicochemical properties. Lipophilic statins, *i.e.*, simvastatin, mevastatin, lovastatin, and pitavastatin, can easily cross cell membranes by diffusion, while hydrophilic statins, *i.e.*, pravastatin, need special membrane transporters[12,15,17,18]. Another difference arises due to their molecular structure. Synthetic statins, *i.e.*, rosuvastatin, pitavastatin, atorvastatin and lovastatin, possess a fluorophenyl group which confers them the ability to form an additional linkage to HMG-CoA reductase; therefore, exhibiting a more potent inhibition. On the other hand, simvastatin, pravastatin, mevastatin and lovastatin are obtained through fungal fermentation, and contain a decalin ring[13,15,18]. In addition, simvastatin and lovastatin are used as inactive prodrugs which makes them 100-fold more lipophilic than pravastatin. After oral administration, these prodrugs are metabolized by CYP enzymes to a hydroxy-acid active form[19].

PRECLINICAL STUDIES EVALUATING THE ANTICANCER EFFECTS OF STATINS

Since the first reports in the late 1990s on the ability of statins to influence cancer progression, their anticancer properties have been extensively documented in a wide range of cancer cell lines and tumor-bearing animal models. Several preclinical studies support the anticancer effects of statins against various types of tumors, including liquid tumors such as myeloma and leukemia, and solid tumors[20]. The possible underlying mechanisms that account for the anticancer effects have been reported in numerous *in vitro* studies. It has been shown that their anticancer properties result from the suppression of tumor growth, induction of apoptosis and autophagy, inhibition of cell migration and invasion, and inhibition of angiogenesis[21-23].

This chapter outlines the current state of knowledge concerning the anticancer effects of statins from *in vitro* and *in vivo* preclinical studies. However, due to the vast data available in the literature regarding this subject, we will focus on presenting the most recent reports.

In vitro studies

Some of the most recent results from *in vitro* studies on statin anticancer activity are presented in Table 1. By examining the results reported in the literature, several conclusions can be drawn, which are in agreement with findings previously reported by Osmak *et al*[11], Ahmadi *et al*[24], and others.

Firstly, it appears that the antitumor potential depends on the physicochemical properties of the statins, more precisely their lipophilicity. The chemical structure of the molecule dictates the solubility of the statin, which in turn will affect the pharmacokinetic profile[19,23,25]. The lipophilicity promotes access to different tissues, including cancer cells. Statins are taken up into cells by the organic anion-transporting polypeptide OATP1B1 mainly expressed by hepatocytes and for lipophilic statins also by passive diffusion through the membrane. As a result, hydrophilic statins show an increased affinity for hepatic tissue, but not for other tissues. However, lipophilic statins achieve higher levels in extrahepatic tissues where they interfere with the synthesis of cholesterol[19,24,26]. Several *in vitro* studies on various cancer cell lines have reported lower anticancer efficacy for hydrophilic statins as opposed to lipophilic statins. Beckwitt *et al*[27] assessed the anticancer activity of four statins, namely atorvastatin, simvastatin, rosuvastatin, and pravastatin, on four types of cancer cell lines derived from primary tumors: Breast (MCF-7 and MDA-MB-231), prostate (DU-145), brain (SF-295), and melanoma. Atorvastatin displayed the highest antitumor effect, while pravastatin had the lowest efficacy at suppressing tumor growth in all the above-mentioned cell lines. Furthermore, rosuvastatin was less potent than atorvastatin, even though the former shows similar affinity for the enzyme HMG-CoA reductase. Simvastatin, on the other hand showed similar efficacy to atorvastatin[27]. Consistent with these findings, another study demonstrated that lipophilic simvastatin significantly inhibited the proliferation of esophageal adenocarcinoma OE-19 cells and esophageal squamous cell carcinoma Eca-109 cells, at concentrations of 30 μmol/L, accompanied by the down-regulation of COX-2 and PGE2 in both cancer cell lines, in a dose-dependent manner. However, hydrophilic pravastatin had no obvious suppressive effect on tumor growth in the two investigated esophageal cancer cell lines[28]. In pancreatic cancer cell lines (PA-TU-8902, MiaPaCa-2, BxPC-3), except for pravastatin, all investigated lipophilic statins, at a concentration of 12 μmol/L,

Table 1 *In vitro* studies on the anticancer potential of statins

Cancer type	Cancer cell line	Statin	Observations	Changes in intracellular signaling pathways	Ref.
Hepatoma	HepG2, Hep3B	Simvastatin	Inhibition of cell growth in a dose- and time-dependent manner; G0/G1 cell cycle arrest; Apoptosis	AMPK activation and STAT3/Skp2 axis suppression, inducing p21 and p27 accumulation	[21]
Ovarian cancer	Hey, SKOV3	Atorvastatin	Dose-dependent antiproliferative effect (1-250 μmol/L); Decrease in size and density of the cancer cells, and colony forming ability (at 150 μmol/L); G1-phase cell cycle arrest and S-phase decrease (at 150 μmol/L); Induction of apoptosis; Increased ROS levels in a dose-dependent manner; Induction of autophagy; Inhibition of cell adhesion and invasion	Inhibition of Akt/mTOR and activation of MAPK pathway; Decreased Mcl-1 expression, variable effect on Bcl-2 expression, increased cleaved PARP protein expression; Increased expression of cellular stress protein (PERK and Bip) (at 150 μmol/L); Reduced expression of VEGF protein and MMP-9	[22]
Breast cancer	SUM149, SUM159, MDA-MB-231	Simvastatin	Inhibition of proliferation, decrease in S-phase and increase in G1/S-phase arrest; Suppression of cell migration; Decrease in tumor sphere formation	Down-regulation of phosphorylated FOXO3a in SUM149 and SUM159 cells; Variable effect on total FOXO3a expression	[43]
Endometrial cancer	ECC-1, Ishikawa, primary cultures of endometrial cancer cells	Simvastatin	Dose-dependent antiproliferative effect in both cancer cell lines (0.01-50 μmol/L), and in 5/8 primary cultures; G0/G1-phase cell cycle arrest, decreased S-phase in ECC-1 cells; Decreased HMG-CoA reductase activity; Induction of apoptosis; Increased DNA damage, cellular oxidative stress; Reduced cell adhesion and invasion	Inhibition of MAPK pathway, differential effects on the Akt/mTOR pathway; Increased cleaved caspase-3, decreased Bcl-2 expression, unmodified Mcl-1	[20]
Osteosarcoma	MNNG/HOS	Simvastatin	Dose- and time-dependent antiproliferative effect (0.5-64 μmol/L); Dose-dependent morphological changes in treated cells: Cell shrinkage, loss of intercellular contact, reduced cell adherence, floating shapes; Dose-dependent suppression of cell migration, G0/G1-phase cell cycle arrest (16 μmol/L), and apoptosis	Dose-dependent down-regulation of MMP-2 and MMP-9; Down-regulation of cyclin D1, CDK2 and CDK4, up-regulation of CDKIs, p21 Cip1 and p27 Kip1; Decrease in PI3K and phospho-Akt expression, while total AKt remained unmodified, up-regulation of Bax and cleaved PARP expression, decreased Bcl-2 expression	[44]
Lung adenocarcinoma	A549, H1299, PC9, HCC827, H1975, H1435, PE8sc, CL1-0, Bm7, and immortalized normal lung epithelial cells (HBEC3KT)	Simvastatin	Higher cytotoxicity against LC cells with p53 mutation; Dose-dependent apoptosis; Reduced lipid rafts in mutant p53-bearing LC cells; Reduction in the migration distance; Promotes the nuclear transport of mutant p53 in Bm7 and H1435 cells	Increased levels of cleaved PARP and cleaved caspase-3; No difference in the level of LC3-II; Decreased level of p53, and increased level of high molecular weight HSP-40	[45]

MAPK: Mitogen-activated protein kinase; ROS: Reactive oxygen species; HMG-CoA reductase: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; AMPK: AMP-activated protein kinase; STAT3: Signal transducer and activator of transcription 3; skp2: S-phase kinase associated protein 2; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin; PARP: Poly (ADP-ribose) polymerase; VEGF: Vascular endothelial growth factor; MMP: Matrix metalloproteinase; CDK: Cyclin-dependent kinase; CDKI: Cyclin-dependent kinase inhibitor; PI3K: Phosphoinositide 3-kinase; LC3: Microtubule-associated protein 1A/1B-light chain 3.

displayed significant antiproliferative activity. Cerivastatin and simvastatin proved to be the most effective in suppressing tumor growth, followed by fluvastatin and lovastatin[29]. Jiang *et al*[25] also proved the superior anticancer effect of lipophilic statins on BC (MDA-MB-231, MDA-MB-432, MDA-MB-435) and brain cancer (A172, LN443, U87, U118, U251), compared to hydrophilic rosuvastatin and pravastatin. Furthermore, the research group proved that the *in vitro* IC_{50} of cerivastatin and pitavastatin can be achieved at therapeutic doses of 0.2-0.4 mg/d for cerivastatin, and 1-4 mg/d for pitavastatin, respectively. The clinical relevance of these observations is that for these two statins, the doses needed to inhibit tumor growth are in the same range as those used to control cholesterol levels[25].

Secondly, statins exhibit anticancer effects with varying sensitivity depending on the type of tumor, as not all types of cancer cell lines are sensitive to statins. In one

study, atorvastatin was tested against seven different types of solid tumors, including ovarian (IGROV1, OVCAR3), breast (HS-578T, T47D), prostate (PC-3, DU-145), colon (HCT-116, KM-12), lung (HOP-92, NCI-H322M), brain (SF-295, SF-539) cancers, and melanoma (SK-MEL-5, MDA-MB-435). Atorvastatin affected the proliferation of these tumor cells differentially: The growth of some cell lines was fully or partially suppressed, while other cells were insensitive to atorvastatin treatment (at a concentration of 10 μmol/L)[30]. These results suggest that the pharmacological effect is influenced by the genetic background of the cancer cells[25]. Using gene expression data from the above-mentioned fourteen cancer cell lines, and the results obtained from the sensitivity assay, Raghu *et al*[31] were able to produce and validate a genetic signature which identifies statin-sensitive cells. Moreover, they demonstrated that statin-resistance is linked to increased E-cadherin (E-cad) expression on cancer cells. E-cad expressing cancer cells, namely epithelial and mixed epithelial-mesenchymal cancer cells (characteristic of primary and metastatic tumors) are inhibited at much higher statin concentrations than mesenchymal cancer cells (characteristic of circulating metastatic tumor cells). In this sense, Ishikawa *et al*[32] screened atorvastatin against four cancer cell lines (two PC, and two LC cell lines) with different expressions of vimentin and E-cad. The sensitivity of the cells to atorvastatin decreased from sensitive mesenchymal PC-3 and HOP-92 cells to less sensitive epithelial NCI-H332M cells, and lastly resistant mixed epithelial-mesenchymal Du-145 cells. This suggests that statins preferentially inhibit the growth of mesenchymal-like cancer cells which are responsible for cancer dissemination and metastasis[32]. In a recent study, Hong *et al*[33] tested the sensitivity of eight gastric cancer (GC) cell lines to simvastatin, in order to identify potential biomarkers of statin sensitivity. Half of the cell lines (SNU5, SNU719, SNU16, AGS) responded to simvastatin treatment, while the remaining (MKN45, SNU620, SNU668, NCL-N87) proved insensitive. Furthermore, the expression of thiamine pyrophosphokinase-1 (TPK1) was significantly increased in the simvastatin-sensitive cell cultures, suggesting that the TPK1 gene could be a valuable predictive biomarker of statin anticancer therapy efficacy[33].

Lastly, given their anticancer potential, a large number of studies have proposed the association of statins to standard chemotherapy agents. In most cases, an additive or synergistic effect was observed for the combination therapy. Henslee *et al*[34] investigated the antitumor effect of various statins (fluvastatin, atorvastatin, simvastatin, lovastatin, mevastatin, and pravastatin) in combination with doxorubicin, paclitaxel, or topotecan as a new treatment strategy in natural killer cell leukemia. Fluvastatin and atorvastatin inhibited cell growth in a dose-dependent manner, but in combination with different chemotherapy agents, a significantly greater cytotoxic effect was observed compared to the single drug treatment. In another study, simvastatin and mevastatin exhibited strong synergistic effects with doxorubicin against CRC. The cytotoxicity of doxorubicin was greatly enhanced in doxorubicin-resistant LoVo cancer cells, but there was little change in sensitive LoVo cells. Both statins promoted the accumulation of doxorubicin in LoVo cells, possibly through the induction of MDR1 gene expression which codes for P-glycoprotein[35]. Furthermore, simvastatin and atorvastatin, at a concentration of 12.5 μmol/L, which did not induce significant cytotoxicity, enhanced the effect of bortezomib in multiple myeloma U266 cells. In addition, simvastatin displayed superior activity to atorvastatin[36].

Several studies were conducted to evaluate the antitumor potential of lovastatin in combination with chemotherapy drugs such as cisplatin, 5-fluorouracil, daunorubicin, enzastaurin, and temozolomide, in various tumor cell lines. Lovastatin enhanced the cytotoxic effect of these drugs[24,37]. Enhanced antitumor effects were reported for simvastatin in combination with cisplatin, doxorubicin, gefitinib, vemurafenib/selumetinib in nasopharyngeal carcinoma, bladder cancer, non-small cell LC, and melanoma, respectively[24,38,39].

The combined treatment of a statin (atorvastatin, pitavastatin) and a tyrosine kinase inhibitor (TKI) such as gefitinib, erlotinib, or sorafenib, displayed increased antitumor activity in TKI-resistant hepatocellular carcinoma (HCC), and non-small cell LC[40-44].

In vivo preclinical studies

In addition to their *in vitro* efficacy, statins have been shown to exhibit anticancer effects *in vivo*, in various models of cancer in animals. The anticancer effects of statins have been mostly demonstrated in xenograft animal models, after the inoculation of cancer cells in rodents. Jiang *et al*[25] investigated the anticancer effect of pitavastatin and fluvastatin on glioblastoma in a xenograft mouse model. The results showed that pitavastatin was superior to fluvastatin in inhibiting tumor growth, on account of the different physicochemical and biopharmaceutical properties of the two molecules (lipophilicity, rate and efficacy of absorption *etc*.). At the same time, this study

indicated that intraperitoneal injection improved the efficacy of pitavastatin, compared to oral administration, although the oral dose was higher[25].

Ali *et al*[40] reported the efficacy of atorvastatin as an antitumor agent against non-small cell LC in NSG mice. Daily atorvastatin administration suppressed tumor growth in mice carrying TKI-resistant PC-9GR, H1975 and H1703 xenografts, by 59%, 48% and 57%, respectively, compared to their vehicle counterparts. The reduced tumor sizes in the atorvastatin-treated group corresponded to loss of Cav1 and GLUT3, induced pro-apoptotic Bax, and lowered tumor cholesterol content. Furthermore, glucose levels were significantly reduced following atorvastatin treatment, compared to vehicle treatment. The antitumor activity of atorvastatin *in vivo* was also verified in a transgenic mouse model expressing the clinically relevant EGFR T790M/L858R mutation, in which at treatment termination, atorvastatin-exposed animals showed an approximately 33% decrease in tumor mass compared to vehicle-treated transgenic mice. These data support the promise that statins are candidate drugs for TKI-resistant non-small cell LC treatment[40].

Of all the HMG-CoA reductase inhibitors, simvastatin is one of the most investigated and documented statins. The anticancer effect of simvastatin was demonstrated in xenograft mouse models of osteosarcoma, HCC, and CRC, following intraperitoneal or oral administration[21,45,46]. According to the immunohistochemical analysis of tumor tissues, simvastatin exhibited its tumor growth suppressing effects by increasing p21 and p27 expression, and AMPK activation, decreasing Skp2 expression and STAT3 phosphorylation[21], or upregulating the expression of BMP2[46].

Many animal studies suggest that statins suppress BC progression. Ahern *et al*[47] reported that simvastatin, administered orally, impairs the growth of human breast tumor xenografts in mice by increasing PTEN expression and inducing apoptosis. In another study, a significant antitumor effect was observed in mice bearing ErbB2 transformed MCNeuA mammary cancer, after the daily oral intake of simvastatin or fluvastatin. Even though both statins significantly inhibited tumor growth *in vivo*, fluvastatin was slightly more effective than simvastatin. Immunohistochemical studies on these MCNeuA tumors demonstrated that the *in vivo* antitumor effect was due to a statin-induced decline in tumor cell proliferation (decreased Ki67 staining) and survival (increased cleaved caspase-3 staining)[48].

The effects of statins and their underlying cellular mechanisms in the chemoprevention of CRC in suitable animal models of both sporadic and colitis-associated CRC have been reported by Pikoulis *et al*[49].

In addition to tumor xenograft animal models, the anticancer efficacy of statins has also been reported in chemical-induced tumors in animals. Li *et al*[47] reported the ability of statins to reverse adriamycin-induced cancer stem cell properties and metastasis in osteosarcoma by down-regulating KLF4 using a BALB/c (nu/nu) mouse model. Animals treated only with adriamycin showed a large increase in tumor incidence, supporting the idea that adriamycin can promote tumorigenesis of osteosarcoma cells. Tumor incidence in the adriamycin plus simvastatin group was much lower than that of the adriamycin group. Immunohistochemical analysis demonstrated that simvastatin blocks the adriamycin-mediated activation of KLF4 and CD133, and its tumor-initiating ability[50].

Statins have also been shown to reduce metastasis *in vivo*, in various types of cancer. Liu *et al*[51] demonstrated, in a nude mouse model, that simvastatin significantly prevents the formation of osteolytic lesions caused by the metastasis of human A549 LC cells to the bone. This effect of simvastatin may be the result of its action on colonized LC cells in the bone, inhibiting the production and secretion of osteoclastogenic factors. It was shown that simvastatin attenuated the expression of CD44, a cell surface antigen enriched in epithelial tumor-initiating and metastatic cancer cells, which regulates the migration and invasion of LC cells. Simvastatin could increase the levels of p53 in A549 cells to repress the expression of CD44, and down-regulate MMP2 and MMP9[51].

Furthermore, studies have demonstrated that statins increase the efficacy of chemotherapy *in vivo*. Atorvastatin restored sensitivity to different anticancer agents (*i.e.*, temozolomide, sorafenib) in mouse models of glioblastoma and HCC[41,52]. The overexpression of ABC transporters is generally associated with resistance to chemotherapy, which is prominently mediated by transporters like ABCB1, ABCC1, and ABCG2. Atil *et al*[53] conducted experiments on two xenograft mouse models to demonstrate the antitumor activity of simvastatin by ABCB1 down-regulation and apoptosis induction. CD-1 Nu/Nu mice inoculated with rhabdomyosarcoma or neuroblastoma cells received clinically relevant simvastatin concentrations and showed marked induction of apoptosis in both tumor tissues, indicated by PARP and caspase-3 cleavage. ABCB1 down-regulation was found in the liver and tumor tissues

but did not reach significance in neuroblastoma. The extend of apoptosis was comparable to that induced by cyclophosphamide, and was further amplified by the combination of the two drugs[53].

THE CLINICAL EXPERIENCE WITH STATINS IN ONCOLOGY

Statins are widely used for the control of hypercholesterolemia; thus, extensive data are available regarding cancer incidence and mortality in patients using statins. Despite this, the reported results are quite controversial. Initially, an increase in cancer incidence and cancer-related mortality was reported, but in recent years an opposite or lack of effect was observed in cancer patients using stains[54]. A possible explanation for these questionable results stems from the primary goal of the clinical trials, in which statins were considered for their effect on cardiovascular morbidities, and not on cancer incidence. These controversial results were first observed in animal studies, where a dose-response dependence on the onset/suppression of particular cancers was reported[8]. For more conclusive results regarding the impact of statin use on the incidence of cancer, the forthcoming clinical trials should include the identification of preexisting cancers and other confounding factors[55].

Preclinical studies on statin anticancer efficacy highlighted the need to reach concentrations in the micromolar range in order to inhibit cell proliferation, concentrations that are unattainable in clinical practice without inducing side effects. Given this fact, statin use as single therapy for cancer treatment is questionable, but their association with standard chemotherapeutic agents for a synergistic or additive effect seems to be a feasible strategy for cancer therapy[8]. The statins currently available on the market are atorvastatin, rosuvastatin, pravastatin, pitavastatin, simvastatin and fluvastatin[54]. The anticancer potential of statins has been demonstrated mostly in PC and BC, but their effects on other solid malignancies, such as LC, CRC, and GC, are also noteworthy.

PC

PC is the most common type of cancer and the leading cause of cancer-related death among men, in many countries[56]. Acute or chronic inflammation is the main cause not only of carcinogenesis but also the progression of PC. Thus, drugs and diets that suppress the inflammatory response or modulate the immune status have been reported to be beneficial for PC. Statins have great potential in preventing PC progression, as some studies have shown that statin use is associated with a reduction in PC risk[57]. On the other hand, the onset of PC is tightly associated with risk factors like obesity, hypertension, increased levels of testosterone, race, and family history[58]. Some of these risk factors are also linked to cardiovascular diseases and their related complications, as previously mentioned in this review. At present, the most efficient therapy for PC is androgen deprivation[58]. This therapy is based on the ability of PC cells to synthesize androgen hormones *de novo* due to the high levels of circulating cholesterol and on the *de novo* synthesis of cholesterol. Since cholesterol is a precursor in androgen synthesis, by inhibiting this pathway, statin therapy was considered a potential strategy to improve the outcomes in PC. In cancer, cholesterol is not only associated with the synthesis of sexual hormones, but is also responsible for cell growth, progression, proliferation and migration. Thus, statins can improve the outcomes of PC therapy by increasing the survival rate and decreasing the progression and recurrence of the tumor[59-62]. In addition, due to their anti-inflammatory properties, statins have the ability to inhibit the overexpression of androgen receptors, which in turn leads to suppression of cell growth[59,60].

It was also observed that androgen deprivation combined with radiotherapy increases the survival rate[58]. *In vivo* and *in vitro* studies revealed a synergistic effect between statin therapy and radiotherapy, mainly by cell death. This combination therapy caused a 30% reduction in mortality among statin users diagnosed with PC[63].

The results regarding the effect of statins on PC risk were obtained mainly as secondary data from clinical trials evaluating the use of statins in primary or secondary prevention of cardiovascular disease[64]. A retrospective study published in 2014 showed that postdiagnostic use of statins was associated with a decreased risk of PC mortality and all-cause mortality, especially in patients who used statins before diagnosis[62]. Prospective studies examining the link between statin use and the risk of PC suggested that statins may not reduce the risk of PC but may lower the risk of advanced or high-grade disease[65]. However, as prostate-specific antigen (PSA) is the primary method for PC screening, and statin use is associated with lower PSA levels,

this confounds the associations between statins and the risk of being diagnosed with PC. To clarify this, the association between baseline statin use and the risk of overall, high-grade (Gleason ≥ 7) or low-grade (Gleason ≤ 6) PC *vs* no cancer was examined in a post-hoc secondary analysis of REDUCE, a prospective multinational randomized controlled trial of dutasteride *vs* placebo among men with elevated PSA and a negative PC biopsy at baseline. The conclusion of the study was that statins were not associated with the risk of being diagnosed with PC or high-grade disease[66]. The data from the REDUCE study were also used to test the correlation between statin users and prostate volume (PV) change over time, determined from transrectal ultrasonography performed to guide prostate biopsy at baseline, and 2- and 4-years after randomization. Statins were found to modestly attenuate PV growth, with a magnitude in line with previously reported PSA-lowering effects for these drugs (approximately 4%).

Taking into account the conflicting evidence regarding the role of statins in PC chemoprevention, a recently published cohort study performed in the United States evaluated the association of statin use with PC in terms of Gleason score (reflecting the aggressiveness of PC), time and dose dependence, based on electronic medical records. The conclusion of the study was that statins might be associated with a reduced PC risk only when used for a relatively longer time, and the risk reduction was higher for patients with higher Gleason score. Additionally, lipophilic statins were more protective than hydrophilic statins[67].

Statins, administered preoperatively, were also evaluated in clinical studies for their influence on disease recurrence, proliferation index or tumor biomarker status, in men undergoing radical prostatectomy[68-70]. The statins investigated in these trials were simvastatin and atorvastatin, but the results have still to be published.

BC

BC is the most frequently encountered cancer among women, and the number of clinical trials assessing the putative clinical benefit of statins in BC is increasing. Recently, a direct association between cholesterol blood levels and the incidence of BC was observed. A high level of LDL increases BC cell proliferation and induces gene changes that are not favorable for the prognosis of BC[71]. A 72% risk reduction in the onset of BC was observed among statin users, especially in estrogen-negative BC cases. This result has been reported in long-term statin therapy[72]. Additionally, statins were shown to reduce BC patient mortality, but the benefit appears to be dependent on statin type and follow-up time. Thus, lipophilic statins showed a stronger protective effect in BC patients, reflected by a significantly increased recurrence-free survival and an improved overall survival. On the other hand, hydrophilic statins only slightly improved all-cause mortality. Furthermore, the protective effect was observed only in groups with less than 4 years of follow-up[73,74]. Also, it has been shown that statins did not increase the risk of BC[75].

More recent clinical studies have included the evaluation of tumor biomarkers capable of predicting statin response, in their design and analysis plan. Thus, a clinical trial investigated the effects of short-term (two weeks) administration of atorvastatin, at the maximum recommended dose, on the levels of conventional BC pathological biomarkers, *i.e.*, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2), as well as the cell cycle regulators cyclin D1 and p27. While ER, PR and HER2 expression remained stable following treatment, a significant decrease in cyclin D1 expression and a significant increase in p27 expression were observed. The results of this study suggested that cell cycle regulatory effects contributed to the antiproliferative effects of statins in BC[76]. Another ongoing clinical trial is exploring the relationship between the short-term use of oral simvastatin and changes in the expression of Ki-67 (a candidate biomarker of breast tumor proliferation), in women with clinical stage 1 or 2 primary invasive BC, but the results of this trial have not yet been published[77]. The same biomarker was the focus of another clinical trial, in which the administration of atorvastatin (80 mg/d) for two weeks led to a decrease in cell proliferation rate[47].

Another objective in recent clinical studies is to exploit the antitumor activity of statins in combination with preoperative standard chemotherapy, associated or not with zoledronate (zol). For this purpose, atorvastatin was evaluated in a clinical trial of patients with triple negative BC. The clinical trial is to finish in 2020, and the expected outcomes are the efficacy endpoint and the proportion of responsive patients after 6 months of treatment, at surgery[78]. In another pre-surgical study, fluvastatin and atorvastatin were administered in high doses to BC patients with baseline overexpression of HMG-CoA reductase. Inhibition of cell proliferation was observed. There were no differences between lipophilic and hydrophilic statins, which suggested

that all statins act by inhibiting the mevalonate pathway[79].

Exposure to potentially cardiotoxic BC therapies, including anthracyclines, trastuzumab, and radiation therapy, coupled with host factors, place patients at an increased risk of developing cardiovascular diseases (CVD) compared to non-cancer controls. Overall survival outcomes are significantly worse in patients who develop CVD, and cardiovascular death may even exceed the risk of cancer death in the long-term. In this context, there is a current trend to establish cardioprotective strategies at the time of cancer therapy initiation, and statins are among the proposed drugs[80]. The Preventing Anthracycline Cardiovascular Toxicity with Statins trial explores a new clinical paradigm to manage BC: Primary prevention of anthracycline-based adjuvant therapy-related left ventricular (LV) dysfunction using pre-treatment with statins. Thus, 279 patients with early stage BC or lymphoma, with normal baseline left ventricular ejection fraction (LVEF), and treated with anthracyclines, will receive 40 mg atorvastatin or placebo at the start of chemotherapy, and will be continued for 24 mo. The primary endpoint is LVEF maintenance at 24 mo. This study will also quantify measures of cardiac and vascular remodeling, including strain, wall thickness, LV volumes, fibrosis, and pulse wave velocity[81]. Another ongoing study will evaluate the use of simvastatin for prophylactic cardioprotection[23]. Besides anthracyclines, trastuzumab is another effective drug used to treat HER2+ BC, but it is associated with a risk of cardiac dysfunction. A retrospective case-control study based on electronic chart review of consecutive women with HER2+ BC, treated with trastuzumab-based therapy was carried out to evaluate whether exposure to statins during cancer treatment would have a lower decline in LVEF and lower incidence of cardiotoxicity, compared to those who were not exposed to statins. The results showed that the concomitant use of statins was associated with a lower risk of cardiotoxicity[82]. In another clinical study, the topical administration of atorvastatin 1% gel twice daily during radiotherapy significantly reduce itching, breast edema and pain in patients under treatment[83].

CRC

Accumulating evidence suggests that statins may have a role in CRC prevention and treatment, but associations between individual statin characteristics, their doses and CRC have not yet been defined. Rho and Ras proteins are overexpressed in this type of cancer, and by inhibiting their synthesis through the mevalonate pathway, cancer proliferation and invasion are suppressed[84].

In many studies, statin use was associated with a 30% to 50% risk reduction in developing CRC[84-86]. A study analyzing data from the National Health Insurance Service-Health Screening (NHIS-HEALS) cohort in Korea, conducted by NHIS from 2002 to 2015, showed that statins might have different preventive activity against CRC, depending on the anatomical site of the tumor, and patient sex. Thus, the risk of developing CRC was lower in statin users with hypercholesterolemia, especially proximal colon cancer in men and rectal cancer in both sexes[87]. A meta-analysis of existing comparative studies published between 1990 and 2016 investigated the association between statin use and the risk of colorectal adenoma. According to this publication, statin use was associated with a reduced risk of advanced adenoma, but did not significantly reduce the risk of any adenoma. It appears that statins may prevent CRC by acting at the later stages of progression, rather than at the early stages of adenoma initiation and development[88]. As a result of affecting the later stages of the adenoma-carcinoma sequence, statins reduce the aggressiveness and invasiveness of CRC. This hypothesis is supported by various studies which report that statin therapy is associated with improved CRC-specific survival[89].

Simvastatin was clinically evaluated in addition to standard XELIRI/FOLFIRI chemotherapy regimens, to assess whether it confers a clinical benefit to patients with previously treated metastatic CRC. However, the proposed treatment did not improve progression-free survival nor did it increase the toxicity of the conventional chemotherapy regimen[90].

Gynecologic cancers

Endometrial and ovarian cancers are the most common types of gynecological malignancies[91,92]. In most cases, it was observed that the mevalonate pathway plays a major role in the development of these two types of cancer. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are inhibited by statins, are involved in the modification of several regulatory proteins, including the Ras and Rho protein family. Changes in Ras and Rho protein expression are responsible for 20% and 40% of cases of endometrial and ovarian cancer, respectively[93]. Conflicting results have been reported by different studies regarding these two types of cancer. One study

concluded that while statin use reduced the risk of developing endometrial cancer, there was no influence on ovarian cancer[93]. Other studies reported a 50% risk reduction of developing ovarian cancer in statin users[94]. A meta-analysis published in 2018 evaluated the association between postdiagnostic statin use and ovarian cancer mortality, and, based on the analysis of eight cohort studies of ovarian cancer patients, a significant protective effect on overall and cancer-specific survival was observed[95].

HCC

HCC is the most common liver cancer with a high mortality worldwide[96]. A meta-analysis concluded that statin therapy can reduce the incidence of HCC by almost 37% in a time- and dose-dependent manner. It is worth noting that this result was observed only among statin users and not when other cholesterol lowering agents were used, implying that HCC risk reduction is more likely attributed to the pleiotropic effects of statins rather than to their cholesterol lowering properties[97]. Bearing this in mind, two clinical trials investigated the influence of pravastatin therapy on patient survival after transarterial embolization (TAE) of HCC. Due to its high affinity for hepatic tissue, pravastatin prolonged the survival of patients with HCC[98,99]. In addition, pravastatin also demonstrated hepatoprotective and tumor progression suppressive effects when administered in conjunction with TAE[99].

GC

Similar to HCC, GC has a decreased long-term survival rate, and occurs primarily due to specific lifestyle features[100]. A phase II clinical trial evaluated the antitumor effect of a high dose of lovastatin in advanced GC. No clinically significant response was observed, even if prior preclinical studies suggested otherwise and the highest dose of lovastatin was administered[101]. Another two clinical trials investigated the impact of adding a statin, namely simvastatin or pravastatin, to chemotherapy, on tumor progression rate or survival rate. Simvastatin was chosen for its effectiveness in a wide variety of cancers, while pravastatin was selected for its hydrophilic profile, which makes it available in high concentrations in peripheral tissues such as gastric tissue. However, no improvements in the outcome were obtained in either of the two trials[102,103]. The lack of efficacy against GC can be ascribed to the use of a low dose of statin or to extensive hepatic metabolism[101,102,104].

Other types of cancer

As the number of studies investigating the potential clinical benefits of statins in various types of cancer is very high, the conclusions of several recent meta-analyses addressing the most explored applications in cancer treatment are summarized in Table 2[105-110]. Based on the synergism reported for combinations of statins and cytotoxic drugs in preclinical studies, several clinical trials investigated the effect of adding statins to anticancer treatment in various types of cancer. Other clinical studies explored the impact of statin use on patient survival. However, the results of these studies were, to some extent, contradictory. The published meta-analyses are useful for an integrated conclusion, but a consensus whether statins are useful in oncology has not yet been reached.

In 2018, Abdullah *et al*[111] concluded that the lack of success encountered especially in prospective clinical studies may be due to a poor design of these studies. The authors pointed to the necessity of including in subsequent clinical trials, several crucial factors (*i.e.*, the administered dose, schedule, choice of statin, and diet), previously identified in preclinical trials as essential for statins to be effective, in order to improve the outcome of cancer patients[111].

CONCLUSION

It has been proven that in addition to its major implication in the promotion of cardiovascular diseases, cholesterol also plays a significant role in the onset of cancer. Based on these findings, statins, potent inhibitors of HMG-CoA reductase, used as first-line medication in the treatment of hypercholesterolemia, were considered for cancer treatment. *In vitro* and *in vivo* studies performed on many types of cancer highlighted the beneficial effects of statins in cancer prevention/treatment. The observed effects were highly dependent on statin physicochemical properties, potency, dose and treatment length. In most cases, lipophilic statins were preferred as they could easily cross cell membranes, and are efficiently taken up by cancer cells. Taking into consideration the results from preclinical studies and the high number of statin

Table 2 A summary of recent meta-analyses evaluating the benefits of statins in various types of cancer

Cancer type	No. of clinical trials and subjects included	Objective	Results	Ref.
Active cancer	Ten studies, 1881 individuals with stage 3 or higher disease	To evaluate the randomized controlled trials of statins in addition to standard anticancer therapy	The addition of statins to standard anticancer therapy did not improve overall survival or progression-free survival	[105]
Solid cancer	Eight randomized controlled trials, 1760 patients	To evaluate the effect of statins added to systemic anticancer therapy in patients with solid cancer	The addition of statins to chemotherapy did not significantly increase the incidence of grade 3-5 adverse events, did not improve the overall response rate and failed to prolong the progression-free survival and overall survival compared with that of chemotherapy alone	[106]
Pancreatic cancer	Six retrospective cohort studies, 12057 patients were included	To explore the association between statin and metformin use and overall survival of pancreatic cancer patients	Statin use was associated with a significantly improved overall survival (but with a significant publication bias)	[107]
	Twenty-six studies, more than 3 million participants, 170000 pancreatic cancer patients	The relationship between statin use and the risk of pancreatic cancer	Statins have a protective effect on pancreatic cancer	[108]
Kidney cancer	Twelve studies, 18105 patients	To evaluate the association between statin use and kidney cancer survival outcomes	Statin use was not associated with significant recurrence-free survival or progression-free survival; statin use was associated with marked improvements in cancer-specific survival and overall survival	[109]
Lung cancer	Seventeen studies, 98445 patients	To analyze the impact of statins on mortality and survival of LC patients	Statins were potentially associated with a decreased risk of mortality and an improvement of overall survival in observational studies but not in randomized controlled trials; Statins potentially enhanced the effects of tyrosine kinase inhibitors and chemotherapy on the overall survival of patients with non-small cell LC	[110]

users, observational clinical studies were performed in order to establish a direct linkage between statin therapy and risk reduction in the onset/recurrence of cancer. Studies have shown that there is a direct correlation between statin use and a reduced risk of cancer onset, or improvement in cancer outcomes. Most studies focused on PC, BC and CRC, because cholesterol plays a major role in these cancers, and reported statistically significant results. The positive results obtained from animal and clinical studies encouraged scientists to search for new directions in cancer treatment. Currently, statins are evaluated in many ongoing clinical trials on cancer patients. According to the published results, statin therapy shows some benefits in several types of cancer, with an increased survival rate, but other studies reported no effect. Therefore, more studies are needed to clarify these controversial results.

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REVIEW

Novel molecular targets in hepatocellular carcinoma

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Abstract

Globally, hepatocellular carcinoma (HCC) is a leading cause of cancer and cancer-related deaths. The therapeutic efficacy of locoregional and systemic treatment in patients with advanced HCC remains low, which results in a poor prognosis. The development of sorafenib for the treatment of HCC has resulted in a new era of molecular targeted therapy for this disease. However, the median overall survival was reported to be barely higher in the sorafenib treatment group than in the control group. Hence, in this review we describe the importance of developing more effective targeted therapies for the management of advanced HCC. Recent investigations of molecular signaling pathways in several cancers have provided some insights into developing molecular therapies that target critical members of these signaling pathways. Proteins involved in the Hedgehog and Notch signaling pathways, Polo-like kinase 1, arginine, histone deacetylases and Glypican-3 can be potential targets in the treatment of HCC. Monotherapy has limited therapeutic efficacy due to the development of inhibitory feedback mechanisms and induction of chemoresistance. Thus, emphasis is now on the development of personalized and combination molecular targeted therapies that can serve as ideal therapeutic strategies for improved management of HCC.

Key words: Hepatocellular carcinoma; Prognosis; Arginine deprivation; Cancer stem cells; Glypican-3; Hedgehog signaling pathway; Histone deacetylases; Personalized medicine; Molecular targeted therapy; Notch signaling pathway; Polo-like kinase 1; Tumour-associated antigens

Core tip: Hepatocellular carcinoma (HCC) remains a critical concern worldwide due to the severity of disease outcome. The primary cause is the low efficacy of current therapeutic

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regimens available to treat advanced HCC. This review provides details on novel potentially vulnerable targets in the oncogenic signaling pathways associated with HCC development and progression, which should be targeted to develop molecular combination therapies to improve disease management. Moreover, the identification and establishment of novel biomarkers would complement this process in assisting timely management of the disease *via* powerful personalized drug regimens.

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INTRODUCTION

Cancer of the liver is the sixth most commonly diagnosed cancer worldwide, and is responsible for 4.7% of all new cancer cases and 8.2% of all cancer-related deaths[1]. Although the five-year survival rate of liver cancer have improved from an abysmal 3% four decades ago to 18%, it is still significantly lower than the survival rates observed in many other solid cancers with a high global incidence, including breast (90%), colorectal (65%), and prostate (98%) cancers[2]. Three quarters of liver cancer patients present with hepatocellular carcinoma (HCC); while the other subtypes include cholangiocarcinoma, angiosarcoma, hepatoblastoma, and other non-cancerous liver diseases. The most common cause of HCC is hepatitis B virus (HBV) or hepatitis C virus (HCV) infection which is responsible for more than 90% of HCC cases in developing countries and nearly half the number of cases in developed countries[3]. Other risk factors include aflatoxin B_1 consumption, alcoholic liver disease, non-alcoholic fatty liver disease, smoking, autoimmune hepatitis, hemochromatosis, obesity, and diabetes. Importantly, in countries endemic for HBV, the introduction of a new universal vaccination program aided by mass screening has been shown to significantly reduce the rate of HBV-induced HCC in children and young adults[4,5]. Nevertheless, patients with early HCC are always asymptomatic or develop nonspecific complaints such as abdominal pain, enlarged abdomen, jaundice, and weight loss which results in HCC being initially undetected. Consequently, the management of high risk groups using routine serum α-fetoprotein monitoring and abdominal ultrasonography is important for better control over disease progression[6]. For the management of early and intermediate HCC, liver resection, orthotopic liver transplantation, thermal ablation including radiofrequency ablation and microwave ablation, transarterial therapies including, radioembolization with yttrium-90 and transarterial embolization with chemotherapeutic agents, and selective internal radiotherapy are potentially curative[6-8]. Although a 5-year survival rate of 50%-75% can be achieved, these curative therapies are only applicable for HCC patients with a smaller tumour size and adequate liver function[7,9-13]. Moreover, for patients presenting with advanced HCC, neoadjuvant and adjuvant systemic therapies are prescribed to reduce the rate of recurrence or the development of extrahepatic metastases; however, systemic chemotherapy has been reported to have a low tumour response rate and is commonly associated with the development of chemoresistance in advanced HCC[14-17].

The most actively used first-line systemic therapeutic agent approved for patients with nonresectable advanced HCC is sorafenib, an oral multikinase inhibitor targeting Raf, epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), FMS-like tyrosine kinase-3 and c-kit[18,19]. At least two large-scale, randomized, placebo-controlled drug trials independently confirmed the effectiveness of sorafenib treatment in inhibiting tumour growth and angiogenesis in advanced HCC; although, the median increase in the overall survival period of HCC patients treated with sorafenib was just under 3 mo as compared to the placebo group[20,21]. Moreover, prolonged exposure of HCC cells to sorafenib has been shown to induce resistance, caused by activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway, resulting in enhanced tumour growth and the development of distant metastases[22,23]. Considering this predicament in managing HCC using sorafenib alone, it is essential to explore alternative options such as investigating potentially druggable molecular targets or the administration of alternative drug regimens, to achieve an improved disease outcome. Recently, the

FDA approved lenvatinib (Lenvima) as an alternate first-line therapeutic agent demonstrated a non-inferior role in improving the overall survival of HCC patients relative to sorafenib[19,24]. Furthermore, for HCC patients not benefitting from sorafenib, regorafenib or nivolumab and ipilimumab are the approved second-line therapeutic agents[25,26]. Treatment with lenvatinib was found to have improved secondary endpoints including a higher objective response rate, longer progression-free survival and longer time to progression than patients treated with sorafenib alone[19], HCC patients not responding to first-line sorafenib treatment were found to have a better overall survival following the administration of second-line drugs[25,27,28]. Due to the limited options available for the systemic treatment of HCC patients, there is an immediate requirement to develop novel therapeutic compounds with high efficacy to improve disease management. In this review, we explore some of the novel molecular targets currently known in HCC. Emphasis will also be paid to the development and clinical application of personalized molecular targeted therapies as powerful therapeutic strategies to improve prognosis in HCC.

POTENTIAL DRUGGABLE MOLECULAR TARGETS IN HCC

An important aspect of cancer therapeutics is the development of targeted therapy that makes use of chemical compounds designed to regulate the activity of specific molecular targets involved in critical oncogenic signaling pathways that ultimately govern the proliferation, growth, survival and distant metastatic dissemination of cancer cells. Consequently, targeted therapy has the advantage of delivering focussed and powerful suppression of cancer development and progression, albeit with a lower toxicity to non-malignant cells; which is a common pitfall associated with systemic chemotherapy and radiotherapy. With an increase in our understanding of the molecular biology of HCC, many such druggable molecular targets associated with HCC genesis and progression have been identified. Key targets include: (1) Intracellular signaling proteins such as those involved in the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, ras/raf/mitogen-activated protein kinase (MAPK) pathway, Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway and Wnt/β-catenin pathway; (2) Angiogenic factors such as VEGF, fibroblast growth factor (FGF), angiopoietins, platelet-derived endothelial cell growth factor (PD-ECGF), heparanase, matrix metalloproteinases (MMPs), PDGFR, and COX-2; (3) Peptide growth factors and their receptors such as EGF and its receptor (EGFR), hepatocyte growth factor (HGF) and its receptor (c-Met), insulin-like growth factor (IGF) and its receptor (IGFR) and transforming growth factor-alpha (TGF-α); (4) Cell cycle regulators such as cyclins and cyclin-dependent kinases (CDKs); and (5) Transcription factors such as nuclear factor-kappa B and activator protein 2. The details of these targets have been comprehensively reviewed elsewhere[29-36]. Examples of the therapeutic agents against these molecular targets, currently in phase II/III clinical trials for the treatment of HCC are summarized in Table 1. However, the anti-tumour activity as well as the primary outcome measures, such as time to progression and overall response rate and safety level, exhibited by most of these compounds are either equivalent or significantly less than the effectiveness of sorafenib in HCC[37-40]. Consequently, it is important to identify novel molecular targets that are druggable in HCC. Table 2 summarizes potential pipeline compounds targeting novel targets that are a part of oncogenic signaling pathways in several cancers, including HCC. Given the importance of these oncogenic pathways in HCC development, these pipeline compounds hold promise as novel therapeutic strategies in HCC treatment. Hence, the following section specifically focuses on these targets to understand their role in HCC pathogenesis.

Hedgehog signaling pathway

The Hedgehog (Hh) pathway is an evolutionarily conserved signaling cascade that plays a critical role in early embryonic development and adult tissue homeostasis. Under normal circumstances, the adult liver does not manufacture the Hh protein unless the organ is undergoing regeneration after a partial hepatectomy[41]. However, recent evidence suggests that dysregulation of Hh signaling contributes to the development of HCC[42-44]. In its oncogenic role, the Hh protein impairs the inhibitory activity of patched homolog-1 (Ptch), resulting in the release of the proto-oncoprotein smoothened (Smo) from Ptch[42]. The released Smo subsequently induces the nuclear translocation of glioma-associated oncogene homolog (GLI) transcription factor, resulting in increased transcription of regulatory genes such as, cyclins and β-catenin

Table 1 Summary of current molecular targeted compounds under phase II/III clinical studies for the treatment of hepatocellular carcinoma

Drug	Targets	Descriptions	Ref./ClinicalTrials.gov identifier
Phase II			
Bevacizumab	VEGF	Monoclonal antibody Inhibits tumour growth of HCC cell line or patient-derived HCC xenografts Shows significant antitumour activity in patients with non-metastatic HCC, but serious bleeding complications occurs in 11% of patients.	[116-118]
Cediranib	VEGFR	Tyrosine kinase inhibitor Shows high toxicity and ineffective for patients with unresectable or metastatic HCC	[119]
Cetuximab	EGFR	Human-mouse chimeric monoclonal antibody Shows no obvious response in patients with advanced HCC	[120]
Dovitinib	c-KIT, Flt-3, FGFR, VEGFR	Multikinase inhibitor Significantly prolongs survival and inhibits primary tumour growth and lung metastasis in HCC xenograft models Shows less antitumour activity than sorafenib as a frontline systemic therapy for HCC	[38,121]
Erlotinib	EGFR	Tyrosine kinase inhibitor Shows modest prolonged progression-free survival and overall survival in patients with unresectable HCC	[122,123]
Gefitinib	EGFR	Tyrosine kinase inhibitor Inhibits tumour growth of HCC xenografts in mouse model	NCT00071994, [124]
Selumetinib	MEK1	Tyrosine kinase inhibitor Suppresses tumour growth of HCC xenografts in mouse model Shows inadequate antitumour activity with no radiographic response and short progression-free survival in patients with locally advanced or metastatic HCC	[125,126]
Phase III			
Brivanib	FGFR, VEGFR	Tyrosine kinase inhibitor Inhibits tumour growth of patient-derived HCC xenografts by increasing apoptosis, reducing microvessel density and decreasing VEGFR phosphorylation Shows promising antitumour activity in patients with advanced HCC	[127-129]
Linifanib	PDGFR, VEGFR	Receptor tyrosine kinase inhibitor Inhibits tumour growth of HCC xenografts in mouse model Shows similar overall survival in patients with advanced HCC as compared with sorafenib	[39,130,131]
Sunitinib	c-Kit, Flt-3, PDGFP, VEGFR	Multi-targeted receptor tyrosine kinase inhibitor Inhibits tumour growth of patient-derived HCC xenografts by increasing apoptosis and reducing microvessel density Shows significantly poorer overall survival than sorafenib in patients with advanced HCC, and shows more frequent and severe toxicity in treated patients	[132-134]
TSU-68 (Orantinib)	FGFR, PDGFR, VEGFR	Tyrosine kinase inhibitor Suppresses the tumour growth of subcutaneously co-injected HCC cell lines (Huh7/WI-38) xenografts	[135-137]

	Orantinib combined with TACE shows no improvement in overall survival in patients with unresectable HCC

EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; Flt-3: FMS-like tyrosine kinase-3; HCC: Hepatocellular carcinoma; MEK1: Mitogen-activated protein kinase (MAPK) kinase; PDGFR: Platelet-derived growth factor receptor; TACE: Transcatheter arterial chemoembolization; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

which promote cell cycle progression, a higher rate of cell proliferation and an associated tumour growth in HCC. Moreover, activation of the Hh signaling pathway also enhances the metastatic potential of HCC cells through focal adhesion kinase (FAK)/AKT and ERK-mediated production and activation of MMP-2 and MMP-9[45,46]. In addition to the Hh protein, mRNA levels of Ptch and GLI were found to be over-expressed in HCC and have been reported to serve as potential biomarkers to determine disease recurrence and overall survival following curative surgery[47]. In addition, blocking of the Hh signaling pathway by a Smo inhibitor (Vismodegib) has been found to exert anti-proliferative effects in HCC cells[42,48], suggesting that targeting the Hh signaling pathway is a potential therapeutic option for HCC patients.

Notch pathway

The Notch cell-cell signaling cascade is highly conserved and regulates cell fate, cell proliferation and cell death in several developmental and physiological processes[49]. Four Notch proteins are found in mammals and they are transmembrane proteins composed of a large extracellular domain for ligand binding and a cytoplasmic Notch intracellular domain (Nicd) for signal transduction. Mammalian Notch ligands include Delta-like ligand (DLL)1, DLL3, DLL4, Jagged1 and Jagged2 which are also membrane-bound. Therefore, activation of the Notch signaling pathway is mediated by ligand-receptor interaction between adjacent cells which leads to a conformational change in Notch receptors. After γ-secretase-induced cleavage of the Notch receptor, cytoplasmic Nicd is released and then translocated to the nucleus. Nuclear Nicd functions as a transcription factor to cause the transcription of its target genes including, HES-family members p21 and c-Myc[50].

Dysregulation of the Notch signaling pathway is observed in several types of cancers, including HCC. Aberrant expression of Notch receptors and its ligand Jagged1 has been detected in HCC tissues when compared with the adjacent non-malignant mucosae[51-54]. Activation of Notch signaling has also been reported to induce HCC tumour formation in mice[55]. Moreover, Notch signaling also contributes to enhancement of the oncogenic effects of HBV and HCV in HCC pathogenesis[56-58]. Several studies have verified that targeting critical members of the Notch signaling pathway represents a potential therapeutic avenue for HCC treatment. Giovannini *et al*[59] demonstrated that selective ablation of the Notch protein in combination with chemotherapeutics such as doxorubicin results in increased DNA damage, cellular apoptosis, and a concurrent decrease in cell cycle progression in HCC cells. Treatment with γ-secretase inhibitors (GSI) was found to inhibit growth of HCC cells *in vitro*[60,61]. Zhou and colleagues inhibited the Notch signaling pathway using DAPT which suppressed the invasion of HCC cells by impacting signaling of the extracellular signal-regulated kinases 1 and 2 (ERK1/2), thereby repressing the activity of MMP2, MMP9 and VEGF[62]. Active clinical studies on the use of GSIs such as MK-0752 and RO4929097 demonstrated a significant anti-tumour effect in different cancer models[63-66], which suggests its therapeutic potential in treating HCC.

Polo-like kinase 1

Polo-like kinase 1 (Plk1) is a serine/threonine kinase with peak expression during the mitotic phase of the cell cycle[67]. Plk1 functions as a cell cycle regulator promoting mitosis by modulating the activities of cell division cycle 25 homolog C (Cdc25C) and CDK1/Cyclin B[68,69]. Overexpression of Plk1 overrides the mitotic checkpoint which results in immature cell division and genetic instability leading to aneuploidies and tumour development[70]. In HCC, activation of Plk1 by HBx, a hepatitis B viral protein, was found to impair the DNA damage checkpoint and DNA repair pathways causing increased genetic instability and malignant transformation[71]. Consequently, Plk1 has been reported to be upregulated in numerous cancers, including HCC. In addition, a higher expression of Plk1 was found to predict poor prognosis in HCC[72-74]. Silencing Plk1 inhibited proliferation of HCC cells *in vitro* and *in vivo* by inducing G2/M arrest and enhanced apoptosis[75-77], suggesting that targeting Plk1 with small molecule inhibitors is a potential strategy for the treatment of HCC. Gilmartin *et al*[78] described a

Table 2 Summary of potential pipeline compounds targeting novel molecular targets in several cancers

Drug	Descriptions	Phase	Type of tumour	Ref./ClinicalTrials.gov identifier
Hh signaling pathway				
Erismodegib, (LDE-225)	Smo antagonist	0	Pancreatic cancer	NCT01694589
	In vitro and *in vivo* test results on HCC cells are not available	I	Advanced solid tumours	NCT00880308
		I	SCLC	NCT01579929
		II	Advanced or metastatic basal cell carcinoma	NCT01327053
		I/II	Medulloblastoma	NCT01125800
Vismodegib	Smo antagonist	I	HCC and lymphoma	NCT01546519
	Promotes regression of liver fibrosis and HCC tumour growth in a murine model of primary liver cancer[138]	I	Advanced or metastatic basal cell carcinoma	
	Shows no obvious response in patients with hepatic impairment[139]	II	Ovarian cancer	NCT00739661
Notch signaling pathway				
MK-0752	γ-secretase inhibitor	I	Advanced solid tumour	[63]
	In vitro and *in vivo* test results on HCC cells are not available	I	Brain and central nervous system tumours	[64]
RO4929097	γ-secretase inhibitor	I	Refractory metastatic or locally advanced solid tumours	[65]
	Prevents tumour development and decreases liver fibrosis in mouse model[141]	II	Metastatic colorectal cancer	[66]
Plk1				
HMN-214	Stilbene derivative interferes with the subcellular spatial distribution of Plk1 at centrosomes	I	Advanced solid tumours	[79]
	In vitro and *in vivo* test results on HCC cells are not available			
GSK461364	Reversible ATP-competitive Plk1 inhibitor	I	Advanced solid tumours and non-Hodgkin's lymphoma	[81]
	In vitro and *in vivo* test results on HCC cells are not available			
Arginine deprivation				
ADI-PEG-20	Arginine deiminase	I	Pediatric ASS-deficient tumour	NCT01528384
	Shows its safe and efficacious in stabilizing the progression of advanced HCC in an Asian population	II	SCLC	[142], NCT01266018
	Shows no overall survival benefit in second line setting for patients with advanced HCC	II	Advanced melanoma	[143]
		II	Malignant pleural mesothelioma	NCT01279967
		II/III	Advanced HCC	[87,144]
BCT100/ Peg-rhArg1	Recombinant human arginase I	I	Leukemia and lymphoma	NCT01551628
	Inhibits tumour growth of HCC xenografts in mouse model[85]	I/II	Advanced HCC	[90], NCT01092091
HDACs				
Resminostat	HDACs (1, 3 & 6) inhibitor[145]	I/II	Advanced HCC	[146]
	Combined with sorafenib shows no significant efficacy advantage over sorafenib monotherapy in patients with advanced HCC in East Asian populations	II	Hodgkin's lymphoma	NCT01037478
		I/II	Advanced colorectal carcinoma	NCT01277406
Chidamide	HDACs inhibitor (1, 2, 3 & 10)[147]	I	Advanced solid tumours and lymphomas	[148]
	Inhibits proliferation of HCC cells *in vitro*[95]			

Panobinostat,(LBH-589)	Pan-HDAC inhibitor Inhibits tumour growth and lung metastasis of HCC xenografts in mouse model[149]	I	Prostate carcinoma	[150]
		I	Advanced solid tumours	[151]
		II	Refractory metastatic renal cell carcinoma	[152]
Glypican-3				
Codrituzumab (GC33)	Anti-GPC3 monoclonal antibody Inhibits tumour growth of HCC xenografts in mouse model[102] Shows no clinical benefit in advanced HCC patients who has failed prior systemic therapy	I	Advanced or metastatic HCC	[153]

HCC: Hepatocellular carcinoma; HDAC: Histone deacetylase; Plk1: Polo-like kinase-1; SCLC: Small cell lung cancer; Smo: Proto-oncoprotein smoothened.

reversible ATP-competitive Plk1 inhibitor with a very high selectivity for Plk1 relative to other Plk subtypes or a panel of 48 other kinases that included CDK2/Cyclin A, MEK and serine/threonine kinase NEK2. Moreover, the authors demonstrate that the inhibition of Plk1 resulted in a dose-dependent arrest of cell cycle progression, leading to cell culture growth inhibition and tumour regression in xenograft models; while the toxicity of the drug in slow dividing non-cancerous cells was minimal. Therefore, GSK461364 offers the feasibility to overcome the limitation of traditional chemotherapy. Other phase I/II clinical studies of Plk1 inhibitors also demonstrated an anti-tumour effect by causing tumour regression and inhibition of tumour growth[79-82]. These studies suggest that Plk1 may be a potential therapeutic target in the treatment of HCC.

Arginine deprivation in arginine-driven HCC

Arginine is a semi-essential amino acid biosynthesized from citrulline in the urea cycle through the action of argininosuccinate synthetase (ASS-1), argininosuccinate lyase (ASL) and ornithine transcarbamylase (OTC)[83]. HCC is auxotrophic for arginine as it lacks the expression of ASS-1, ASL and/or OTC[84,85]. Therefore, enzymes capable of removing arginine can function as potential therapeutic agents in HCC. ADI-PEG-20 is an arginine deiminase (ADI) which has been shown to induce HCC regression through arginine depletion in ASS-deficient tumours[86,87]. For ASS-positive but OTC-deficient HCC, a recombinant human arginase I (rhArg1) has been shown to be potent in inhibiting HCC tumour growth[84,88-90]. A recent study by our group demonstrated that treatment with a pegylated rhArg1, BCT100, inhibits proliferation of HCC cells through an enhanced caspase-dependent apoptosis and induction of S-phase cell cycle arrest[85]. Moreover, the drug also inhibited xenograft tumour growth in a dose-dependent manner. At the molecular level, arginine deprivation was observed to inhibit the Wnt/β-catenin and Akt/mTOR signaling pathways with a concurrent downregulation of survivin and X-linked inhibitor of apoptosis (XIAP) expression[85]. Therefore, human recombinant arginase may be a potential agent in arginine-driven tumours such as HCC.

Histone deacetylases

One of the key regulatory mechanisms of gene expression is *via* epigenetic post-translational modifications of histone proteins. Among other covalent modifications, acetylation of the histones is a critical physiological process that is regulated by a balance between the activities of histone acetyltransferases and histone deacetylases (HDACs). Contrary to the acetyltransferases, HDACs work by removing acetyl groups from the lysine amino acid on the histone protein to increase the net positive charge on the histone tails, resulting in high-affinity binding between the histones and the DNA backbone. High HDAC activity results in a condensed and a transcriptionally inactive chromatin[91]. Moreover, aberrant expression of HDAC family members has been observed in multiple steps of cancer development including, cell proliferation, autophagy and cell cycle progression (HDAC 1, 2, 3 and 8), apoptosis (HDAC 1 and 2), differentiation (HDAC 3, 4, 5, and 8), angiogenesis (HDAC 4, 6, 7 and 10), migration (HDAC 6), and chemosensitivity (HDAC 1). The functional roles played by each family member of HDACs have been reviewed elsewhere in greater detail[92]. Dysregulated expression of HDACs has been found to correlate with a poor disease outcome in several cancers including HCC[92-94]. Specifically, upregulation of HDAC 3

and 5 mRNA expression was observed to be associated with DNA copy number gains in HCC[93]. Several HDAC inhibitors (HDACi) have been shown to have an anti-proliferative effect on HCC cells *in vitro* and *in vivo*. Panobinostat, a pan-HDAC inhibitor, has been found to enhance apoptosis and inhibit tumour growth in HCC cells through down-regulation of the anti-apoptotic protein survivin[93]. Chidamide, a benzamide type inhibitor of HDAC 1, 2, 3 and 10 subtypes, inhibits HCC cell growth by inducing cell cycle arrest at G0/1 phase by the up-regulation of p21[95]. Although most of the studies of HDACi in HCC are still at the pre-clinical stage, HDACi in HCC therapy has great potential.

Glypican-3

The glypican (GPC) family represents a group of cell-surface heparan sulphate proteoglycans which interact with growth factors, act as a co-receptor and modulate growth factor activity. Glypican-3 (GPC3), a carcinoembryonic antigen, promotes cell proliferation by modulating fibroblast growth factor 2 (FGF2) activity[96] and canonical Wnt signaling[97]. Interestingly, GPC3 is a transcriptional target of c-Myc, while the expression of c-Myc is under the regulation of GPC3[98]. This positive feedback loop between GPC3 and c-Myc also determines the oncogenic behaviour of GPC3. GPC3 is a diagnostic marker for HCC which is over-expressed in 70% of cases, while its expression is correlated with a poor outcome[99,100]. Silencing GPC3 in HCC cells induced apoptosis *via* the Bax/Bcl-2/cytochrome c/caspase-3 signaling pathway[101]. An antibody against GPC3 has also been developed, and it has been shown to cause antibody-dependent cell-mediated cytotoxicity in HCC cells[102]. In addition, due to its highly specific expression in HCC tumours, but not in the normal hepatocytes or benign hepatocellular mass lesions[103], GPC3 serves as a tumour-associated antigen which is an ideal target for immunotherapy. Tumour immunotherapy is the use of the host tumour-specific immune response to selectively target the tumour-associated antigens present on tumour cells. A phase I trial of a GPC3-derived peptide vaccine demonstrated measurable immune response and antitumor efficacy which correlated with overall survival in advanced stage HCC patients[104].

CANCER STEM CELLS AS THERAPEUTIC TARGETS FOR HCC TREATMENT

Cancer stem cells (CSCs) are a subpopulation of cancer cells possessing stem cell-like properties. Briefly, CSCs are tumour-initiating cells in the bulk of tumours that are capable of self-renewal and can divide and differentiate into multiple cell lineages. Markers of CSCs in HCC include ALDH, CD13, CD44, CD90, CD133, CD326 (EpCAM), and OV6, and a side population (SP) determined through an adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter[105,106]. CSCs also play a crucial role in tumour recurrence, metastasis and chemoresistance. A recent study reported that circulating $CD45^{-}CD90^{+}CD44^{+}$ CSCs can predict post-hepatectomy HCC recurrence[107]. Importantly, while systemic chemotherapy is effective in killing differentiated, fast-growing cancer cells, it induces chemoresistance and enriches the population of CSCs which significantly increases the risk of disease recurrence and metastasis. Ma *et al*[108] reported a CSC population in HCC characterized by their CD133 phenotype which were shown to survive chemotherapy of doxorubicin and fluorouracil with preferential expression of survival proteins involved in the AKT and Bcl-2 pathway. The authors further demonstrated that treatment with an AKT1 inhibitor significantly reduced the expression of these survival proteins, thereby enhancing the chemosensitivity of $CD133^{+}$ CSCs. In a different study, CD133+ cells were also observed to contribute to radio-resistance in HCC in a mouse xenograft model[109]. Other molecular pathways including TGF-β, Wnt, Notch and Hh, that are deregulated in HCC were also found in CSCs[105,110,111]. Therefore, molecular therapy that is targeted towards CSCs can assist in preventing tumour-initiation, recurrence, metastasis or even chemoresistance in HCC.

PERSONALIZED AND COMBINED MOLECULAR TARGETED THERAPIES IN HCC

Development of HCC is a multi-step process and the mechanisms involved in the initiation, progression and metastasis are not completely understood. Recent studies

have demonstrated the role of multiple signaling pathways that contribute to the pathogenesis of HCC. Although no single pathway is deemed dominant, the inhibition of a single pathway may induce a feedback mechanism within an alternate pathway resulting in a low response rate to monotherapy. For example, rapamycin up-regulates the expression and phosphorylation of PDGFRβ and the subsequent activation of the AKT and MAPK pathway through the PDGFRβ-dependent feedback loop results in rapamycin resistance[112]. Therefore, emphasis is focussed on a personalized and combined molecular targeted therapy as an ideal therapeutic strategy for HCC.

An *in vitro* study demonstrated that the level of EGFR expression predicts the cell line response to sorafenib treatment and the addition of gefitinib or erlotinib (EGFR inhibitors) or cetuximab (a monoclonal antibody against EGFR) significantly enhances the efficacy of sorafenib and a synergistic anti-proliferative effect is also demonstrated[113]. Therefore, by screening the EGFR status, we can predict the tumour's response to sorafenib treatment, and the addition of an EGFR inhibitor may help sensitize the tumour's response to sorafenib. However, an *in vivo* orthotopic model failed to demonstrate a synergistic anti-tumour effect of combination treatment with erlotinib and sorafenib[114]. A recent press release also reported that a large scale phase III clinical trial on the efficacy of combining erlotinib with sorafenib treatment in HCC (SEARCH trial, NCT00901901) failed to demonstrate any additional benefit on the overall survival of patients with unresectable HCC over sorafenib treatment alone[115]. Although these studies failed to show a clinical impact of one combined treatment in HCC, presently several clinical studies are evaluating alternate combination based molecular targeted therapies, examples of which are summarized in Table 3. Importantly, the success of personalized therapies in HCC heavily depends on the identification of novel biomarkers that provide critical information pertaining to the progress of the disease. As small tissue biopsy or fine-needle aspiration biopsy specimens are easily obtained, evaluation of biomarkers associated with crucial signaling pathways within these specimens can provide indications for treatment of these patients with drug combinations with/without locoregional therapies to maximize tumour response and survival rates.

CONCLUSION

HCC has been a cause of concern for a long time owing to a high rate of mortality and an overall poor outcome associated with the disease. Molecular investigations have indicated the dysregulation of several critical signaling pathways that contribute to the genesis and progression of HCC. Hence, the role of molecular therapy targeting pivotal members within these signaling pathways is undisputed. While monotherapy is frequently associated with a low tumor response rate and chemoresistance events, there is a need to explore and develop personalized and combined molecular targeted therapies as a powerful therapeutic strategy in HCC. Additionally, an increase in the discovery and clinical application of novel biomarkers that can speak volumes about the developing tumor would provide important information for guiding the clinician on the usage of appropriate personalized therapies in HCC.

Table 3 Clinical study of combined molecular targeted therapy based on sorafenib treatment for hepatocellular carcinoma

Drug + Sorafenib	Phase	Ref./ClinicalTrials.gov identifier
VEGF inhibitors		
Bevacizumab	I/II	NCT00867321
Lenvatinib	I/II (HCC)	NCT01271504
mTOR inhibitor		
Everolimus (RAD001)	II	NCT01005199
	I/II	[154]
Temsirolimus	I/II	NCT01335074, NCT01687673, NCT01008917
HDAC inhibitors		
Resminostat	II (Advanced HCC)	NCT00943449
Panobinostat	I (HCC)	NCT00823290
Anti-GPC3 antibody		
GC33	I	NCT00976170
MEK1 inhibitor		
Selumetinib (AZD6244)	I/II	NCT01029418
HGFR inhibitor		
Tivantinib (ARQ197)	I	NCT00827177
TNF-a secretion inhibitor		
Lenalidomide	I	NCT01348503
TRAIL receptor 1 antibody		
Mapatumumab	I/II	NCT00712855, NCT01258608

HGFR: Hepatocyte growth factor receptor; TNF-α: Tumour necrosis factor-α; TRAIL: Anti-TNF-related apoptosis-inducing ligand; HCC: Hepatocellular carcinoma; VEGF: Vascular endothelial growth factor; mTOR: Mammalian target of rapamycin.

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ORIGINAL ARTICLE

Retrospective Study

Effectiveness of a novel, fixed dose combination of netupitant and palonosetron in prevention of chemotherapy induced nausea and vomiting: A real-life study from India

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Abstract

BACKGROUND

A new, oral fixed dose combination of highly selective neurokinin-1 receptor antagonist, netupitant with 5HT3 receptor antagonist, netupitant and palonosetron (NEPA) was approved in India for prevention of chemotherapy induced nausea and vomiting (CINV).

AIM

To assess effectiveness of NEPA in real-world scenario.

METHODS

We retrospectively assessed the medical records and patient dairies of adult patients who received highly emetogenic or moderately emetogenic chemotherapy (HEC/MEC) and treated with NEPA (Netupitant 300 mg + Palanosetron 0.50 mg) for prevention of CINV. Complete response (CR) was defined as no emesis or no requirement of rescue medication in overall phase (0 to 5 d), acute phase (0-24 h) and delayed phase (2 to 5 d).

RESULTS

In 403 patients included in the analysis, mean age was 56.24 ± 11.11 years and 55.09% were females. Breast cancer (25.06%) was most common malignancy encountered. HEC and MEC were administered in 54.6% and 45.4% patients respectively. CR in overall phase was 93.79% whereas it was 98.01% in acute CINV and 93.79% in delayed CINV. Overall CR in HEC and MEC groups was 93.63% and 93.98% respectively. CR was more than 90% in different chemotherapy cycles except in group of patients of cycle 4 where CR was 88.88%.

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CONCLUSION
NEPA is a novel combination that is effective in preventing CINV in up to 93% cases treated with highly emetogenic or moderately emetogenic chemotherapy. This study brings the first real-life evidence of its effectiveness in India population.

Key words: Chemotherapy induced nausea vomiting; Netupitant; Palonosetron; Cancer; Chemotherapy

Core tip: A fixed-dose combination of Netupitant (300 mg) and Palonosetron (0.50 mg) indicated for the prevention of acute and delayed phase of nausea-vomiting in patients on highly and moderately emetogenic chemotherapeutic regimen was recently approved in India. There was no data on the effectiveness of this fixed dose combination in Indian patients in real world setting,the pervious data available was part of regulatory trial conducted in controlled environment, which may not give the real picture of the usage of the molecule in clinical setting. So to look for the effectiveness of the molecule in real world setting this study was conducted among.

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INTRODUCTION

Chemotherapy induced nausea and vomiting (CINV) is one of the most feared adverse events in various cancer chemotherapy regimens[1]. Evidence suggests that the incidence of acute CINV varies from 30% to 90% and that of delayed CINV is reported to be 28%-50%[2-4]. Rates of nausea (28.8% to 53.5%) and vomiting (9.4% to 19.2%) in the overall phase reported from Asia Paicifc region after first cycle of chemotherapy were varied[5]. A study from North India observed the CINV prevalence of 25.5%[6]. These data suggest that CINV may affect upto half of all the patients receiving highly-emetogenic or moderately-emetogenic chemotherapies (HEC/MEC).

Pathomechanistically, serotonin and substance P are major neurotransmitters involved in acute and delayed CINV. Serotonin binds to 5HT3 receptor present mainly in the gastrointestinal tract and Substance P binds with neurokinin-1 (NK1) receptors in the nucleus tractus solitarius and induces vomiting. Therefore, targeting serotonergic and neurokinin pathways are helpful in prevention of CINV[7]. The European Society of Medical Oncology and the Multinational Association of Supportive Care in Cancer guidelines recommend 5HT3 receptor antagonist, dexamethasone and NK1 receptor antagonist in acute CINV, whereas later two are advised in delayed CINV[8]. Recently, a new, oral fixed dose combination (FDC) of netupitant (highly selective NK1 receptor antagonist, 300 mg) with Netupitant and palonosetron (5HT3 receptor antagonist, 0.5 mg) (NEPA) was approved in India[9]. NEPA + DEX has been found to be clinically superior to monotherapy of palonosetron + DEX in preventing both acute and delayed CINV[10,11]. Being a recent and novel FDC antiemetic with limited evidence in Indian setting, there is need to further understand its efficacy and safety. Hence, we planned this observational study to determine efficacy of NEPA in prevention of CINV.

MATERIALS AND METHODS

Study design

This single-centre, retrospective study was conducted in patients treated with HEC/MEC.

Ethics

Study was initiated after the approval from independent ethics committee and was conducted according to good clinical practice and applicable regulatory guidelines.

Setting

This study was conducted in tertiary care centre in Hyderabad, India. This centre provides super-specialty services in management of various malignancies. It caters to the urban, semi urban and rural population.

Participants

Adults aged > 18 years of either sex who were treated with HEC/MEC and prescribed NEPA irrespective of the number of chemotherapy cycles from June 2019 to December 2019 were identified from the patient database at our centre. Any patient treated with low-emetogenic chemotherapy or those who received chemotherapy with minimal emetogenic potential were excluded.

Treatment schedule in participants

After identifying the patients from the database, their demographic and baseline data mentioned in medical records was captured in structured case record form. Demographic data included age, gender, and clinical data on type of chemotherapy, current number of cycles, *etc*. were noted. As a standard practice, the given treatment schedule was followed in all patients for prevention of CINV.

Before initiating chemotherapy, all patients were treated with a single oral capsule of netupitant 300 mg and palonosetron hydrochloride 0.5 mg. After 60 min, chemotherapy was initiated. Dexamethasone (12 mg intravenous once) was concomitantly administered intravenously in all patients. Data on nausea and vomiting was captured by patients in patient diaries which were available with their medical records. From these diaries, events of nausea and vomiting were identified during first 24 h and over day 1 to day 5. Events that occurred within first 24 h were considered as acute CINV and those between day 2 and day 5 were considered as delayed CINV (Figure 1).

Outcome measurement

The main outcome assessed was complete repose (CR) to NEPA. CR was defined as no emesis or no requirement of rescue medication. CR was determined in acute phase (0-24 h), delayed phase (24-120 h) and in overall phase (0-120 h). Overall CR was primary outcome measure. Effect of study drug was also evaluated by emetogenicity of chemotherapy as high and moderate as well as in by the cycle of chemotherapy.

RESULTS

Baseline characteristics

In total, 403 patients were identified and analysed. Baseline characteristics of the study patients are shown in (Table 1). Mean age of the participants was 56.24 ± 11.11 years with majority being in age group of 51 to 65 years (51.36%). Proportion of females was slightly higher than males (55.09% *vs* 44.91% respectively). Among study participants, most common malignancy was of breast (25.06%) followed by colon (15.63%), oral cavity (10.66%) and others as shown in (Table 1). 54.6% patients had received HEC whereas remaining were treated with MEC. Also, patients were in different cycles of chemotherapy regimens as shown in (Table 1).

Outcome assessment

CR in overall population: For overall phase, the CR in our study was 93.79%. CR in acute and delayed phase CINV was 98.01% and 93.79% respectively (Table 2).

CR as per emetogenic potential of chemotherapy: We further analysed the CR according the chemotherapy regimen. In participants who received HEC (n = 220), overall CR was observed in 93.63% whereas 97.27% had CR in acute phase, and 93.63% had CR in delayed phase. Similarly, in patients receiving MEC (n = 183), overall response was seen in 93.98% whereas CR in acute and delayed CINV was 98.90% and 93.98% respectively.

CR as per number of chemotherapy cycles: All the enrolled participants were on

Table 1 Baseline characteristics of enrolled patients

Characteristics	Observations
Age (yr)	
mean ± SD	56.24 ± 11.11
Age groups	
≤ 35	16 (3.97)
36-50	97 (24.06)
51-65	207 (51.36)
66-80	75 (18.61)
Gender	
Male	181 (44. 91)
Female	222 (55.09)
Type of cancer	
Breast	101 (25.06)
Colon	63 (15.63)
Oral	43 (10.66)
Lung	29 (7.19)
Gall bladder	24 (5.95)
Epiglottis	13 (3.2)
Cervix	12 (2.97)
Rectum	12 (2.97)
Others[1]	106 (26.03)
Chemotherapy	
Highly emetogenic	220 (54.6)
Moderately emetogenic	183 (45.4)
Chemotherapy cycles	
1	75 (18.61)
2	89 (22.08)
3	30 (7.44)
4	90 (22.33)
5	52 (12.90)
> 5	67 (16.62)

Data presented as mean±standard deviation or frequency (%); Baseline demographic characteristics of patients enrolled in the study, distribution of there age (mean ± standard deviation), gender, type of cancer, type of chemotherapy and the chemotherapy cycle.
[1]Others- Includes following cancers-Endometrial; Larynx, Stomach; B cell lymphoma; Ewing's Sarcoma; Tonsil; Osteoblastoma; Mediastinal lymphadenopathy; Peri ampullary; Testis; Pyloric antrum; Pyriform fossa; Oropharynx; Ovary; Pancreas.

various cycles of chemotherapy (Tables 1 and 3). Overall CR was 90% or more in all groups of chemotherapy cycles except in the group of patients with 4 cycles in whom overall CR was 83%. Similarly, the CR in acute CINV was over 90% in all chemotherapy cycle groups except patients who had 4 chemotherapy cycles in whom CR in acute CINV was 88.88%. Acute CINV CR was 100% in patients who had 5 chemotherapy cycles. CR in the delayed CINV phase was similar to overall CR in all chemotherapy cycle groups.

Table 2 Outcome assessments

Population	Number of participants	Acute phase, Number of participants (%)	Delayed phase, Number of participants (%)	Overall phase, Number of participants (%)
Overall	403	397 (98.01)	378 (93.79)	378 (93.79)
Highly emetogenic chemotherapy	220	214 (97.27)	206 (93.63)	206 (93.63)
Moderately emetogenic chemotherapy	183	181 (98.90)	172 (93.98)	172 (93.98)

Complete response rate in acute delayed and overall phase among patients on highly and moderately emetogenic chemotherapy regimen.

Table 3 Complete response rate among enroled patients

Chemothrapy cycle	Number of participants	Acute phase-number of participants (%)	Delayed phase-number of participants (%)	Overall phase-number of participants (%)
1	75	73 (97.33)	68 (90.66)	68 (90.66)
2	89	88 (98.87)	86 (96.22)	86 (96.22)
3	30	28 (93.33)	27 (90.00)	27 (90.00)
4	90	80 (88.88)	75 (83.00)	75 (83.00)
5	52	52 (100.00)	51 (98.07)	51 (98.07)
> 5	67	65 (97.01)	63 (94.02)	63 (94.02)

Complete response rate in acute, delayed and overall phase among patients enrolled in various cycles of chemotherapy.

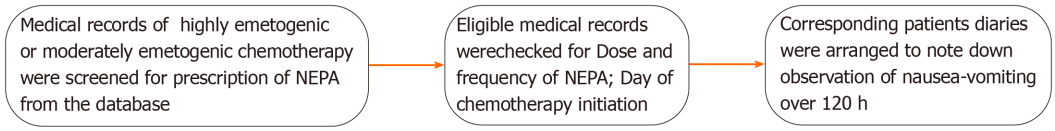

Figure 1 Study flow chart. Study flow: Medical records and patient dairies were evaluated for incidence and severity of nausea-vomiting, time period was form the time chemotherapy was administered to 120 h (day 1 to day 5) to look for complete response in acute, delayed and overall phase. NEPA: Netupitant and palonosetron.

DISCUSSION

Combination of netupitant and palonosetron is first of its own kind FDC for prevention of CINV. In this study, we demonstrated that NEPA was effective in preventing CINV as shown by CR of 93.79% in overall and delayed phase with CR of 98.01% in acute phase. Compared to the finding of Hesketh *et al*[10] who observed CR in 89.6% patients, CR in our study was substantially higher. This is probably attributable to the differences in participants in two studies as Hesketh *et al*[10] included patients receiving HEC only. Badalamenti *et al*[12] (2019) also reported overall CR in first chemotherapy cycle to be 88.9%[12]. This indicates overall excellent efficacy of NEPA in preventing acute and delayed phase CINV. The combination has also been found to be more effective than monotherapy with palonosetron. In randomized, double-blind, study involving patients on MEC, Aapro *et al*[13] demonstrated that the CR in overall phase, acute phase and delayed phase was 74.3%, 88.4% and 76.9% in NEPA group and 66.6%, 85.0% and 69.5% with palonosetron monotherapy. Dexamethasone was co-administered in both treatment groups[13]. Hesketh *et al*[10] also reported NEPA was superior to palonosetron in preventing CINV in patients receiving HEC[10]. This suggest that NEPA is highly effective in preventing CINV in any level of emetogenic chemotherapy. Further, CINV due to chemotherapy can lead to reduced quality of life, impairment in home and occupational activities, may add to increased cost and cause organ damage in the long run, preventing CINV is one of the primary goals of therapy[14-17]. Therefore, single oral dose of NEPA can contribute the improved quality

of life of patients receiving chemotherapeutic regimens.

We observed persistent CR in patients from different number of chemotherapy cycles suggesting that effectiveness of NEPA is not affected in repeated administration or initiating at any chemotherapy cycle. The overall CR in first cycle was similar to those who had more than five chemotherapy cycles. Similar finding was observed by Gralla *et al*[18] in evaluation of patients receiving HEC or MEC. They found consistent overall CR which was 81%, 86%, 91%, 90%, 92% and 91% in cycles 1, 2, 3, 4, 5, and 6 of chemotherapy[18]. Combined with our observation, the evidence is clear that NEPA is highly effective in preventing CINV over multiple cycles of HEC/MEC. This has important clinical implications as single dose is effective and there is no need of repeat administration or rescue medications. With improved patient education, compliance to chemotherapy regimens can be improved substantially with appropriate intake of antiemetics[19].

We observe certain strengths and limitations in our study. Study has inherent limitations of retrospective design. We assessed the response acute and delayed phase but its efficacy in anticipatory, breakthrough, and refractory CINV in Indian population require further assessment. Although efficacy in low emetogenic chemotherapy was not assessed, NEPA is expected to be efficacious in these group of patients as it had proved its efficacy in HEC/MEC. Further, age and gender difference in efficacy as well as efficacy in different tumours can be assessed to identify population that can get most benefited with use of NEPA. Also, we did not compare the efficacy with existing therapies which would have provided more insights in understanding the benefits with NEPA. Nonetheless, our initial experience with NEPA suggests its effective utility in preventing CINV in HEC/MEC.

A novel FDC of netupitant and palonosetron has been approved for prevention of CINV. We observed that this FDC is effective in preventing CINV in patients receiving HEC/MEC with complete response rate of 93.79% with near complete response in acute phase of CINV. Also, the response was maintained irrespective of HEC or MEC administration as well as repose was consistent across number of chemotherapy cycles. Thus, in real-world setting, we find that NEPA is effective for preventing CINV over multiple cycles of highly or moderately emetogenic potential chemotherapy regimens. These finding need to be further confirmed in larger, randomized, comparative studies.

ARTICLE HIGHLIGHTS

Research background

Chemotherapy induced nausea and vomiting (CINV) is one of the most feared adverse events with patient receving chemotherapy regimens. Pathomechanistically, serotonin and substance P are major neurotransmitters involved in acute and delayed CINV, targeting both optimizes CINV control. NEPA, an oral fixed dose combination Netupitant (300 mg) and Palonosetron (0.50 mg), was recently approved in India for the management of CINV. Hence there was a need to evaluate the effectiveness of NEPA in Indian setting in real world scenario.

Research motivation

To analyse the effectiveness of NEPA in prevention of CINV among Indian patients who have received highly and moderately emetogenic chemotherapy regimen.

Research objectives

To elucidate the clinical effectiveness of NEPA, in terms of the complete response in acute-delayed and overall phase of nausea-vomiting irrespective of the chemotherapy cycle. Thereby, we hope to generate the real world evidnce on the usefulness of NEPA in the management of CINV patients in India.

Research methods

Medical records and patient diaries of adults cancer patients who were treated with highly emetogenic or moderately emetogenic chemotherapy and received NEPA irrespective of the number of chemotherapy cycles from June 2019 to December 2019 were retrieved. Relevant clinical variables such as presence or absence of nausea-vomiting and if present, the severity of nausea on visual analog scale and cycle wise distribution of the data were captured.

Research results

The study demonstrated that complete response in overall phase was 93.79% whereas it was 98.01% in acute CINV and 93.79% in delayed CINV. Overall complete response in highly emetogenic chemotherapy group of patients was 93.63% and in moderately emetogenic group of patients was 93.98%.

Research conclusions

We found that the oral fixed dose combination of netupitant 300 mg and palonosetron hydrochloride 0.5 mg is effective in preventing CINV in patients receiving highly or moderately emetogenic chemotherapy regimen in the real world setting. Also, the response was consistent across number of chemotherapy cycles.

Research perspectives

This study demonstrated the clinical effectiveness of NEPA among Indian patients in managing CINV, and serves as an impetus for future research.

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ORIGINAL ARTICLE

Observational Study

Mutational analysis of *Ras* hotspots in patients with urothelial carcinoma of the bladder

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Author contributions: Tripathi K contributed to the conception and design of the study; Tripathi K performed the experiments and participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Goel A and Singhai A revised the article critically for important intellectual content; Garg M contributed to the conception and design of the study and revised the article critically for important intellectual content; all the authors approved the final version of the article to be published.

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Institutional review board statement: This study was approved by the appropriate institutional research ethics committee and was performed in accordance with the ethical standards as laid down in the 1964

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Abstract

BACKGROUND

Mutational activation of *Ras* genes is established as a prognostic factor for the genesis of a constitutively active RAS-mitogen activated protein kinase pathway that leads to cancer. Heterogeneity among the distribution of the most frequent mutations in *Ras* isoforms is reported in different patient populations with urothelial carcinoma of the bladder (UCB).

AIM

To determine the presence/absence of mutations in *Ras* isoforms in patients with UCB in order to predict disease outcome.

METHODS

This study was performed to determine the mutational spectrum at the hotspot regions of *H-Ras*, *K-Ras* and *N-Ras* genes by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing followed by their clinical impact (if any) by examining the relationship of mutational spectrum with clinical histopathological variables in 87 UCB patients.

RESULTS

None of the 87 UCB patients showed point mutations in codon 12 of *H-Ras* gene; codon 61 of *N-Ras* gene and codons 12, 13 of *K-Ras* gene by PCR-RFLP. Direct DNA sequencing of tumor and normal control bladder mucosal specimens followed by Blastn alignment with the reference wild-type sequences failed to identify even one nucleotide difference in the coding exons 1 and 2 of *H-Ras*, *N-Ras* and *K-Ras* genes in the tumor and control bladder mucosal specimens.

Declaration of Helsinki and its later amendments or comparable ethical standards. Ethical clearance was obtained from Bioethics Cell, Institutional Ethics Committee (IEC), KGMU (Reference no. 89th ECM II A/P8), Lucknow, India.

Informed consent statement: Subjects were not required to give informed consent as the analysis used anonymous data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All the authors of the study declare no potential conflict-of-interest.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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CONCLUSION

Our findings on the lack of mutations in *H-Ras*, *K-Ras* and *N-Ras* genes could be explained on the basis of different etiological mechanisms involved in tumor development/progression, inherent genetic susceptibility, tissue specificity or alternative *Ras* dysfunction such as gene amplification and/or overexpression in a given cohort of patients.

Key words: Coding exons; Oncogenic activation; Polymerase chain reaction - restriction fragment length polymorphism; Point mutations; *Ras* genes; Urothelial carcinoma of bladder

Core tip: Mutant *Ras* has been shown to be associated with drug resistance, enhanced metastasis and shorter survival of patients. Due to reported heterogeneity among the distribution of the most frequent mutations in *Ras* isoforms in different patient populations with urothelial carcinoma of the bladder, it is necessary to examine these patients for *Ras* mutations in order to predict disease outcome. Our findings on the lack of *Ras* mutations could be explained on the basis of different etiological mechanisms involved in tumor development, inherent genetic susceptibility, tissue specificity or alternative *Ras* dysfunction including gene amplification or overexpression in a given cohort of patients.

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INTRODUCTION

Urinary bladder cancer is the second most common genitourinary cancer globally and its occurrence has very high gender variability (http://cancerindia.org.in/globocan-2018-india-factsheet/). It is the sixth most common cancer in men and the seventeenth most common cancer in women. The etiology of bladder cancer is very complex. Among many factors, tobacco chewing/smoking and environmental or occupational exposure to a number of carcinogens have been identified as the most important risk factors for bladder cancer[1-3].

Urothelial carcinoma of the bladder (UCB) originates in the cells of the innermost layer of bladder urothelium and accounts for approximately 90% of all bladder cancers. Clinically, two distinct forms of UCB namely, non-muscle invasive bladder cancer (NMIBC in 75%-80% of patients) and muscle invasive bladder cancer (MIBC in 20%-25% of patients) develop along papillary and non-papillary pathways[4]. Patients diagnosed with NMIBC can be successfully treated. Nevertheless, these tumors have a higher tendency to recur (50% to 90%) and 15% progress to invasive and metastatic tumors. Morbidity and mortality are associated with the high grade, non-papillary, muscle invasive form of the disease. Molecular studies to characterize the genotypic differences in the pathogenesis of NMIBC and MIBC may improve the diagnostic/prognostic outcome of the disease.

Rat sarcoma viral oncogene homolog (*Ras*) belongs to the family of small G proteins with intrinsic GTPase activity that governs various cellular signal transduction pathways. Alterations in the expression or functions of (*Ras*) genes caused by various point mutations within the gene have been established as prognostic factors in the genesis of a constitutively active RAS-mitogen activated protein kinase pathway that leads to cancer. Point mutations within the hotspot regions of *Ras* gene lead to reduced intrinsic GTPase activity, the protein is locked into a constitutively active state and results in aberrant cell signaling even in the absence of external signals[5]. *In vitro* and *in vivo* studies on tumor regression upon withdrawal of *Ras* expression indicate that mutant *Ras* is a therapeutically useful drug target even in advanced metastasis[6]. Mutant *Ras* gene has been shown to be associated with drug resistance, enhanced metastasis, poor prognosis and shorter survival of patients[7].

Approximately 30% of human cancers are known to harbor genomic mutations in

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the three functional isoforms of Ras genes (*H-Ras*; located at 11p15.5; *K-Ras*; located at 12p12.1; and *N-Ras*; located at 1p13.2). The most common mutational hotspots in the codons for amino acid residues 12, 13 or 61 are confined to exon 1 or 2 of *H-Ras* (G12V, G12S, G12A, G12D, G13D, Q61R); *K-Ras* (G12D, G12S, G12R, G12A, G12V, G12C, G13D); and *N-Ras* (G12D, Q61N, Q61L, Q61K). Tissue and organ specificities of *Ras* gene activation have been reported to vary with mutated codon and type of *Ras* gene isoform. *K-Ras* mutations occur frequently in non-small cell lung, colorectal, and pancreatic carcinomas; *H-Ras* mutations are common in bladder, kidney, and thyroid carcinomas; while *N-Ras* mutations have been identified in melanoma, hepatocellular carcinoma, and hematologic malignancies[8,9].

Published studies provide conflicting results regarding the frequency distribution of *Ras* mutational spectrum in UCB patients[10-14]. Out of a total of 11.67% mutations in exon 1 of *K-Ras*, maximum mutations were reported at codon 12 in bladder cancer patients[15]. Iranian patients with bladder cancer did not exhibit any mutation in the hotspot codons (12, 13, and 61)[16]. Various studies have examined 45%, 46.7% and 39% of *H-Ras* mutations in codon 12 in bladder cancer patients[17-19]. Due to the reported heterogeneity in the distribution of the most frequent mutations in *Ras* isoforms in bladder cancer specimens, it is necessary to examine the presence/absence of mutations in order to predict disease outcome[10,11].

Speculating the role of mutant *Ras* in bladder tumorigenesis, the present study has been conducted to determine its clinical impact by examining the relationship between clinical histopathological variables in UCB patients and the mutational spectrum. Frequency distribution and prevalence of mutations in the hotspot regions of *H-Ras* codon 12 (glycine to valine/serine/alanine/aspartic acid), *K-Ras* codon 12 (glycine to valine/aspartic acid/serine/arginine/alanine/cysteine), *K-Ras* codon 13 (glycine to aspartic acid) and *N-Ras* codon 61 (glutamine to lysine/arginine) were examined by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) in a cohort of 87 North Indian UCB patients and 23 controls with normal bladder mucosa. The results were confirmed by direct DNA sequencing of coding exons 1 and 2 of *H-Ras*, *K-Ras* and *N-Ras* genes.

MATERIALS AND METHODS

Patients and controls

Patients were enrolled in the Urology OPD at King George's Medical University (KGMU), Lucknow during 2018-2019. All 87 patients examined had symptoms of hematuria as a major sign followed by urinary frequency or irritative symptoms and were assessed for primary tumor. Patients underwent bimanual examination under anesthesia before and after endoscopic surgery (biopsy or transurethral resection) or histological verification of the absence or presence of tumor. Imaging of the chest, abdominal ultrasound and computed tomography of the abdomen (whenever required) were performed to detect common metastatic sites as well as lymph node involvement. Tumor tissues from 42 NMIBC (stage pTa-pT1) and 45 MIBC (stage pT2-pT4) were obtained after transurethral resection of the bladder tumor. Tissues were collected in RNAlater, snap frozen and stored at -80°C for future use. Clinical data on the UCB patients and pathological classification/records based on pathological TNM staging were provided by the Department of Urology and Department of Pathology, KGMU, Lucknow. After informed consent, normal bladder mucosal tissues were collected from 23 benign prostate hyperplasia (BPH) patients during cold cup biopsy. These patients underwent transurethral resection of the prostate for BPH and had known bladder lesions. Pathologists independently diagnosed and classified bladder tumors according to World Health Organization and International Society of Urologic Pathology 2004 classification system[20]. Ethical clearance was obtained from Bioethics Cell, Institutional Ethics Committee, KGMU (reference no. 89th ECM II A/P8).

DNA extraction

Genomic DNA was extracted from 87 UCB and 23 control bladder mucosal tissues using proteinase K and phenol-chloroform extraction, followed by ethanol precipitation, and was quantified and then stored at -20°C.

PCR-RFLP

PCR was performed to amplify DNA segments which span (1) codon 12 of *H-Ras* gene; (2) codon 12 and codon 13 of *K-Ras* gene; and (3) codon 61 of *N-Ras* gene in 87 UCB and 23 bladder mucosal tissues. The primer sequences used are listed in Table 1.

Table 1 Primer sequences used for polymerase chain reaction - restriction fragment length polymorphism

Gene	Target codon	Strand	Primer sequences
H-Ras	12	+	5′GACGGAATATAAGCTGGTGG 3′
		-	5′AGGCACGTCTCCCCATCAAT 3′
K-Ras	12 and 13	+	5′ACTGAATATAAACTTGTGGTAGTTGGACCT 3′
		-	5′TTCTCCATCAATTACTACTTGCTTCCTGTA 3′
N-Ras	61	+	5′GACATACTGGATACAGCTGGC 3′
		-	5′CCTGTCCTGATGTATTGGTC 3′

+: Forward strand; -: Reverse strand.

PCR was carried out with 200 ng of DNA, 10 pmol of primer(s), and Emerald Amp max PCR master mix (TaKaRa, Clontech) using a thermal cycler (T100™, BioRad, United States). Cycling conditions included initial denaturation at 98°C for 20 s, followed by 30 cycles of [denaturation: 98°C for 10 s, annealing: 60°C (for *H-Ras* and *N-Ras*) and 58°C for *K-Ras* for 30 s, and extension: 72°C for 30 s] followed by a final extension at 72°C for 5 min.

Restriction endonucleases MspI (Thermo Scientific), BstNI (Thermo Scientific), HphI (Thermo Scientific), and MScI (Thermo Scientific) were used to digest amplified PCR fragments containing codon 12 of *H-Ras*, codon 12 of *K-Ras*, codon 13 of *K-Ras*, and codon 61 of *N-Ras*, respectively. Buffers and incubation conditions (37°C for 1-16 h) were used according to the manufacturers' recommendations. The digested and undigested fragments were subjected to electrophoresis on 3% agarose gel. A summary of the *Ras* gene assays is described in Table 2.

Direct DNA sequencing

Coding exons 1 and 2 each of *H-Ras*, *N-Ras* and *K-Ras* were amplified by the laboratory developed primer pairs (Table 3). Primers were designed for the GenBank reference sequence of *H-Ras*, *N-Ras* and *K-Ras* (accession numbers: NM_001130442.1.1, NM_004985.4.1, NM_002524.4.1, respectively) by Primer plus software. The 200 ng of DNA was amplified with 10 pmol primer using the Phusion high-fidelity PCR kit (Thermo Scientific). The thermal profile included initial denaturation at 98°C for 40 s, followed by 35 cycles of (1) *H-Ras*: [denaturation: 98°C for 5 s, annealing: 63.2°C (for exon 1) and 64.8°C (for exon 2) for 10 s, and extension: 72°C for 15 s]; (2) *N-Ras*: [denaturation: 98°C for 5 s, annealing: 62.1°C (for exon 1) and 61.4°C (for exon 2) for 10 s, and extension: 72°C for 15 s]; (3) *K-Ras*: [denaturation: 98°C for 5 s, annealing: 61.8°C (for exon 1) and 61.3°C (for exon 2) for 10 s, and extension: 72°C for 15 s]; followed by a final extension at 72°C for 7 min. Amplified PCR products were electrophoresed on 2% agarose gel, eluted and purified with a QIAquick® PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing reactions were performed for both the DNA strands by the BigDye™ Terminator v1.1 Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Monza, Italy) on a total of 10 ng of purified PCR products. Sequence analysis was performed using a 3500 Genetic Analyzer. The files/electropherogram obtained were analyzed by seq scap_v5.2 software. Sequence results of bladder mucosa were aligned with the reference sequences (mentioned above) using Blastn. Furthermore, DNA sequences of the respective regions in bladder tumor specimens were compared with that of wild-type sequences to examine the presence/absence of mutations in the coding exons 1 and 2 of the *H-Ras*, *N-Ras* and *K-Ras* genes.

RESULTS

Clinical histopathological summary of patients

The mean age of the patients included in the study was 58.3 years (range: 25-83 years) and 44 (50.17%) patients were older than 60 years. The male to female ratio was 11:1. Of 87 patients, 66 (75.86%) had a positive history of either smoking or a tobacco chewing habit. Twenty of 87 tumors (22.98%) were more than 3 cm in size, whereas 67 tumors (77.01%) were less than 3 cm. Pathologically, 42/87 (48.27%) tumors were

Table 2 Summary of *Ras* gene assays

Gene	Target codon	Restriction enzyme/site	Fragment size		
			Undigested	Mutant after digestion	Wild-type/normal after digestion
K-Ras	12 Glycine (GGT) to Valine (GTT)/Aspartic acid (GAT)/Serine (AGT)/Arginine (CGT)/Alanine (GCT)/Cysteine (TGT)	MvaI (BstNI) CC↓ WGG GGW↑ CC	144 bp	144 bp	115 bp and 29 bp
K-Ras	13 Glycine (GGC) to Aspartic acid (GAC)	HphI GGTGAN8↓ CCACTN7↑	144 bp	101 bp and 43 bp	144 bp
H-Ras	12 Glycine (GGC) to Valine (GTC)/Aspartic acid (GAC)/Serine (AGC)/Alanine (GCC)	MspI (HpaII) (C↓ CGG) (GGC↑ C)	420 bp	420 bp	390 bp and 30 bp
N-Ras	61 Glutamine (CAA) to Arginine (CGA)/Lysine (AAA)/Leucine (CTA)	MlsI (MscI) (TGG↓ CCA) (ACC↑ GGT)	65 bp	65bp	44 bp and 21 bp

bp: Base pair.

Table 3 Primer sequences used in direct DNA Sequencing

Gene	Strand	Coding exon	Primer sequences	Length of amplified fragment
K-Ras	+	1	F 5'-TTAACCTTATGTGTGACATGTTCTAA-3'	378 bp
K-Ras	-	1	R 5'-CCCTGACATACTCCCAAGGA-3'	
K-Ras	+	2	F 5'- TCAAGTCCTTTGCCCATTTT-3'	375 bp
K-Ras	-	2	R 5'- TGCATGGCATTAGCAAAGAC-3'	
N-Ras	+	1	F 5'-GCCCAAGGACTGTTGAAAAA-3'	477 bp
N-Ras	-	1	R 5'-TGCATAACTGAATGTATACCCAAAA-3'	
N-Ras	+	2	F 5'-GGCAGAAATGGGCTTGAATA-3'	424 bp
N-Ras	-	2	R 5'-CCTAAAACCAACTCTTCCCATAA-3'	
H-Ras	+	1	F 5'-GTGGGTTTGCCCTTCAGAT-3'	386 bp
H-Ras	-	1	R 5'-TCTAGAGGAAGCAGGAGACAGG-3'	
H-Ras	+	2	F 5'-CAGGACACAGCCAGGATAGG-3'	492 bp
H-Ras	-	2	R 5'-ACATGCGCAGAGAGGACAG-3'	

F: Forward strand (+); R: Reverse strand (-); bp: Base pair.

classified as NMIBC and 45/87 (51.72%) as MIBC. According to the histopathological classification, 80.95% (34/42) non-muscle invasive tumors were of low grade and 19.04% (8/42) were of high grade. All MIBC patients had a high grade tumor. Of 87 tumors, 26/87 (29.88%) were recurrent type, while the remaining 61/87 (70.11%) were identified as primary tumors (Table 4).

Point mutation detection in H-Ras

PCR-RFLP was carried out to examine the point mutation in codon 12 of *H-Ras* gene in 87 bladder tumor tissues and 23 normal bladder mucosal tissues. Digestion of the wild-type amplicon of 420 bp by MspI gave rise to two bands of 390 bp and 30 bp. The presence of a point mutation at codon 12 results in loss or modification of the endonuclease recognition site which is indicative of the translational change of glycine

Table 4 Clinicohistopathological profile of patients with urothelial carcinoma of bladder

Clinicohistopathological variables	*n* (%)
Total no. of patients	87 (100)
Age (yr) mean, range	58.3, 25-83
$n < 60$	44 (50.17)
$n \geq 60$	43 (49.42)
Gender	
Male	81 (93.10)
Female	8 (9.19)
Hematuria	
Present	87(100)
Absent	Nil
No information	Nil
Smoking/Tobacco chewing status	
Smokers	66 (75.86)
Non-smokers	21 (24.1)
Tumor grade	
Low	34 (39.04)
High	53 (60.91)
Tumor stage	
Ta-T1 (Low/NMIBC)	42 (48.27)
T2-T4 (High/MIBC)	45 (51.72)
Tumor type	
Primary	61 (70.11)
Recurrent	26 (29.88)
Tumor Size	
> 3 cm	20 (22.98)
< 3 cm	67 (77.01)

NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer.

(GGC) to serine (AGC)/valine (GTC)/alanine (GCC)/aspartic acid (GAC). In our study, none of the tumors were examined for the presence of point mutation at codon 12 of *H-Ras* gene (Figure 1A).

Direct DNA sequencing of the coding exonic region 1 spanning the codons 12, 13 and exon 2 containing hotspot codon 61 of *H-Ras* gene was performed. Blastn results of DNA sequences in all the tumor specimens showed 100% alignment with that of the wild-type. Electropherogram analysis did not identify the presence of any point mutations in exons 1 and 2 of *H-Ras* genes in the tumor specimens (Figure 1B and C).

Point mutation detection in N-Ras

Tumor specimens from 87 UCB patients and 23 normal bladder mucosal tissues were examined by PCR-RFLP for the presence or absence of specific point mutations at codon 61. The presence of a point mutation at codon 61 may result in the conversion of glutamine (CAA) to lysine (AAA)/arginine (CGA)/leucine (CTA). The proper restriction site (*TGG↓CCA)* was created by changing only one nucleotide in a forward primer just before the start of codon 61. Restriction digestion of the wild-type amplicon of 65 bp by enzyme MscI resulted in its cleavage into 21 bp and 44 bp (Figure 2A). The present study failed to detect the presence of point mutations in 87 UCB and 23 normal mucosal specimens.

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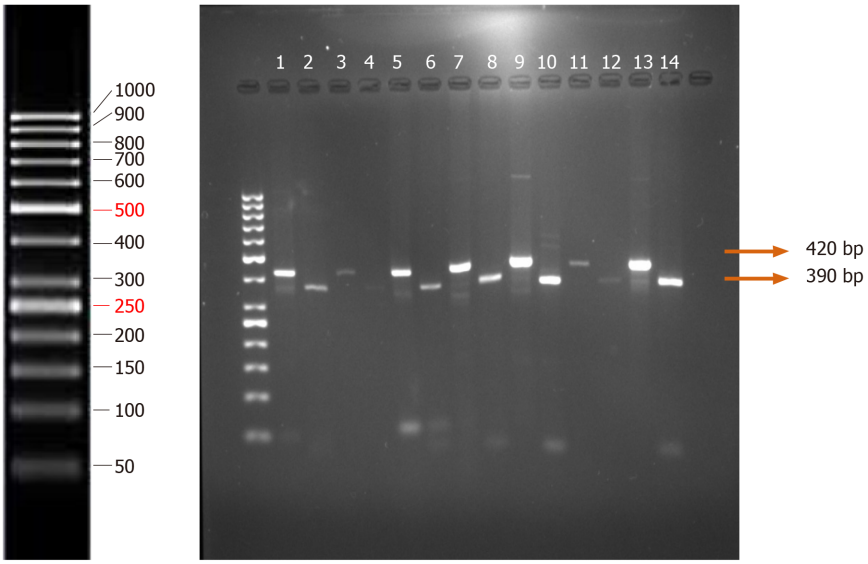
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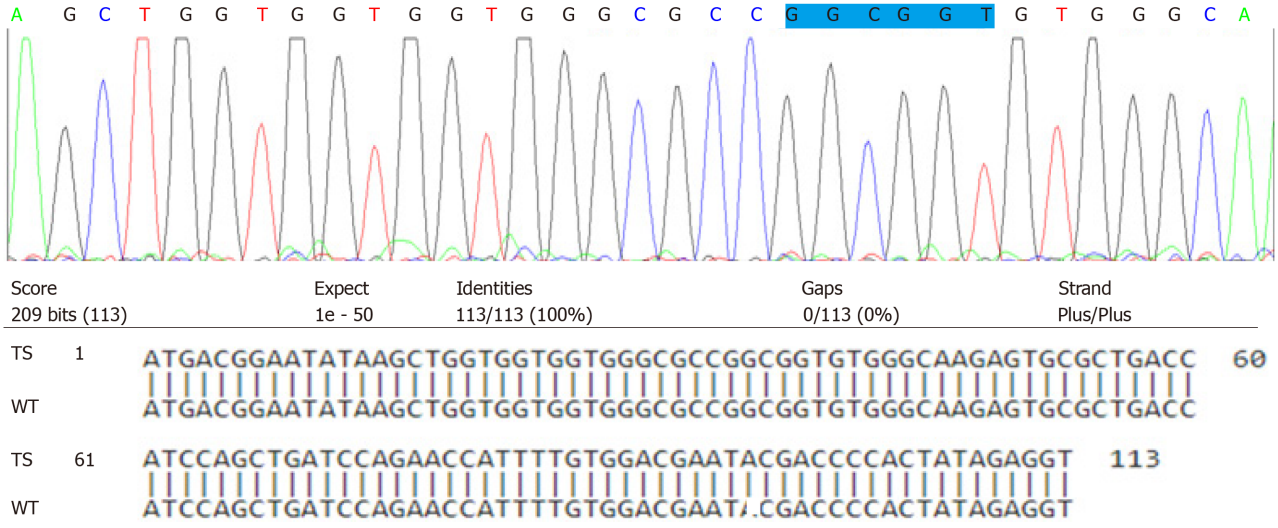
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Gaps
0/113 (0%)
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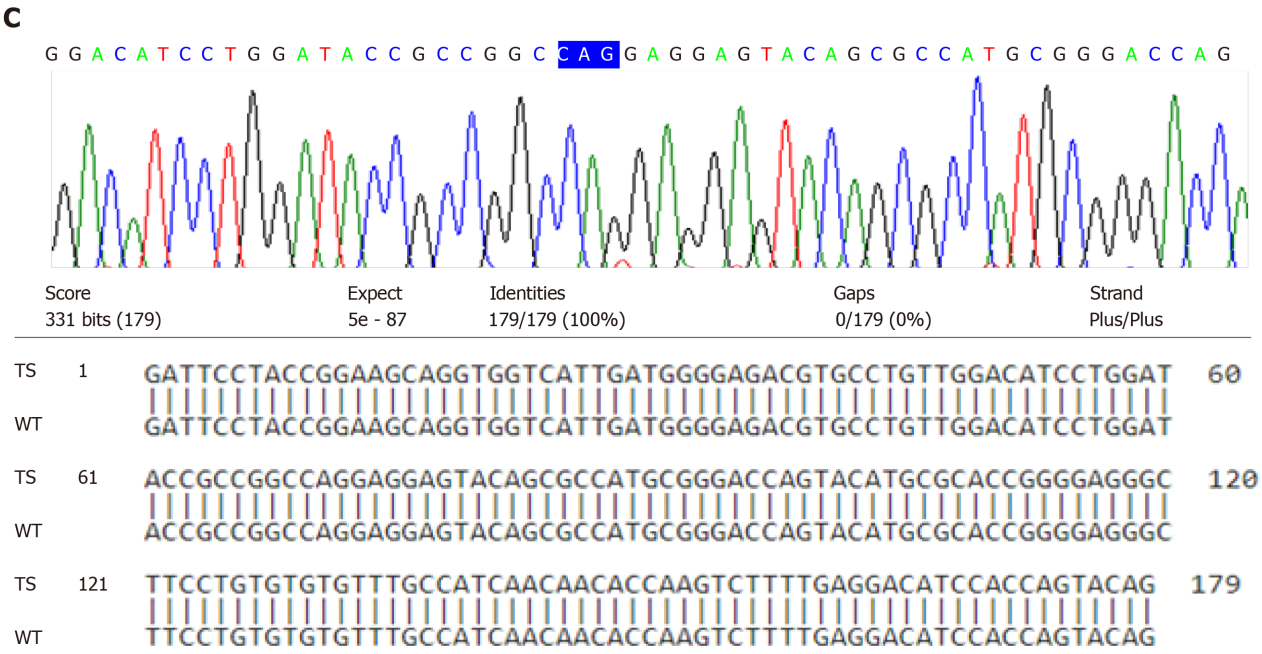

Figure 1 *H-Ras* gene point mutation analysis in patients diagnosed with urothelial bladder cancer. A: Polymerase chain reaction (PCR) - restriction fragment length polymorphism of codon 12: Undigested amplified PCR product (420-bp); MspI-cut PCR product (390-bp and 30-bp); Lanes 1, 3, 5, 7, 9, 11 and 13: Undigested products; and Lanes 2, 4, 6, 8, 10, 12 and 14: Digested products. Lanes 1 and 2 represent the band pattern in normal bladder mucosal tissues whereas lanes 3, 4; 5, 6; 7, 8; 9, 10; 11, 12; and 13, 14 represent the band patterns in tumor specimens; B: Direct DNA sequencing of *H-Ras* coding exon 1 in bladder tumors and normal bladder tissues. Codons 12 and 13 are highlighted; C: Direct DNA sequencing of *H-Ras* coding exon 2 in bladder tumors and normal bladder tissues. Codon 61 is highlighted. TS: Tumor specimen; WT: Wild-type.

Direct DNA sequencing was performed to detect the point mutations in *N-Ras* coding exons 1 and 2 spanning codons 12, 13; and 61, respectively. Sequencing results in the wild-type and tumor specimens were analyzed and compared. The presence of point mutations in the hotspots of codon 12 and 13 of exon 1 and codon 61 of exon 2 of *N-Ras* gene was not detected in any of the bladder specimens (Figure 2B and C).

Point mutation detection in K-Ras

PCR amplification followed by RFLP was carried out to determine the presence of point mutations in codons 12 and 13 in *K-Ras* gene in 87 UCB and 23 normal bladder mucosal tissues. A primer was designed to create a restriction site just before the start of codon 12. Restriction digestion of the wild-type amplicon of 144 bp by enzyme BstNI resulted in its cleavage into 115 bp and 29 bp. The presence of a point mutation at codon 12 results in loss of the recognition site which is indicative of the translational change of glycine (GGT) to valine (GTT)/aspartic acid (GAT)/serine (AGT)/arginine (CGT)/alanine (GCT)/cysteine (TGT). The presence of a point mutation at codon 12 in *K-Ras* gene was not observed (Figure 3A).

Enzyme HphI was used to cleave the restriction site (GGTGA7/8↓) at codon 13 which is indicative of the conversion of glycine (GGC) to aspartic acid (GAC) in *K-Ras* gene. This site does not exist in the wild-type but tends to appear in mutants. The wild-type amplicon yielded a fragment of 144 bp when cut by HphI. Nevertheless, the presence of a mutation at codon 13 would yield two fragments of 101 bp and 43 bp oligonucleotides on restriction digestion (Figure 3B). PCR-RFLP failed to identify any mutational change in codon 13 of *K-Ras* gene in tumor and normal bladder mucosal tissues.

The results of direct DNA sequencing and Blastn of coding exons 1 (spanning codons 12 and 13) and 2 (spanning hotspot codon 61) of *K-Ras* genes in tumor and normal bladder mucosal tissues exhibited 100% alignment. DNA sequencing analysis verified the results of PCR-RFLP. No point mutations in the hotspots of exonic regions 1 and 2 of the *K-Ras* gene were observed (Figure 3C and D).

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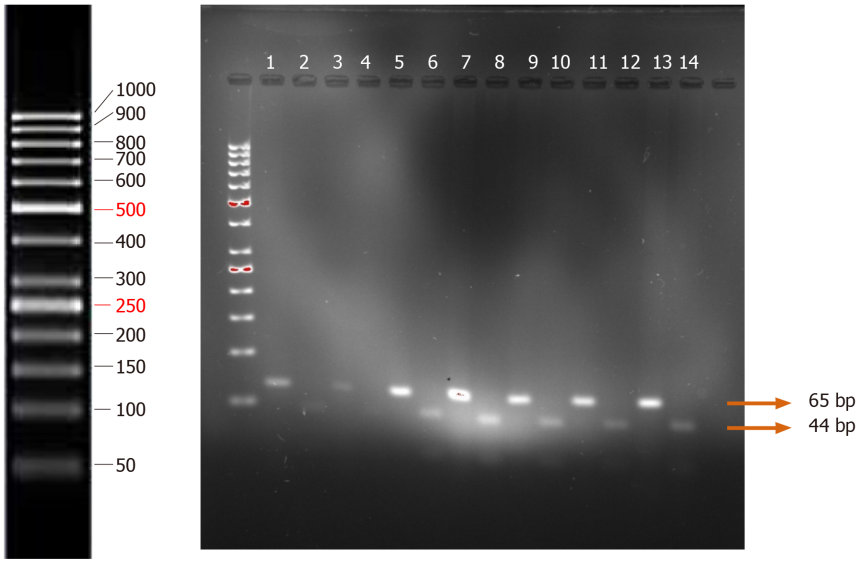
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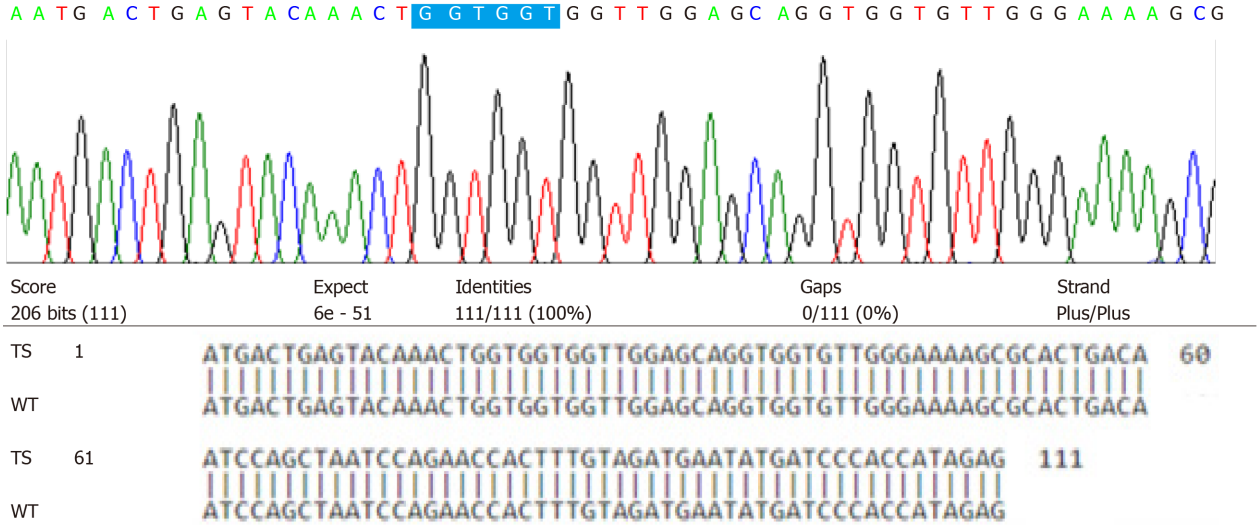
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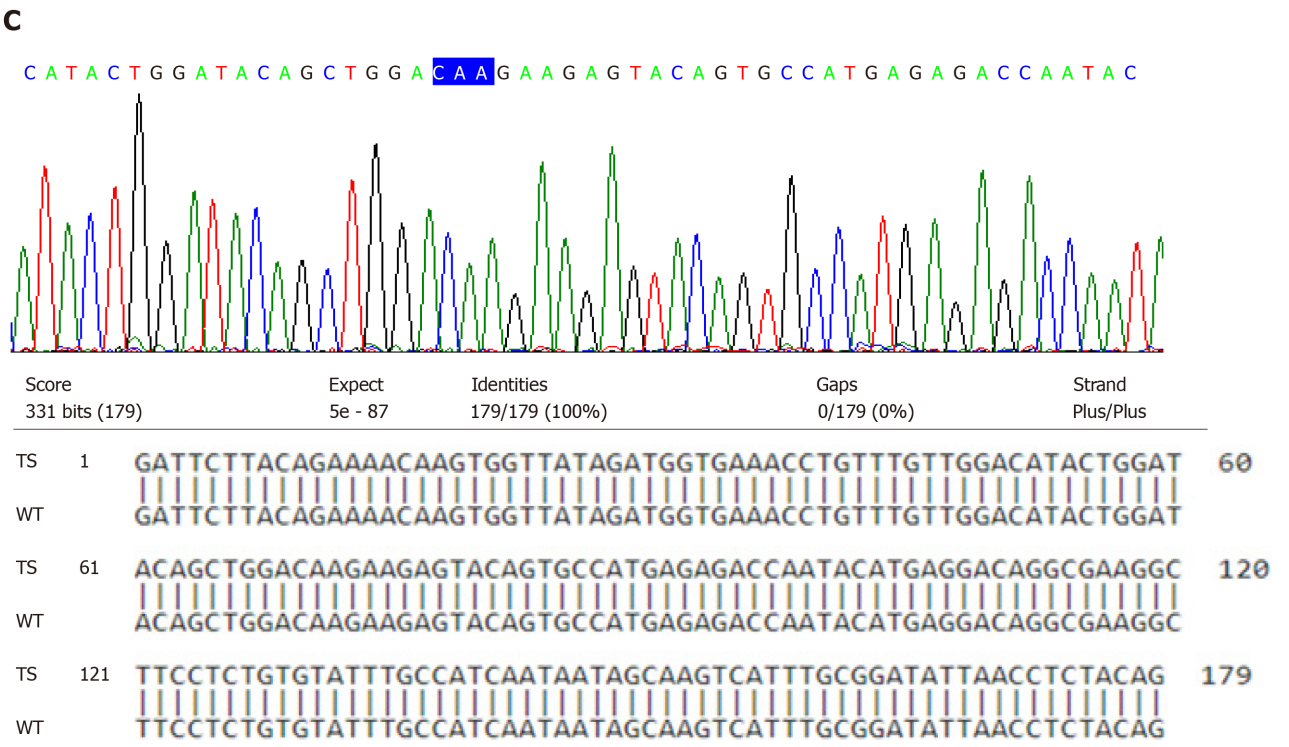


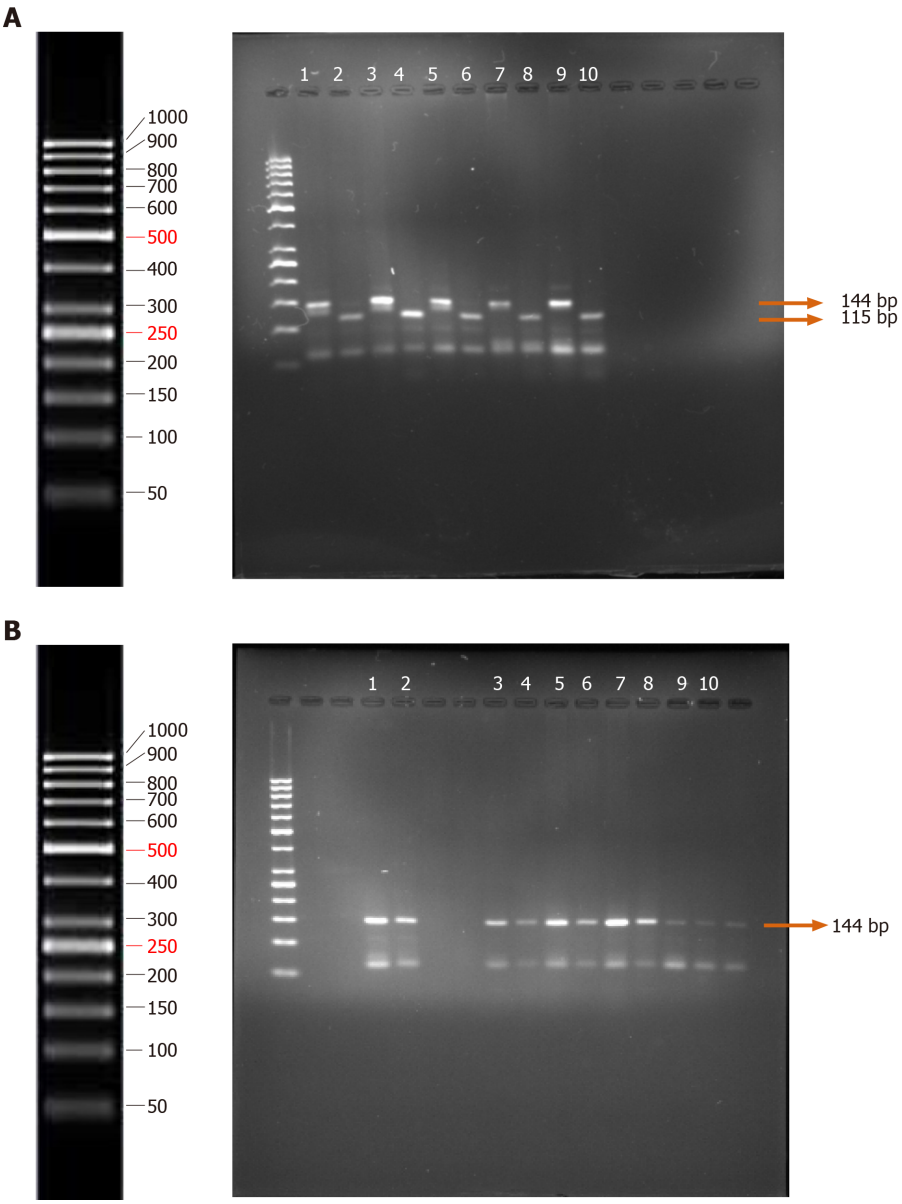
Figure 2 *N-Ras* gene point mutation analysis in patients diagnosed with urothelial bladder cancer. A: Polymerase chain reaction (PCR) - restriction fragment length polymorphism of codon 61. Undigested amplified PCR product (65-bp); MscI-cut PCR product (44-bp and 21-bp); Lanes 1, 3, 5, 7, 9, 11, and 13: Undigested products; and Lanes 2, 4, 6, 8, 10, 12 and 14: Digested products. Lanes 1 and 2 represent the band patterns in normal bladder mucosal tissues whereas lanes 3, 4; 5, 6; 7, 8; 9, 10; 11 12; and 13, 14 represent the band patterns in tumor specimens; B: Direct DNA sequencing of *N-Ras* coding exon 1 in bladder tumors and normal bladder tissues. Codons 12 and 13 are highlighted; C: Direct DNA sequencing of *N-Ras* coding exon 2 in bladder tumors and normal bladder tissues. Codon 61 is highlighted. TS: Tumor specimen; WT: Wild-type.

DISCUSSION

Considerable experimental evidence has demonstrated the significance of continual expression of mutant *Ras* in tumor maintenance. Withdrawal or suppression of *Ras* expression impairs the *in vitro* growth of *Ras*-mutant human cancer cell lines and tumor regression in mouse models driven by inducible mutant *Ras*. These findings indicate that mutant *Ras* is a therapeutically useful drug target even in advanced metastatic tumors[6].

Studies of a variety of tumors have demonstrated the prevalence of specific point mutations in the hotspots of *Ras* isoforms. These point mutations are known to transform *Ras* proto-oncogene into an oncogene and prevent normal deactivation of *Ras* proteins. Activated *Ras* proteins are associated with drug resistance, enhanced metastasis, poor prognosis and shorter survival of patients[7]. The present study examined the mutational spectrum at the hotspot regions of *H-Ras* codon 12, *K-Ras* codons 12, 13 and *N-Ras* codon 61 by PCR-RFLP followed by direct DNA sequencing of the coding exons 1 and 2 of the three *Ras* isoforms in 87 UCB patients and their clinical impact if any.

The incidence of *Ras* mutations varies, and greatly depends on the tissue or cell type from which the cancer cells are derived. Although *Ras* mutations occur in 75% to 95% of pancreatic carcinomas and in 50% of colon carcinomas, they are rare in several other neoplasms[15]. The *H-Ras* mutation was first detected in the human bladder cancer cell line T24. Subsequent studies demonstrated the frequent occurrence of *H-Ras* mutations in urinary tract tumors compared to mutations in *K-Ras* or *N-Ras* genes[21]. A number of studies has reported *H-Ras* mutations with variable frequencies in urinary bladder cancer specimens. Fitzgerald *et al*[22] reported mutations in the *H-Ras* gene in 44% of urine sediments from bladder cancer patients. Czerniak *et al*[17] observed *H-Ras* mutation specifically at codon G12 in 45% of bladder cancers. Zhu *et al*[19] and Buyru *et al*[18] showed 46.7% and 39% point mutations in *H-Ras* at codon 12, respectively. Cattan *et al*[23] detected only 1% of such alterations in bladder cancer patients[23]. In constrast, Przybojewska *et al*[24] observed *H-Ras* mutations in 84% of patients with bladder cancer using PCR-RFLP. In contradiction to many earlier published studies, we did not find mutations at *H-Ras* codon G12 (glycine to valine/serine/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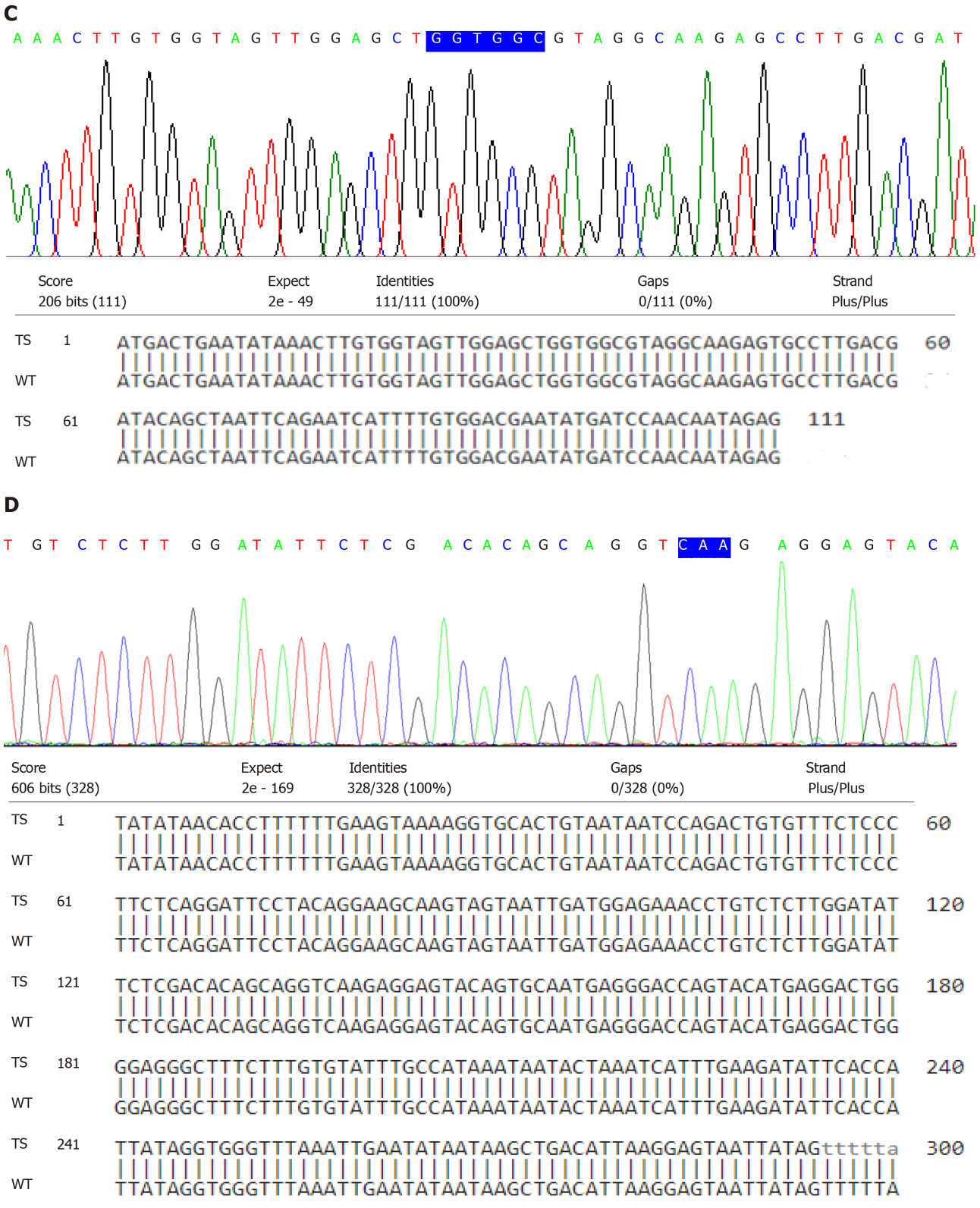

Figure 3 *K-Ras* gene point mutation analysis in patients diagnosed with urothelial bladder cancer. A: Polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) of codon 12. Undigested amplified PCR product (144-bp); BstI-cut PCR product (115-bp and 29-bp); Lanes 1, 3, 5, 7 and 9: Undigested products and Lanes 2, 4, 6, 8 and 10: Digested PCR products. Lanes 1 and 2 represent the band patterns in normal bladder tissues whereas lanes 3, 4; 5, 6; 7, 8; and 9, 10 represent the band patterns in tumor specimens; B: PCR-RFLP of *K-Ras* codon 13. Undigested amplified PCR product (144-bp); Hph I-cut PCR product (101-bp and 43-bp); Lanes 1, 3, 5, 7, 9, and 11: Undigested products and Lanes 2, 4, 6, 8 and 10: Digested PCR products. Lanes 1 and 2 represent the band patterns in normal bladder tissues whereas lanes 3, 4; 5, 6; 7, 8; 9, 10; and 11 represent the band patterns in tumor specimens; C: Direct DNA sequencing of *K-Ras* coding exon 1 in bladder tumors and normal bladder tissues. Codons 12 and 13 are highlighted; D: Direct DNA sequencing of *K-Ras* coding exon 2 in bladder tumors and normal bladder tissues. Codon 61 is highlighted. TS: Tumor specimen; WT: Wild-type.

alanine/aspartic acid) or in the coding exons 1 and 2 in a cohort of North Indian

urothelial bladder cancer patients.

N-Ras gene mutations have mainly been associated with hematopoietic malignancies and melanoma[25,26]. Results of the study by Przybojewska *et al*[24] revealed the frequent prevalence (80%) of *N-Ras* gene mutations at codon Q61 (glutamine to lysine) in bladder tumor tissues. These tumor tissues were obtained following infiltration of urinary bladder walls as well as peripheral blood specimens from confirmed bladder cancer patients[24]. Of the total mutations detected in *N-Ras* gene, 60% of mutations were observed in codon 61 in a cohort of North Indian patients[26]. A strong association between the percentage of mutations in *Ras* genes and smoking status of patients (total of 78% mutations) and the age of patients (more than 60 years with total of 80% mutations) was observed. However, no association between the percentage mutation distribution and tumor stage/grade was reported[27]. Jebar *et al*[10] examined one mutation in codon 12 and three mutations in codon 61 in 98 urinary bladder tumors and 31 bladder cell lines. Our findings on the lack of *N-Ras* gene mutations in bladder cancer patients are not in accordance with published studies. Heterogeneity in the results/genomic alterations could be attributed to the differences in ethnicity, exposure to different environmental carcinogens, genetic susceptibility to carcinogens and or tissue specificity.

High frequency of *K-Ras* gene mutations has been detected in many forms of cancer, including pancreatic cancer (80%-90%) and adenocarcinoma of the lung (60%)[17,22]. Cancers of the lung, large intestine (including colon, rectal and anal), pancreas and biliary tract exhibited higher frequency of mutations in *K-Ras* gene[28]. Observed similarities in the percentage distribution of mutations in *K-Ras* codons in lung cancer (58%) and bladder cancer (47%) could be due to the effects of tobacco consumption. Tobacco is considered an important risk factor for both of these cancers and can induce local somatic mutations in genes[13]. These studies did not report an association between *K-Ras* mutations and tumor stage/grade[13]. Unlike the majority of tumors that harbor an activated *K-Ras* gene, changes in *K-Ras* gene have been observed as a rare event in urinary bladder tumors[24]. Studies examined the percentage mutation prevalence at codon G12 (glycine to valine/aspartic acid/serine/ arginine/ alanine/cysteine) and codon G13 (glycine to aspartic acid) in *K-Ras* gene as an infrequent event in bladder cancer[29,30]. A study by Nanda *et al*[15] identified 11.67% tumors which harbored *K-Ras* mutations as well as a significant correlation of the *K-Ras* mutant status with the smoking history of patients, high tumor grade, lymph node involvement and tumor recurrence. Yan *et al*[31] reported the ability of mutant *K-Ras*, but not *H-Ras*, to confer metastatic phenotype in cells by interfering with the maturation of cell surface integrins and disrupting cell-cell adhesion. A recently published study reported a higher prevalence of point mutations in all the *Ras* isoforms in NMIBC (27%) compared to MIBC (9.4%) patients.

In contrast to earlier published studies, our findings on the lack of *H-Ras*, *K-Ras* and *N-Ras* gene mutations in urothelial bladder cancer patients provide evidence for the tissue specific activation of *Ras* isoforms.

Discrepancies/heterogeneity in the frequency distribution of mutations at hotspot regions/codons in different isoforms of *Ras* gene among different cohorts of UCB patients belonging to different ethnic groups are reported in many published studies. Observed heterogeneity among different studies could be explained on the basis of different etiological mechanisms involved in disease development/progression, inherent genetic susceptibility or alternative *Ras* dysfunction such as gene amplification and/or overexpression.

In conclusion, *Ras* mutations are the most common genetic alterations known in human cancers. Single base changes/point mutations in codon 12, 13 and 61 of the three closely related isoforms of the *Ras* gene family namely, *H-Ras*, *K-Ras* and *N-Ras* cause loss of intrinsic GTPase activity and thereby confer oncogenic functions. Oncogenic activation of *Ras* genes is involved in urothelial malignancies.

The present study was conducted to determine the clinical impact of mutant *Ras* by examining the relationship of clinical histopathological variables in 87 UCB patients with the mutational spectrum at the hotspot regions of *H-Ras*, *K-Ras* and *N-Ras* genes by PCR-RFLP and direct DNA sequencing.

The current observations rule out the possible role of the mutations examined in the above-mentioned hotspot regions in *Ras* gene activation. Our findings on the lack of mutations in *H-Ras*, *K-Ras* and *N-Ras* genes could be explained on the basis of different etiological mechanisms involved in disease development/progression, inherent genetic susceptibility, tissue specificity or alternative *Ras* dysfunction such as gene amplification and/or overexpression.

ARTICLE HIGHLIGHTS

Research background

Mutational activation of *Ras* genes has been established as a prognostic factor for the genesis of a constitutively active RAS-mitogen activated protein kinase pathway that leads to cancer.

Research motivation

Due to the reported heterogeneity among the distribution of the most frequent mutations in *Ras* isoforms in different patient populations with urothelial carcinoma of the bladder (UCB), it is necessary to determine the presence/absence of mutations in order to predict disease outcome.

Research motivation

The present study was conducted to determine the mutational spectrum at the hotspot regions of *H-Ras*, *K-Ras* and *N-Ras* genes.

Research objectives

PCR-RFLP and direct DNA sequencing were employed to determine the presence or absence of mutations in the *Ras* isoforms and their clinical impact, if any, in 87 UCB patients.

Research methods

None of the 87 UCB patients showed point mutations in codon 12 of *H-Ras* gene; codon 61 of *N-Ras* gene and codons 12, 13 of *K-Ras* gene by PCR-RFLP. Direct DNA sequencing of tumor and control bladder mucosal specimens followed by Blastn alignment with the reference wild-type sequences failed to identify even a single nucleotide difference in the coding exons 1 and 2 of *H-Ras*, *N-Ras* and *K-Ras* genes in the tumor and normal bladder mucosal specimens.

Research results

Our findings on the lack of mutations in *H-Ras*, *K-Ras* and *N-Ras* genes could be explained on the basis of different etiological mechanisms involved in tumor development/progression, inherent genetic susceptibility, and or tissue specificity in a given cohort of patients.

Research conclusions

Gene amplification and/or overexpression of *Ras* could further explain an alternative mechanism of its dysfunction in *Ras* driven cancers.

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SYSTEMATIC REVIEWS

Management of neuroblastoma in limited-resource settings

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Abstract

BACKGROUND

Neuroblastoma (NB) is a heterogeneous disease with variable outcomes among countries. Little is known about NB in low- and middle-income countries (LMICs).

AIM

The aim of this review was to evaluate regional management protocols and challenges in treating NB in paediatric oncology units in LMICs compared to high-income countries (HICs).

METHODS

PubMed, Global Health, Embase, SciELO, African Index Medicus and Google Scholar were searched for publications with keywords pertaining to NB, LMICs and outcomes. Only English language manuscripts and abstracts were included. A descriptive review was done, and tables illustrating the findings were constructed.

RESULTS

Limited information beyond single-institution experiences regarding NB outcomes in LMICs was available. The disease characteristics varied among countries for the following variables: sex, age at presentation, MYCN amplification, stage and outcome. LMICs were found to be burdened with a higher percentage of stage 4 and high-risk NB compared to HICs. Implementation of evidence-based treatment protocols was still a barrier to care. Many socioeconomic variables also influenced the diagnosis, management and follow-up of patients with NB.

CONCLUSION

Patients presented at a later age with more advanced disease in LMICs. Management was limited by the lack of resources and genetic studies for

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improved NB classification. Further research is needed to develop modified diagnostic and treatment protocols for LMICs in the face of limited resources.

Key words: Neuroblastoma; Limited resources; Management; Outcomes; Low- and middle-income countries

Core tip: Neuroblastoma (NB) is a childhood malignancy of the sympathetic system that accounts for a large percentage of the childhood malignancy mortality. The heterogenous presentation contributes to various treatment challenges especially in low- and middle-income countries (LMICs). NB in LMICs has not been investigated beyond single institutions, but the limited reports differ from those in high-income countries (HICs). The incidence of NB in LMICs has been reported to be lower than HICs, but the disease presents with a higher incidence of high-risk and advanced disease. Furthermore, the limited resources in these countries contribute to the challenges in the management of NB that leads to a high mortality rate. The genetic profile of NB in LMICs is also not known due to limited capacity to perform genetic investigations. This article aims to comprehensively describe NB in LMICs.

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INTRODUCTION

The burden of disease in low- and middle-income countries (LMICs) is predominantly infectious in origin[1,2]. Yet, it is shifting towards non-communicable diseases such as congenital diseases, malignancies and road traffic incidents[2,3]. To date, the focus in research has been on communicable paediatric diseases with the World Health Organization's initial integrated management of childhood illness programme being one example[2]. Building the capacity of health care professionals to identify childhood malignancies has not been optimal[4]. This possibly explains the 28%-49% childhood malignancy gap reported between LMICs and high-income countries (HICs)[5].

Neuroblastoma (NB) data from HICs are well documented, whereas data from LMICs are limited. NB, predominantly a childhood malignancy, remains a major contributor to childhood cancer mortality and accounts for up to 15% of paediatric malignancy-related deaths[6]. Even with increased-intensity treatment in HICs, the five-year overall survival (OS) remains approximately 60%[7]. However, there is a major divide between HICs and LMICs due to the advances in diagnostics, treatment options and outcomes of NB in HICs[8].

Because of the variability of NB symptoms, they can easily be misdiagnosed as infections, bone marrow failure, neuropathology and obstructive enteropathies in LMICs by primary health care workers. Nurse-led primary care clinics or general practitioners may not have the expertise to recognise rare diseases in children and are often the first contact versus HICs where the first contact is usually more experienced health care workers[9].

Early diagnosis is crucial and necessitates a high index of suspicion with appropriate risk stratification and treatment[5]. The prognosis of NB is determined by a set of well-described prognostic factors that include patient factors (age at diagnosis), biochemical factors (lactate dehydrogenase and ferritin), tumour-related factors (primary site, tumour histology and stage), biological factors (MYCN amplification, ploidy and loss of chromosome 1p) and management factors (post-induction metastatic remission and degree of resection)[10,11]. NB pathophysiology and biological features, predominantly MYCN status, loss of chromosome 1p and ploidy, determine the spontaneous regression or aggressive growth and spread of metastases but do not explain the international difference in characteristics completely[6]. Similarly, notable differences in outcomes have been reported for risk classifications between LMICs and HICs with similar therapies[12-16]. The aim of this narrative review was to evaluate

regional variations in the diagnosis and management of NB in LMICs versus HICs.

MATERIALS AND METHODS

A comprehensive literature review of publications on PubMed, Global Health, Embase, SciELO, African Index Medicus and Google Scholar with medical subject headings pertaining to NB and outcomes relating to LMICs was done. Search terms included (but were not limited to) "neuroblastoma", "limited resources", "low-income", "middle-income" and names of LMICs. The search was conducted from April 2019 to January 2020 with terms adapted according to search engines without limitations on the date or language, provided that English summaries or abstracts were included. Conference proceedings were included. No authors were contacted regarding publications.

Due to the variability in reporting, nonstandard application of definitions in the reported clinical results, heterogeneous data and paucity of information, the authors constructed limited tables to evaluate clinical and/or biological characteristics to report in the descriptive review.

The systemic literature search retrieved 127 articles, abstracts and documents on NB in LMICs. After removing 11 documents for possible duplicated reporting, the 116 remaining documents consisted of 13 cancer registry-based reports and 103 non-registry-based documents. Twenty-three non-registry-based, nonrandomised studies (two prospective studies and 21 retrospective studies) were selected. All 116 articles, including the remaining 83 articles that were not specific to NB but contained epidemiological and non-interventional data on NB, were utilised to draw descriptive conclusions regarding epidemiological elements and outcomes for NB in the respective countries. Despite significant population numbers, certain LMIC regions were underrepresented in this review due to possible publication bias of reports.

RESULTS

Data from Asia (China, India, Pakistan, Thailand and Vietnam)[13,17-22], the Middle East and North Africa (Egypt, Iran, Iraq and Morocco)[23-28] and the Americas (Argentina, Brazil, Chile, Cuba, Mexico and Uruguay)[12,16,29-35] were accessible, but reports from sub-Saharan Africa and the Pacific Ocean were limited to single reports from the French-African Paediatric Oncology Group (GFAOP) and reunion[15,36]. The differences between HICs and LMICs could be evaluated from these reports, but complete management and outcome data for interregional variations among LMIC regions were less robust.

Incidence of neuroblastoma in low- and middle-income countries versus high-income countries according to international cancer registries

In sub-Saharan Africa, the incidence of NB was low, ranging from 0.4 cases per million in Niger to 5.9 cases per million in Kenya[37], compared to HICs such as North America and Europe where the respective incidences were reported as 10.5 and 11.6 cases per million per year in children younger than 15 years [11,38,39]. South Africa reported an incidence of 2.68 cases per million in children under 15 years of age between 1985 and 2007[40]. In Argentina, intraregional variations in incidence were demonstrated with a higher incidence being associated with areas of high socioeconomic status[29]. Yet, the international incidences have remained stable regardless of economic status[41]. As perinatal and low-risk (LR) NB can be asymptomatic and/or spontaneously regress, underdiagnosis of cases is a possible reason[5,37] but the degree of discrepancy is not known.

Epidemiology of neuroblastoma in low- and middle-income countries

Difference in age at presentation: In LMICs, the majority of patients were under the age of 5 years, but the percentages of infants reported for China (16.3%) and India (5.9%) (Table 1) were low. The mean or median age of presentation was delayed in some LMICs. In Thailand, the median was 34.8 mo of age and in India as high as 48 mo of age. The median age of presentation in the 16 paediatric oncology units (POUs) of the GFAOP study was 48 mo as well[15]. The age-standardised rates varied among countries, but the ratio of patients under 12 to 60 mo could be as low as 2.3:1 in Argentina and 1.2: 1 in Brazil compared to an HIC like Germany with a 4:1 ratio

Table 1 Age distribution at diagnosis

Country	*n*	< 12 mo	< 18 mo	< 60 mo	< 120 mo	< 180 mo	Mean	Median
Asia								
China (2008-2013)[17]	59	44%	56%					24
China (2000-2006)[18]	98	16.3%	4.1%	53%	21.5%	4%		48
India (1990-2004)[19]	103	0%-5.9%	77%-98.1%		1.9%		41	-
Pakistan (2015-2016)[20]	70	30%		63%		7%		36
South America								
Argentina (2000-2012)[29]	753	30%	52.2%		12.9%	45.3%		26.4
Brazil (1991-2012)[30]	258	29%	49%		17%	5%	40.5	28.9
Brazil (1990-2000)[16]	125	26%	13%	41%	20%		38.2	33
Middle East and North Africa								
Egypt (2005-2010)[23]	142	24.2%	75.8%					30
Egypt (2001-2010)[24]	53	22.6%	77.4%					
Iran (1974-2005)[25]	219	21.5%		78.5%			40.5	
Iraq (2008-2014)[26]	62	30.6%	50%		16.1%	3.2%	37	
Sub-Saharan Africa								
Ethiopia (2010-2013)[79]	5	0	40%		40%	20%		
Kenya (1997-2005)[44]	22	31.8%	50%		18.2%			60

(Table 2). However, other LMICs such as Cuba (4.8:1), with a good reputation for health care, and Reunion (2.7:1), a French territory in Africa, compared favourably with the United States of America (2.4:1) in this regard (Table 1). The median age of presentation in HICs was reported to be between 17 and 18 mo of age, of whom approximately 40% were diagnosed under 1 year of age[41]. Many studies have reproduced the 18-mo watershed dividing good prognosis (under the age of 18 mo) and poorer prognosis (over the age of 18 mo). Stage 4 patients were per definition below 12 mo of age with a good prognosis. In HICs, 90% of NB patients were younger than 5 years at diagnosis, with a median age at diagnosis of 19 mo, and 37% of patients had been diagnosed as infants[11]. The ATRX-gene is associated with advanced-age presentations, especially over 9 years of age, conferring a poorer prognosis in adolescents and adults[42]. The paucity of genetic studies in LMICs limited the interpretation of gene mutations related to age at diagnosis.

Gender distribution at diagnosis: The GFAOP reported that the male to female ratio for 16 African POUs was 2: 1[15]. In other LMICs, the male predominance as well as the greater male to female ratio was reproducible (Table 3). The ratios varied from 1.06: 1 to 2: 1. Previous studies from Southern Africa reported a ratio of 1.7:1[43] in keeping with the male predominance, while a Mexican study reported a lower NB incidence of 2.5-4.1 cases per million per year, in keeping with the situation in other LMICs, yet the male to female ratio of 1.1:1 was similar to HICs[32]. Kenya also reported a 1: 1 ratio in an LMIC setting[44]. The incidences based on gender have not been explained by other biological features. These findings were in contrast to the reported surveillance, epidemiology, and end results programme data from North America and European data, according to which a slight male predominance with a ratio of 1.1:1 was noted[38,45].

Population variations: Population variations related to epidemiology and pathophysiology contributed to a difference in the presentation of high-risk (HR) disease but not non-HR disease[46]. Independent from social circumstances, certain ethnicities were diagnosed at an older median age (> 20 mo) and had a higher prevalence of stage 4 disease and unfavourable histology tumours (undifferentiated cells)[46]. Studies amongst Alaskan indigenous ethnicities (a heterogeneous group of Eskimos, Native Indians and Aleuts) reported an incidence of 0.7 cases per million[47]. In Australia, Aboriginal and Torres Strait Island children were 1.83 times more likely to die from neuroblastoma than nonindigenous children while only contributing 3.7%

Table 2 Incidences of neuroblastoma according to the age at diagnosis

Country	*n*	< 12 mo	< 60 mo	Ratio < 12: < 60	< 120 mo	< 180 mo	Total incidence
South America							
Argentina (2000-2012)[29]	753	32.9	14.6	2.3: 1	2.8	1.0	8.3
Uruguay (2001-2010)[35]	69	63.1	18.1	3.4: 1	2.3	0	9.1
Chile (2007-2012)[31]	88	21.9	6.7	3.2: 1	2.1	0.3	4.7
Brazil (1998-2002)[33]	372	15.3	12.4	1.2: 1	3.8	1.3	5.9
Central America and the Caribbean							
Mexico (1996-2005)[32]	72	18.5	5.4	3.4: 1	1.1	0.2	3.8
Cuba (2001-2003)[34]	46	3.9	0.8	4.8: 1	0.5	0.2	0.1
Sub-Saharan Africa							
Reunion (2005-2011)[36]	12	44.1	15.8	2.7: 1	4.1	0	9.6

of diagnoses[48]. The lower incidence of NB among indigenous ethnicities was not reproduced in LMICs of South America or the Pacific Islands[49,50].

Variations in tumour characteristics

Difference in stage during presentation: Many LMICs reported stage 4 rates upward of 50%, with India and Pakistan reporting 71.8% and 79% stage 4 tumours respectively (Table 4). Egypt, Pakistan and Iran did not report any patients with stage 1 tumours, while China and India reported 3% and 1% stage 1 diagnosis respectively[18-20,25]. The GFAOP reported metastatic disease for up to 80% of patients except Burkina Faso and Morocco, where it varied from 20% to 50%[15]. Kenya reported the highest percentage of metastatic disease at 92.3%[44]. The data suggested that presentation in LMIC was usually metastatic.

Difference in MYCN amplification: Molecular and genetic diagnostics were not available in the greater number of reports and were recorded as a challenge in the literature[13,15,51]. In the GFOAP study, only North African countries could determine MYCN status[15] with Namibia and South Africa reporting MYCN studies in Southern Africa[44]. MYCN is present in about 20% of tumours[51,52]. Limited data are available on biological studies, especially genetic studies, in LMICs mainly due to resource constraints. In Iran, MYCN amplification was reported in 80% of NB patients, while Vietnam, Argentina and Egypt respectively reported rates of 17.8%, 20% and 20.8% (Table 4)[14,17,19].

Intra-risk group classification variability: Age groups, biological information and treatment protocols were not standardised in the literature, due to the development of classifications and changing treatments during the review period. Of note, risk classification was either not possible or was done retrospectively. Management protocols focus on administering risk-based treatments after identification of the classification of each patient yet many patients were treated on the basis of stage[39]. LMICs concluded that optimal treatment was doubtful due to the suboptimal classification of tumours[9,15,19]. The International Neuroblastoma Risk Group classification and the Children's Oncology Group classification rely on histological and genetic information (mitosis-karyorrhexis index, MYCN amplification, 11q aberration and DNA ploidy) to determine classification[11], which is not available in many resource-limited settings. Even when available, the lack of consistent cytogenetic evaluation, as was the case in Argentina, relegated patients in need of high-intensity treatment to LR categories and suboptimal treatment[12]. Due to the aggressive nature of especially HR NB, palliative rather than curative options have been pursued in LMICs[11]. Yet, variability in outcomes has been described within each risk class, highlighting that individual assessment is probably suboptimal Therefore, the International Society for Paediatric Oncology (SIOP)-Paediatric Oncology for Developing Countries (PODC) has adapted the approach to risk stratification with therapy based on available resources and utilising available diagnostic techniques[11]. The classification relies on age, stage and the common available nonspecific tumour markers ferritin and lactate dehydrogenase for risk classification[11]. Morocco has

Table 3 Distribution of sex at diagnosis

Country	Total	Male	Female	Ratio M: F
Asia				
Pakistan (2015-2016)[20]	70			1.8: 1
India (2000-2017)[64]	85	57 (67%)	28 (33%)	2: 1
India (1990-2004)[19]	103	76 (74%)	27 (26%)	2.8: 1
Thailand (2000-2007)[21]	67	39 (58.2%)	23(34.3%)	1.7: 1
Vietnam (2010-2012)[22]	130	76(58.5%)	54 (41.6%)	1.4: 1
China (2008-2013)[17]	59	35 (59%)	24 (40.1%)	1.5: 1
China (2000-2006)[18]	98			1.3: 1
South America				
Brazil (1991-2012)[30]	258	148 (57%)	110 (43%)	1.3: 1
Brazil (1990-2000)[16]	125	68 (54.4%)	57 (45.6%)	1.2: 1
Argentina (1999-2015)[12]	39	21 (54%)	18 (46%)	1.2: 1
Argentina (2000-2012)[29]	971	509 (52%)	462 (48%)	1.1: 1
Middle East and North Africa				
Iran (1974-2005)[25]	219			1.9: 1
Iraq (2008-2014)[26]	62	37 (59.7%)	25 (40.3%)	1.5: 1
Morocco (2012-2015)[27]	40	26 (65%)	14 (35%)	1.8: 1
Egypt (2005-2010)[23]	142	68 (51.5%)	64 (48.5%)	1.06: 1
Egypt (2001-2010)[24]	53	35 (66%)	18 (35%)	1.9: 1
Egypt (2007-2011)[28]	271	169 (62.4%)	102 (37.6%)	1.65: 1
Sub-Saharan Africa				
Northern Nigeria (2003-2009)[79]	14	10 (71.4%)	4 (28.6%)	2.5: 1
Southern Africa (South Africa and Namibia) (1983-1997)[43]				1.7: 1
Ethiopia (2010-2013)[78]	5	3 (60%)	2 (40%)	1.5: 1
Kenya (1997-2005)[44]	22	11 (50%)	11 (50%)	1: 1

implemented this classification system in the prospective NB protocol and has concluded that it allowed for more accurate diagnosis and systematic treatment[27]. For more accurate comparisons across resource-limited settings, classifications such as the SIOP-PODC classification should be standardly applied.

Variable reporting and treatment priorities

Reports from LMICs were predominantly single-institution reports. A multi-institutional survey by the GFAOP[15] and a review from India including 17 institutions and 11 cities[3] described the epidemiology, heterogeneous management approaches and outcomes of NB in LMICs[5]. Sub-Saharan African countries reported lower incidences of NB (3%-7.5%) among childhood malignancies compared to North-African countries (7%-30%)[15]. The same study identified the limitations of reporting: Plain radiography, ultrasonography, computed tomography and magnetic resonance imaging were available at all centres, but access to imaging studies was variable. None of the sub-Saharan centres had metaiodobenzylguanidine scans. The North African centres had these scans, but only Algeria had consistent access due to government funding[15]. In Honduras and the Philippines, diagnostic resources were available in large cities but were inaccessible to most patients living in rural areas[50]. This is a typical problem in LMICs[53]. An Indian multi-study review concluded that variability in India included treatment protocols, reporting of outcomes and calculation of survival rates[13]. This conclusion could also be applied to other LMICs. Morocco and Argentina were the only LMICs to describe prospective national studies regarding

Table 4 Disease characteristics of neuroblastoma at diagnosis

Country	*n*	Stage 1	Stage 4	Non-MYCN amplified	MYCN amplified	Non-HR	HR
Asia							
China (2008-2013)[17]	59	6.8%	37.3%	55%	45%	53%	47%
China (2000-2006)[18]	98	3%	50%				
India (1990-2004)[19]	103	1%	71.8%				
Pakistan (2015-2016)[20]	70	0%	79%				> 61.1%
South America							
Argentina (2000-2012)[29]	753	12%	55.5%	80%	20%		
Brazil (1991-2012)[30]	258	15%	46%	75%	25%		
Brazil (1990-2000)[16]	125	7%	64%	53%	47%		
Middle East and North Africa							
Egypt (2005-2010)[23]	142	0%	64.7%			24.2%	75.8%
Egypt (2001-2010)[24]	53	0%	67.9%	79.2%	20.8%	32%	68%
Iran (1974-2005)[25]	219	14.5%	53.8%				
Iraq (2008-2014)[26]	62	1.6%	69.4%			45.2%	54.8%
Sub-Saharan Africa							
Kenya (1997-2005)[44]	26	0%	92.3%				

HR: High-risk.

NB[27,29]. This is representative of the diverse, nonstandardised approach to NB in most LMICs. Most studies found a lack of access to biological tests for stratification (based on HIC-validated data), the presentation of advanced disease, poor socioeconomic circumstances and a significant percentage of patients who absconded from treatment[23,24]. Advanced disease and higher than average percentages of HR disease were described (Table 4). The PODC committee of the SIOP has developed adapted guidelines for the management of NB in LMICs[11]. Yet, in the field of paediatric oncology, especially in sub-Saharan Africa, a prioritised, stepwise approach has been advised in limited-resource settings, prioritising pain management, supportive care, comorbid diseases and malignancies with a higher incidence and relatively uncomplicated treatment regimens above rare childhood malignancies[54]. In Africa, only Morocco has published data from standardised prospective NB protocols from four POUs based on the PODC guidelines[27].

Challenges in improving outcomes

Clinical presentation, index of suspicion and misdiagnosis: Because of its heterogeneous clinical presentation, NB can be challenging to diagnose[30]. The presenting signs of NB can be similar to those of non-malignant diseases and can confound recognition of the disease[10,55]. Symptoms of an NB abdominal mass can be misdiagnosed as more common childhood illnesses such as constipation[56]. In LMICs, similar to HICs, the most common presentation reported in 19%-87% of patients was an abdominal mass (Table 5)[18,19,23,30]. Other common presentations were nonspecific abdominal pain (22%-73.5%)[18,30] and fever (25%-65%)[18,19,23,30], metastatic manifestations such as bilateral proptosis (27%-42.4%)[19,23], bone pain (19%)[30] and pancytopaenia, and constitutional symptoms such as loss of weight[56]. The clinical progression of the tumour involves a spectrum of behaviour from aggressive advancement to metastatic disease or spontaneous regression and mature differentiation of cell types such as ganglioneuroma[29,57]. Health care practitioners must have a high index of suspicion for NB with a varied clinical picture[35]. Misdiagnosing NB from other abdominal tumours prevents accurate registration of the diagnosis[29]. In resource-limited settings, the diagnosis of asymptomatic benign clinical types is less common, possibly due to underdiagnosis. Early detection by screening in HICs neither impacted outcomes nor was it cost-effective[57]. While the incidence was increased during active screening of the disease in the European, North American and Japanese context, surgical

Table 5 Most common clinical presentations in low- and middle-income countries

Asia				
China (2000-2006)[18]	Abd pain (73.5%)	Abd mass (54.1%)	Fever (45.9%)	Limb pain (25.5%)
India (1990-2004)[19]	Fever (65%)	Abd mass (54%)	Bone pain (31%)	Proptosis (27%)
South America				
Brazil (1991-2012)[30]	Fever (25%)	Abd pain (22%)	Abd mass (19%)	Bone pain (19%)
Middle East and North Africa				
Egypt (2005-2010)[23]	Abd mass (87%)	Pallor (57.6%)	Fever (45.5%)	Proptosis (42.4%)
Sub-Saharan Africa				
Kenya (1997-2005)[44]	Abd mass (53.8%)	Bone pain (50%)	Proptosis (38.5%)	Fever (19.8%)

interventions were increased without improvement of survival[57].

Access to and assignment of treatment: The number and capacities of POUs varied substantially among LMICs, and capacities also varied among POUs in a single country[50]. Basic paediatric oncology components were not available in the Philippines and Senegal[50], while Venezuela and Egypt had adequate intensive care facilities and even transplant services[50]. This is also true of POUs in South Africa[44]. Furthermore, paediatric services may not even exist in certain countries or often compete with adult services for resources[54].

Current treatment protocols are based on risk stratification[11]. The LMIC reports included treatments over four decades[13,30]. Therefore, outcomes were predominantly reported per stage and, subsequently, as classification systems evolved, research describing the treatment of LR and intermediate-risk (IR) patients but focussing primarily on HR disease as the greatest NB burden was reported.

In many LMICs, NB treatment choices are limited to mainly chemotherapy, surgery and radiotherapy[1]. In HR NB, multimodal therapy is of vital importance for cure and five-year OS of up to 60% (Figure 1).

Due to advanced disease at diagnosis, palliative treatment is often the only plausible option (Figure 1). Other challenges for the management of NB include lack of surgical and radiotherapy skills or equipment as well as lack of chemotherapy[1,11]. Poor outcomes have necessitated the development of palliative strategies, yet many LMICs where drug insecurity is high do not have even basic medicines for palliation[58]. Resources, drug security and expertise in institutions influence treatment decisions to a similar extent as treatment adherence and response to treatment. The ability of facilities to provide supportive care, in terms of antibiotics, intensive care and granulocyte-stimulating factors, influences decision making regarding the intensity of treatment that patients receive[10,11].

Treatment protocols utilised in low- and medium-income countries and outcomes: Over the past decades, guidelines for the treatment of NB have changed as a result of an improved understanding of biological prognostic factors and changing classification systems yet chemotherapy remains based on etoposide and platinum (cisplatin and/or carboplatin) backbones plus dose- and time-intensive administration of chemotherapy[11]. Some approaches include doxorubicin in the regimens, while the SIOP-PODC treatment guidelines for NB are based on settings relating to the level of supportive care and resources available in a POU[11]. Indicators for reporting outcomes were not consistent over the same period. Some studies reported according to stage, while others reported according to risk classification.

The GFAOP administered various local and international protocols based on the standard backbone including doxorubicin[15]. Individual POUs reported a long-term OS of less than 10% for metastatic disease. Tunisia reported an OS of 78% for non-metastatic disease, while Senegal reported an OS (metastatic plus non-metastatic) of 38.9%. The report concluded that with all countries having access to surgical options, the outcomes were "generally poor" and standardised protocols were being developed for multicentre use[15]. In Morocco, a GFAOP member, a national prospective protocol divided into an HR protocol and a non-HR protocol based on the risk-adapted SIOP-PODC treatment guidelines was studied[11,15,27]. Long-term outcomes were not reported, but 60.6% of HR patients experienced a partial or very good partial response, receiving local control with surgery or consolidation therapy[27]. The study concluded that risk

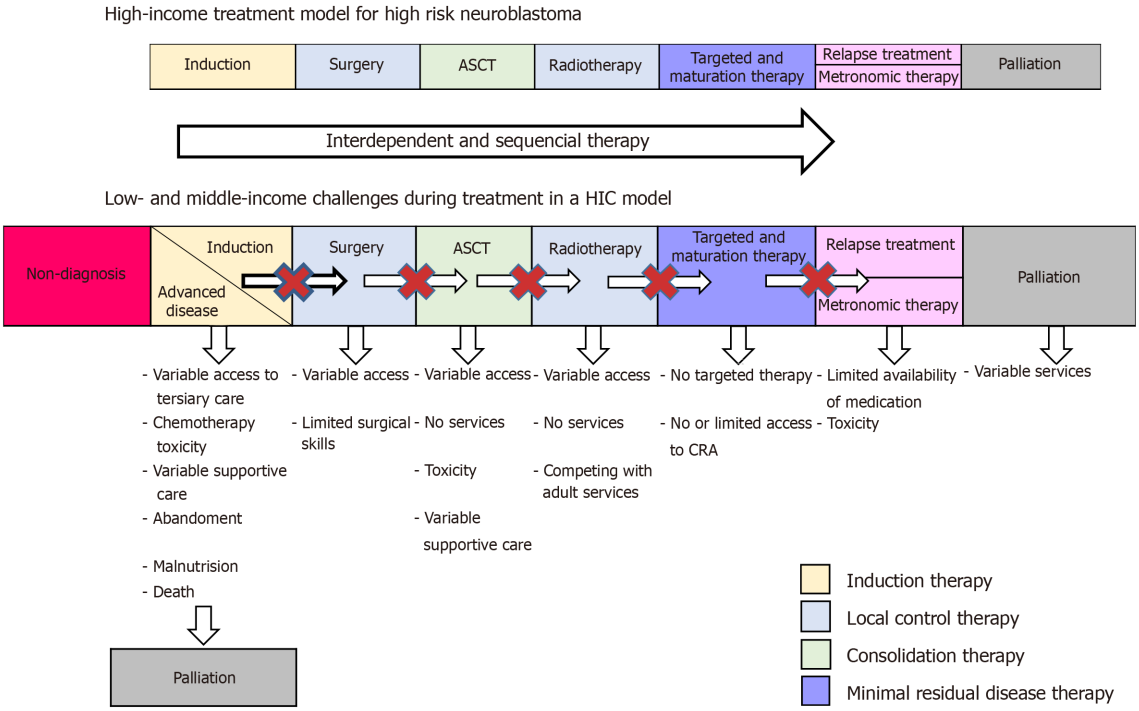

Figure 1 Challenges of non-tumour-related factors during the treatment of high-risk neuroblastoma in low- and middle-income countries.
ASCT: Autologous stem-cell transplant; HIC: High-income country; CRA: Cis-retinoic acid.

stratification and treatment guidelines adapted for LMICs improved the accuracy of diagnosis and access to systematic treatment[27]. The protocol was also suitable for multicentre use[27].

A Chinese study administered OPEC by modifying the Japanese study group protocol[18]. The five-year OS was 80% for stages 1 and 2 and 48.3% and 20% for stages 3 and 4 respectively, which was less than the Japanese outcomes[18].

Egyptian and Indian centres based their HR treatment on the North American CCG-3891 protocols, while other LMIC centres administered chemotherapy according the European protocols from France and the International Society of Paediatric Oncology European Neuroblastoma Research Network (SIOPEN)[59]. Indian institutions followed a non-standardised approach including OPEC/OJEC, doxorubicin-containing and Ifosfamide-containing regimens[13]. Iran and Egypt used OPEC/OJEC regimens[23-25], while Brazil, Thailand and China followed doxorubicin-based regimens[16-18,21,30]. Stage 1 disease had a five-year OS of 100% in Brazil[16], China[17,18] and Thailand[21], while stage 4 OS was under 20%[16,18]. The three-year OS for stage 4 disease in Thailand and China was less than 35%[17,21]. While the outcomes for stage 1 disease were comparable to HICs, the poorer stage 4 outcomes were less optimal than in HICs[10]. The same conclusion was reached in an Indian study with three-year OS and event-free survival for non-metastatic disease of 77% and 54% respectively[60].

Argentina alternated between rapid COJEC and the modified N7 for HR disease according to the SIOPEN HR NBL-1 protocol[12]. The five-year OS was 24%. The study concluded that improved supportive care, optimal treatment and maximising available resources were needed[12]. A second Argentinian study associated lower socioeconomic status with poorer outcomes independent of treatment[29].

In LMICs, no conformity was found in the management of NB amongst regions within countries. Failing to complete one aspect of the sequential treatment protocol relegates the outcome to being suboptimal. This is often the case in LMICs with limited access to health care and limited resources for optimal treatment[61]. It is possible that without genetic factors to distinguish more clearly between IR and HR disease, the IR cohorts in LMICs contain a number of HR patients, thereby affecting outcomes[11].

Main factors affecting outcomes: LMICs have identified treatment-related, tumour-related and social factors that affect the outcomes of children with NB. Delayed diagnosis[30] and inaccurate diagnosis of tumours due to limited radiologic and pathology resources were cited as major obstacles[25,27,60]. The limited ability to perform

biological testing impaired accurate risk stratification[25,27,30,62]. Centres with higher levels of supportive care reported the inability to perform bone marrow transplants as a limitation to improving outcomes[24,60]. The variability of tumours and nonspecific presentation contributed to late diagnosis and the incidence of advanced disease[12,25,27,30,62]. Yet, the greatest problems were the abandonment of treatment and patients lost to follow-up of up to 50%[11,70,62], which were linked to social factors and the distance from treatment centres[12].

Social circumstances and outcomes: A Brazilian study reported intraregional variation in the incidence of NB based on socioeconomic status[33]. The study concluded that patients from regions with a lower socioeconomic status had poorer outcomes[33]. In South African populations, socioeconomic and/or cultural factors related to access to or utilisation of health care services are a possible contributing factor to poorer outcomes[1]. A large proportion of rural inhabitants have restricted access to medical facilities and thus experience a delay in treatment[1,63,64]. A Harvard study concluded that in the United States of America, NB diagnosis was influenced by social circumstances[65]. According to the study, the Human Development Index showed a direct relationship between socioeconomic status and the incidence of NB[65].

Factors influencing health-seeking behaviour: The heterogeneous and aggressive pathophysiology of NB demands prompt response and immediate medical intervention for nonspecific symptoms[66,67]. The economic structure of LMICs influences the affordability of healthcare and parental education[68-70]. These factors determine the promptness of the response to and the action taken with regard to nonspecific symptoms associated with the initial phases of childhood malignancies. The steadfast belief in traditional medicine as a first treatment option and cultural systems in which elders or a single authority figure decide about seeking medical intervention may delay action towards directed care[71,72]. Political stability and government policies have a direct impact on the availability, accessibility and quality of health care systems in treating childhood cancer[73,74].

Research priorities

The focus of research for LMICs should be on creating greater awareness in the diagnosis of NB, improving diagnostics and establishing social support strategies for successful, harmonised management protocols and homogenous treatment facilities to improve outcomes[55,75]. The main priority should be accurate tumour registries to document not only the most common or treatable childhood malignancies but also the rarer tumours such as NB[37]. In resource-limited settings, the need for genetic markers to develop more accurate risk classifications exists, especially to distinguish clearly between IR and HR patients. This is important in the case of stage 2 and stage 4 patients with adverse biology tumours who have in a higher risk classification compared to patients with non-adverse biology tumours[11,25,29]. Genome and exome sequencing have improved the understanding of the pathophysiology of NB in HICs[76]. However, knowledge regarding genetics of NB in the diverse ethnicities in LMICs is limited. A further challenge would be to make treatments and advanced diagnostics, such as liquid biopsies and biological tests, more widely available to all countries, whether HICs or LMICs, to improve diagnostic capacities and outcomes[75]. In advanced disease, palliative research could contribute to a greater understanding of the role of metronomic therapies and disease control in the context of NB[77].

DISCUSSION

Childhood malignancy awareness and advocacy still face great challenges, especially in LMICs, notably countries with large rural populations and great geographical divides, in accurately diagnosing malignancies, especially heterogeneous tumours such as NB. The lack of uniform treatment protocols for this variable disease is still a barrier to care. Epidemiological data are reproducible in different international studies, but data from across the world are not uniform. More research regarding tumour biology, specifically genomics, is needed not only in HICs but also in LMICs to determine underlying differences in molecular biology of the tumours, genetic targets and drug processing of NB patients, especially in heterogeneous populations. This information must then be made available to treatment centres where biological investigation is not possible, ready for clinical application to achieve improved outcomes for NB worldwide.

ARTICLE HIGHLIGHTS

Research background

Neuroblastoma (NB) is a well-documented childhood malignancy with the greatest source of knowledge originating from high-income countries. The management of NB in low- and middle countries (LMIC) is less robust due to various social and resource limitations.

Research motivation

The outcomes of various LMIC during the same period like South America, Francophone/North African countries, Asia and South Pacific Islands was evaluated.

Research objectives

This literature review was to evaluate regional development of management protocols, the challenges in treating NB in paediatric oncology units in LMIC as compared to high-income countries, new laboratory and clinical developments in the treatment of NB.

Research methods

A literature review of publications searched on PubMed, Medline, Global Health, Embase, SciELO and Google Scholar with keywords in keeping with NB and outcomes. Due to the variability in reporting, nonstandard application of definitions in the reported clinical results, heterogeneous data and paucity of information, the authors constructed limited tables to evaluate clinical and/or biological characteristics to report in the descriptive review.

Research results

Childhood malignancy awareness and advocacy still face great challenges, especially in LMICs, in accurately diagnosing malignancies, especially heterogeneous tumours such as NB. The lack of uniform treatment protocols for this variable disease is still a barrier to care. Epidemiological data are reproducible in different international studies, but data from across the world are not uniform.

Research conclusions

More research regarding tumour biology, specifically genomics, is needed not only in high-income countries but also in LMICs to determine underlying differences in molecular biology of the tumours, genetic targets and drug processing of NB patients, especially in heterogeneous populations.

Research perspectives

The focus of research for LMICs should be on creating greater awareness in the diagnosis of NB, improving diagnostics and establishing social support strategies for successful, harmonised management protocols and homogenous treatment facilities to improve outcomes. In resource-limited settings, the need for genetic markers to develop more accurate risk classifications exists. A further challenge would be to make treatments and advanced diagnostics, such as liquid biopsies and biological tests, more widely available to all countries. With advanced disease, palliative research could contribute to a greater understanding of the role of metronomic therapies and disease control in the context of NB.

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CASE REPORT

Concurrent renal cell carcinoma and hematologic malignancies: Nine case reports

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Author contributions: Shields LBE and Kalebasty AR contributed to the conception, design, acquisition, analysis, and interpretation of data; Shields LBE drafted the manuscript; Shields LBE and Kalebasty AR critically revised the manuscript and gave final approval.

Informed consent statement: All patients in this case series provided informed consent for treatment. Several patients died due to complications of their cancer before this case series was written.

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Abstract

BACKGROUND

The presence of renal cell carcinoma (RCC) and hematologic malignancies (HM) in the same patient is rarely observed. Three primary findings have been described in these patients, including male gender and lymphoid malignancy predominance, and the HM are usually diagnosed before or simultaneously with the RCC. There is a lack of evidence about clinical outcomes in this setting. We report the common characteristics of 9 patients diagnosed with concurrent RCC and HM and their clinical course and response to treatment.

CASE SUMMARY

Four (44%) patients were diagnosed with RCC prior to the HM, the diagnosis was simultaneous in 4 (44%) patients, and 1 (11%) patient was diagnosed with the HM prior to the RCC. No patients were treated with cytotoxic chemotherapy or radiation between the diagnosis of RCC and HM. Several unique features were seen in our case series, such as 3 simultaneous cancers in 1 (11%) patient, a splenectomy leading to remission of diffuse large B cell lymphoma without the use of chemotherapy in 1 (11%) patient, chemotherapy and rituximab for lymphoma resulting in a complete response in primary RCC in 1 (11%) patient, and immunotherapy providing an excellent response for primary renal leiomyosarcoma in 1 (11%) patient.

CONCLUSION

These findings highlight the potential role of immune system dysregulation in patients with the diagnosis of RCC and HM whereby the first malignancy predisposes to the second through an immunomodulatory effect. HM have the potential of being confused with lymph node metastasis from kidney cancer. Lymph node biopsy may be necessary at the time of initial diagnosis or in cases of mixed response to therapy. Long-term medical surveillance is warranted when a patient is diagnosed with RCC or HM. Clinicians should be aware of the higher

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prevalence of male gender and lymphoid malignancy with concurrent RCC and HM and that either of these conditions may be diagnosed first or they may be diagnosed simultaneously.

Key words: Oncology; Renal cell cancer; Hematologic malignancy; Lymphoma; Immune system; Immunotherapy; Case report

Core tip: Renal cell carcinoma (RCC) and hematologic malignancies (HM) in the same patient is rare. We report the common characteristics of 9 patients diagnosed with concurrent RCC and HM and their clinical course and treatment response. None of the patients was treated with cytotoxic chemotherapy or radiation between the diagnosis of RCC and HM. Several features in our series strengthen the immune dysregulation theory as the likely mechanism. Long-term medical surveillance is warranted when a patient is diagnosed with RCC or HM. Clinicians should be aware that either of these conditions may be diagnosed first or they may be diagnosed simultaneously.

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INTRODUCTION

Renal cell carcinoma (RCC) and hematologic malignancies (HM) coexisting in a single patient at the same time is a rare phenomenon. RCC is observed in the general population in 12.5 persons per 100000 and HM in 31.8 per 100000[1]. The incidence of RCC and HM occurring in same patient is greater than that expected in the general population[1,2]. A higher than expected incidence of RCC concurrent with non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) has also been reported[2-5]. Epidemiological studies have shown that the observed-to-expected ratio for occurrence of RCC in NHL patients were 1.86 to 2.67[2,3]. Furthermore, it has been reported that patients with the diagnosis of NHL have an increased incidence of RCC, bladder carcinoma, lung carcinoma, brain tumors, and melanoma as well as other HM such as acute myeloid leukemia and HL[6,7]. Three predominant features have been described in these patients: (1) Greater number of males; (2) The majority of HM are lymphoid; and (3) The HM are usually diagnosed prior to or simultaneously with the RCC[8].

Several mechanisms have been proposed to account for concurrent RCC and HM, including genetic mutations common to both RCC and HM, hormonal or environmental factors, viral infections, prior cytotoxic chemotherapy or radiation for malignancy, and immune dysregulation[2,3,5,8-12]. It has been suggested that the first malignancy predisposes to the second through an immunomodulatory effect[1-4]. In this respect, immune dysregulation generates the lymphoma which subsequently leads to the development of solid tumors such as RCC. The surveillance for malignancy and immune dysregulation resulting from immune checkpoint inhibitors in these cases are only theories, and the exact mechanisms are unknown.

In this study, we reviewed the medical records and imaging studies of 9 consecutive individuals who were diagnosed with both RCC and HM and evaluated by the same medical oncologist between June 1, 2013 and December 31, 2019 at our Institution (Table 1). Common characteristics of patients with these 2 conditions are presented, and the potential etiology of simultaneous RCC and HM are discussed. We highlight several distinct features of our cases that strengthen the immune dysregulation theory as the most likely mechanism.

Table 1 Patients with concurrent renal cell carcinoma and hematologic malignancies at our institution

Case	Age at RCC	Grade/Stage/Histology RCC	RCC treatment	Hematologic malignancy	Duration between RCC and HM diagnoses	Treatment for HM
1	68	Grade 2 PRCC with oncocytic features; clinical Stage 1	None	Grade 2 Stage 4 follicular lymphoma	RCC 10 wk before LM	(1) Bendamustine/rituximab; (2) Follow up 49 mo after kidney biopsy; and (3) Still alive with both malignancies in remission
2	70	Stage 3 Grade 4 CCRCC	Radical nephrectomy	Stage 4 mantle cell lymphoma	Simultaneous	(1) Bendamustine/rituximab; imbruvica; bortezomib; rituximab/lenalidomide; and (2) Acute kidney injury, died 54 mo after nephrectomy as result of mantle cell lymphoma; CCRCC in remission
3	55	Stage 1 Grade 2 CCRCC	Radical nephrectomy	Multiple myeloma	RCC 7 wk before LM	(1) Bortezomib/cyclophosphamide/dexamethasone; (2) Autotransplant; (3) Lanelidomide; pazopanib; cabozantinib; nivolumab/ipilimumab; axitinib; lenvatinib/everolimus; atezolizumab/bevacizumab; and (4) Died 70 mo after nephrectomy due to metastatic CCRCC
4	62	Stage 1 Grade 3 CCRCC	Radical nephrectomy	Stage 3 Grade 3 follicular lymphoma	Simultaneous	(1) Cyclophosphamide/doxorubicin hydrochloride/ palonosetron/rituximab/vincristine (CHOP); rituximab; pazopanib, cabozantinib; and (2) Died 64 mo after nephrectomy and lymph node dissection of progressive metastatic CCRCC
5	58	Stage 1 Unclassified RCC	Partial nephrectomy	Stage 4 non-sclerosing Hodgkin's lymphoma	RCC 109 mo before LM	(1) Brentuximab vedotin/doxorubicin/vinblastine/dacarbazine (AAVD); and (2) Follow up 23 mo after lymph node biopsy; (3) Still alive with no evidence of recurrent lymphoma or RCC
6	55	Stage 1 CCRCC	Partial nephrectomy	Stage 1E diffuse large B-cell lymphoma	CCRCC 75 mo before LM	(1) No cytotoxic chemotherapy; (2) Splenectomy; (3) Follow up 112 mo after nephrectomy and 37 mo after splenectomy; and (4) Still alive with no evidence of recurrent lymphoma or CCRCC
7	59	Clinical Stage 1 RCC	None	Low-grade follicular center cell lymphoma of skin	LM 9 wk before RCC	(1) No cytotoxic chemotherapy; (2) Excision of skin lymphoma; (3) Follow up 17 mo after kidney biopsy confirming lymphoma; and (4) Kidney CA on surveillance and no recurrent lymphoma
8	71	Stage 4 Grade 2 leiomyosarcoma of the kidney	Kidney biopsy; radical nephrectomy 9 mo later	B cell lymphoma Stage 1E of the kidney	Simultaneous	(1) Gemcitabine/docetaxel anhydrous; ipilimumab/nivolumab; (2) Follow up 11 mo after biopsy confirming leiomyosarcoma of the kidney; and (3) Still alive with continued response of metastatic kidney cancer on check point inhibitor; no treatment for lymphoma
9	77	Clinical Stage 1 RCC	None	Diffuse large B-cell lymphoma subtype activated B-cell, Stage 2E	Simultaneous	(1) Rituximab/cyclophosphamide/etoposide/vincristine/prednisone; (2) Follow up 12 mo after kidney biopsy confirming lymphoma; and (3) Still alive with no evidence of recurrent RCC or lymphoma

RCC: Renal cell carcinoma; HM: Hematologic malignancy; PRCC: Papillary renal cell carcinoma; CCRCC: Clear cell renal cell carcinoma.

CASE PRESENTATION

Case 1

A 68-year-old man underwent a computed tomography (CT) of the chest, abdomen, and pelvis with Gadolinium contrast which revealed a solid and enhancing hypovascular renal mass, axillary adenopathy, splenomegaly with multiple splenic lesions, and spinal bone involvement (Figure 1A and B).

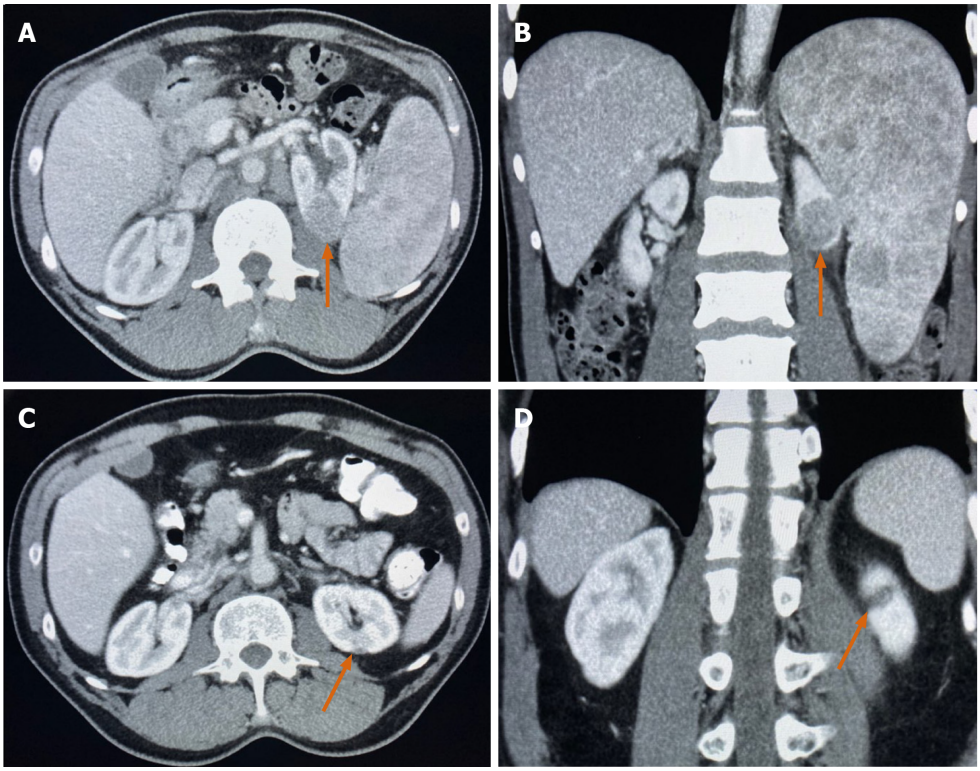

Figure 1 Computed tomography imaging. A and B : Case #1: Computed tomography imaging revealed a renal mass (arrows). Biopsy of the mass was consistent with papillary renal cell carcinoma; C and D: The renal mass resolved after treating the follicular lymphoma with 6 cycles of bendamustine/rituximab (arrows).

Case 2

A 70-year-old man underwent a CT of the abdomen and pelvis with Gadolinium contrast which demonstrated a large mass protruding from the lower pole of the left kidney measuring 10.0 cm × 8.2 cm with enlarged lymph nodes in the left common and external iliac groups and left inguinal area.

Case 3

A 55-year-old man underwent a CT of the abdomen and pelvis with Gadolinium contrast which revealed a 7.0 cm × 5.0 cm left renal mass with bony metastasis of the left acetabulum and superior pubic ramus.

Case 4

A 62-year-old man underwent a CT of the abdomen and pelvis with Gadolinium contrast that demonstrated a 4.2 cm × 3.9 cm mass arising from the posterior right renal cortex and a 1.9 cm retroperitoneal/paraaortic lymph node.

Case 5

A 58-year-old man was diagnosed with Stage 1 unclassified RCC after undergoing a partial nephrectomy. The patient was monitored by CT scan surveillance. A CT of the chest, abdomen, and pelvis performed 105 mo after the partial nephrectomy revealed anterior mediastinal axillary lymphadenopathy (LAD).

Case 6

A 55-year-old man was diagnosed with Stage 1 clear cell RCC (CCRCC) following a partial nephrectomy. The patient was observed by surveillance CT scans. A CT scan performed 75 mo following the partial nephrectomy demonstrated a 5.0 cm splenic mass originally suspected of being metastatic kidney cancer.

Case 7

A 59-year-old man underwent a CT of the chest, abdomen, and pelvis with Gadolinium contrast which revealed a 15.0 mm solid-appearing enhancing lesion at the superior right kidney.

Case 8

A 71-year-old man, with a family history of leukemia in his father, underwent a CT of the chest, abdomen, and pelvis with Gadolinium contrast demonstrated a 5.9 cm × 9.2 cm renal mass with lytic bone lesions. A lumbar magnetic resonance imaging (MRI) with Gadolinium contrast showed multiple T1 hypointense T2 hyperintense enhancing lesions of the lower thoracic and lumbar spine, upper sacrum, and superior medial iliac bones with the largest in the L3 lumbar vertebrae.

Case 9

A 77-year-old man underwent a CT of the abdomen and pelvis with Gadolinium contrast which revealed evidence of a 12.0 cm left upper pole mass with a separate 5.0 cm left lower pole mass with invasion of the spleen and surrounding lymphadenopathy.

FINAL DIAGNOSIS

Case 1

A biopsy of a right inguinal lymph node confirmed Grade 2 Stage 4 follicular lymphoma.

Case 2

The patient was diagnosed with simultaneous Stage 3 Grade 4 CCRCC and Stage 4 mantle cell lymphoma following a radical nephrectomy and retroperitoneal lymph node dissection (LND). Immunohistochemistry confirmed the mantle cell lymphoma in the retroperitoneal lymph node.

Case 3

The patient was diagnosed with Stage 1 Grade 2 CCRCC following a nephrectomy. A gastric resection was also performed during the nephrectomy which revealed a gastrointestinal stromal tumor (GIST). Biopsy of a lytic bone lesion suspected of metastatic kidney cancer revealed a diagnosis of MM.

Case 4

The patient was diagnosed with simultaneous Stage 1 Grade 3 CCRCC and Stage 3 Grade 3 follicular lymphoma following a radical nephrectomy and dissection of interaortocaval lymph nodes.

Case 5

A lymph node biopsy confirmed Stage 4 non-sclerosing HL.

Case 6

The patient underwent a splenectomy that revealed a Stage 1E diffuse large B-cell lymphoma in the spleen.

Case 7

The patient underwent an excisional biopsy of the skin of the right arm that revealed a low-grade follicular center cell lymphoma. Nine weeks later an abdominal MRI demonstrated a solid enhancing mass suggestive of RCC (clinical Stage 1).

Case 8

The patient was diagnosed with simultaneous Stage 4 Grade 2 leiomyosarcoma of the kidney and Stage 1E B cell lymphoma of the kidney by renal biopsy.

Case 9

The patient was diagnosed with simultaneous clinical Stage 1 RCC and Stage 2E diffuse large B-cell lymphoma subtype activated B-cell with involvement of the retroperitoneal lymph nodes. A kidney biopsy confirmed the diagnosis of lymphoma.

TREATMENT

Case 1

The patient was treated with 6 cycles of bendamustine/rituximab which resulted in a complete response of his lymphoma. Interestingly, his primary kidney mass resolved without any further intervention after treating his lymphoma (Figure 1C and D).

Case 2

The patient underwent 13 cycles of bendamustine/rituximab following which a pulmonary metastatic nodule was detected on chest CT, reflecting a progression-free survival (PFS) of 37 mo. He was subsequently treated with 2 cycles of imbruvica and 6 cycles of bortezomib after which progressive disease with cervical and LAD was observed on CT (7 mo PFS). He then had 2 cycles of rituximab/lenalidomide.

Case 3

The patient underwent 4 cycles of bortezomib/cyclophosphamide/dexamethasone followed by an autotransplant. He subsequently underwent 27 cycles of maintenance lanelidomide after which a rib lesion was confirmed as RCC by chest CT (PFS 26 mo). He then had 11 cycles of pazopanib following which there was evidence of another metastatic rib lesion and LAD (PFS 11 mo). The patient underwent 5 cycles cabozantinib and 3 cycles nivolumab/ipilimumab with LAD detected 3 mo later. He was then treated with 4 cycles axitinib with subsequent pulmonary metastatic nodules and LAD by CT (PFS 4 mo). Following 2 cycles of lenvatinib/everolimus, a hepatic metastatic lesion was noted (PFS 3 mo). The patient then underwent 1 cycle of atezolizumab/bevacizumab.

Case 4

The patient underwent 6 cycles of cyclophosphamide, doxorubicin hydrochloride, vincristine, prednisone, and rituximab followed by 3 cycles of maintenance rituximab. A lytic rib lesion was subsequently confirmed as RCC (PFS 14 mo). He was treated with 18 cycles of pazopanib. A T9 vertebral body lesion was subsequently detected (PFS 23 mo), and pulmonary metastatic nodules and LAD were observed 9 mo later on CT. He was treated with cabozantinib with poor tolerance. Immunotherapy with check point inhibitors was not offered due to active seronegative rheumatoid arthritis.

Case 5

The patient underwent 6 cycles of brentuximab vedotin, doxorubicin, vinblastine, and dacarbazine.

Case 6

The patient did not undergo any further systemic treatment for his lymphoma.

Case 7

The decision was made to continue monitoring the kidney mass rather than performing a partial nephrectomy. The patient had radiation to the skin lesion after the excisional biopsy without receiving any systemic therapy.

Case 8

The patient underwent 3 cycles of gemcitabine/docetaxel after which a CT detected a spinal lesion confirmed as progression of osseous metastasis (PFS 3 mo). The patient was then treated with 4 cycles of ipilimumab/nivolumab. Progression was noted in the kidney 7 mo afterwards. He subsequently underwent a radical nephrectomy.

Case 9

The patient underwent 6 cycles of rituximab/cyclophosphamide/etoposide/vincristine/prednisone for his lymphoma which led to a complete response.

OUTCOME AND FOLLOW-UP

Case 1

After 49 mo follow-up since the diagnosis of kidney cancer, the patient is alive with both malignancies in remission.

Case 2
The patient died of acute kidney injury 54 mo after the nephrectomy and LND as a result of mantle cell lymphoma. The RCC remained in remission.

Case 3
The patient died due to metastatic CCRCC, 70 mo after the nephrectomy. His MM remained in remission.

Case 4
The patient died of progressive metastatic CCRCC 64 mo after the nephrectomy and LND.

Case 5
The patient is still alive with no evidence of recurrent lymphoma or RCC, 23 mo after the lymphoma diagnosis.

Case 6
The patient is still alive with no evidence of recurrent lymphoma or CCRCC (follow up 112 mo since CCRCC).

Case 7
The patient is still alive with no recurrent lymphoma 17 mo after the skin biopsy, and the RCC remains under surveillance without any significant radiographic progression.

Case 8
The patient is still alive 4 mo after the nephrectomy with continued response in all metastatic sites of leiomyosarcoma of the kidney on maintenance nivolumab. He did not undergo systemic treatment for the lymphoma.

Case 9
The patient continued to show evidence of primary RCC by imaging with no significant growth over time. The patient is still alive 12 mo later with no evidence of progressive lymphoma.

DISCUSSION
The phenomenon of concurrent RCC and HM has been reported in the literature, with a focus on gender, timing of the diagnosis of RCC and HM, and most common HM (Table 2)[1-5,8,10,11]. All except one of the studies in Table 2 documented a higher number of men with both conditions. Interestingly, in Tihan's and Filippa's study of 15 patients with coexisting RCC and malignant lymphoma, 11 (73%) of their patients were female[5]. Four (50%) studies in the literature in Table 2 reported a higher number of patients diagnosed with HM before RCC. NHL was the most common HM in the majority of these studies. In Dutcher and colleagues' review of 199 cases of RCC and HM in the same patient identified in the literature (n = 173) and in their registry (n = 26) between 1991 and 2016, an association between RCC and HM within families was observed, exemplified by 74 patients with RCC who had 95 family members with HM[8]. These authors suggested a genetic correlation between RCC and B-cell malignancies.

Our case series of 9 patients with concurrent RCC and HM confirms particular aspects in the literature in Table 2, such as the predominance of male gender (all 9 patients in our series) and NHL (7 cases in our series) as the most frequent HM. However, only 1 patient in our series was diagnosed with the HM before the RCC, as either simultaneous diagnosis of the RCC and HM (4 patients) or RCC diagnosis before the HM (4 patients) was more common. We hypothesize that the predilection of male gender in concurrent RCC and HM may be due to the predominant gender in each cancer. In RCC, the male-to-female ratio is almost equal (1.2:1) in patients older than 70 years compared to a 2:1 ratio for patients ages 41 to 60 years old[13]. NHL is significantly more common in males[14]. The lower rate of NHL among females may be explained by direct effects of estrogens on lymphoma cell proliferation or by its effect on anti-tumor immune response[14]. The higher prevalence of males in concurrent RCC and HM may be attributed to more males compared to females affected in both RCC and HM.

Table 2 Concurrent renal cell carcinoma and hematologic malignancies in the literature

Study	Gender (M/F)	Number of patients with HM diagnosed first	Number of patients with simultaneous diagnosis of RCC and HM	Number of patients with RCC diagnosed first	Most common HM
Anderson *et al*[3], 1988, (*n* = 41)	32 (78)/9 (22)	17 (41)	8 (20)	16 (39)	NHL: 41 (100)
Nishikubo *et al*[1], 1996, (*n* = 8)	7 (88)/1 (12)	4 (50)	0 (0)	4 (50)	NHL: 2 (25); CLL: 2 (25); MM: 2 (25)
Tihan *et al*[5], 1996, (*n* = 15)	4 (27)/ 11 (73)	0 (0)	14 (93)	1 (7)	NHL: 15 (100)
Ohsawa *et al*[11], 1998, (*n* = 42)	27 (64)/15 (36)	38 (90)	4 (10)	0 (0)	NHL: 42 (100)
Kunthur *et al*[10], 2006, (*n* = 9)	8 (89)/1 (11)	6 (67)	1 (11)	2 (22)	NHL: 6 (67)
Choueiri *et al*[4], 2008, (*n* = 8)	6 (75)/2 (25)	4 (50)	0 (0)	4 (50)	MM: 8 (100)
Serefhanoglu *et al*[2], 2010, (*n* = 5)	5 (100)/ 0 (0)	2 (40)	3 (60)	0 (0)	CLL: 3 (60)
Dutcher *et al*[8], 2016, (*n* = 26)	18 (69)/8 (31)	16 (62)	2 (5)	8 (31)	NHL: 13 (50)
Current study, 2020, (*n* = 9)	9 (100)/0 (0)	1 (11)	4 (44)	4 (44)	NHL: 7 (78)

HM: Hematologic malignancy; MM: Multiple myeloma; CLL: Chronic lymphocytic leukemia; NHL: Non-Hodgkin's lymphoma; RCC: Renal cell carcinoma.

A host of mechanisms has been reported as playing a role in developing concurrent RCC and HM. As the greatest number of patients with these combined conditions involves diagnosis of HM first[8,11], it has been proposed that immune dysregulation or breakdown of tumor surveillance associated with the lymphoma may lead to RCC[3,10]. An abnormal immune response may either precipitate lymphoma in patients whose RCC was diagnosed first or predispose a patient to developing both malignancies simultaneously[1,9,10]. As both lymphoma is a neoplasm of the immune system and RCC is a solid tumor that possesses an immune responsive behavior, the simultaneous occurrence of these diseases may be due to failure in tumor surveillance caused by the lymphoma that permits the RCC to develop[3]. In other cases, stimulation of the immune system by the RCC may result in lymphocytic proliferation and clonal proliferation which may spur the development of the HM.

We propose that immune dysregulation may be a potential explanation for both RCC and HM occurring in one host, however, this is solely a theory and the exact mechanisms are not known. It may be partly explained by the fact that both RCC and HM are well-known to respond to immunotherapy although they may have different clinical aggressiveness based on histology and grade. Both malignancies may be monitored without treatment at times based on histology, grade, and clinical behavior. Indolent disease may start to progress quickly due to transformation of the tumor to a more agrressive type. It appears in some cases that the host's immune system may partially control the tumor growth resulting in "stable disease" without a need for treatment. In such cases, intrinsic or extrinsic immunosuppression may lead to tumor progression.

There is also an increased risk of RCC in patients with NHL that may be attributed to chemotherapy and radiation used in NHL treatment[2,3,11,12]. Additional etiologies for concurrent RCC and HM include viruses such as Epstein-Barr virus, *Helicobacter pylori*, and human T-lymphotropic virus-1 that have been implicated in lymphomas and carcinomas[2,8]. Interleukin-6 produced by RCC has been shown to stimulate the progression of MM[15]. A common genetic factor may also be involved in concurrent RCC and HM as common chromosomal abnormalities such as 3p and 17p deletions have been observed in both conditions[2,10,16,17]. In addition, *PTEN* germline mutations have been reported in hereditary RCC, and studies have described abnormalities of *PTEN* in T-cell and B-cell HM[8]. *PTEN* abnormalties as a common pathway for the development of RCC and HM in individuals or families remains to be elucidated[8].

The potential similar genetic components in these conditions necessitate a thorough investigation into family histories.

As none of our patients in our series underwent cytotoxic chemotherapy or radiation between the diagnosis of RCC and HM, these treatments did not contribute to the development of either malignancy. Furthermore, as only 1 patient had a family history of leukemia and there were no other definitive environmental, hormonal, or genetic factors, we hypothesize that immune system dysregulation plays the most significant role in the coexistence of RCC and HM. As none of our patients underwent cytotoxic chemotherapy or radiation between the diagnosis of RCC and HM, these treatments did not contribute to the development of either malignancy. Furthermore, as only 1 patient had a family history of leukemia and there were no other definitive environmental, hormonal, or genetic factors, we hypothesize that immune system dysregulation plays the most significant role in the coexistence of RCC and HM.

Several unique features in our patients shed light on the immunological interactions between these 2 conditions. Case #1 did not undergo a nephrectomy or any other treatment for the PRCC which was diagnosed 10 wk before the follicular lymphoma. He underwent cytotoxic chemotherapy (generally not effective in papillary RCC) to treat the latter condition after which both malignancies were in remission. Immune modulation resulting from the lymphoma treatment most likely spurred activation of the patient's immune response leading to resolution of the PRCC. Interestingly, Case #3 was diagnosed with 3 simultaneous malignancies, including CCRCC, MM, and GIST. These conditions are unrelated and arise from different embryonic origins, specifically, carcinoma, myeloma, and sarcoma. Following only a splenectomy and without any systemic therapy in Case #6, his aggressive diffuse large B-cell lymphoma remains in remission.

Case #8 was diagnosed with the exceedingly rare and aggressive leiomyosarcoma of the kidney which accounts for only 0.12% of renal malignancies and is usually detected in women[18-20]. The cause of female predominance is not fully understood, however, it has been suggested that some malignancies are associated with genes located on X chromosomes that avoid X-inactivation[18,21]. Interestingly, a male predominance has been reported with concurrent RCC and HM, while renal leiomyosarcoma is more common in females. The diagnosis of renal leiomyosarcoma in a male with a simultaneous lymphoma makes our case more unique. A nephrectomy is the treatment of choice for renal leiomyosarcoma; chemotherapy has been reported to be of limited success[18,20]. Case #8 had an excellent response of 7 mo to immunotherapy with check point inhibitors.

Our case series also highlights the important role of biopsy in confirming the diagnosis. Although it may not be possible to biopsy each and every metastatic site, clinicians should consider biopsy when in doubt given the possibility of a second malignancy. Kidney cancer does not follow a certain pattern for lymph node metastasis, and it may be difficult to know whether adenopathy is of benign etiology or metastasis from kidney cancer or another malignancy. Clinicians should consider biopsy in cases of "mixed response" to systemic treatment for kidney cancer.

Strides have been made in cancer immunotherapy with the discovery of checkpoint inhibitors which effectively inhibit the immune system[22]. Programmed cell death 1 receptor (PD-1) signaling plays a role in encouraging cancer development and progression by boosting tumor cell survival[23]. It has been reported that blocking PD-1 signaling significantly promotes antitumor immunity, produces favorable clinical responses, and prolongs survival[23]. Developing antibodies that block PD-1 and programmed cell death receptor ligand 1 have been investigated. The checkpoint inhibitors ipilimumab and nivolumab proved invaluable in the treatment of Case #8 as exemplified by the 7-mo excellent response in this rare kidney cancer histology.

Both RCC and HM may usually be monitored based on clinical and histological factors. RCC has risk stratification criteria (International Metastatic RCC Database Consortium criteria and Memorial Sloan-Kettering Cancer Center criteria) which has been used extensively for offering treatment[24,25]. Watchful waiting may be offered in certain circumstances to patients with RCC. These patients generally have a favorable risk, low disease burden, and a single site of metastasis. Other factors such as metastatic site should be considered in cases of surveillance. Lung, adrenal, and pancreatic metastasis may potentially have a slower clinical course compared to liver and bone metastasis. Diligent monitoring may be performed for some asymptomatic patients with low-grade HM who do not have significant cytopenia.

CONCLUSION

Long-term medical surveillance is warranted when a patient is diagnosed with RCC or HM. Clinicians should be aware that either of these conditions may be diagnosed first or they may be diagnosed simultaneously. A thorough evaluation into the patient's history of hormonal or environmental factors, viral infections, and prior cytotoxic chemotherapy or radiation as well as family history of RCC or HM is imperative to delve into the mechanisms that may contribute to these conditions. Further investigation into the immunological dynamics and common genetic abnormalities of RCC and HM may elucidate the relationship between these malignancies.

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CASE REPORT

Proton beam therapy of periorbital sinonasal squamous cell carcinoma: Two case reports and review of literature

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Abstract

BACKGROUND

Sinonasal malignancies are rare but demanding due to complex anatomy, usually late diagnosis, and inconsistent therapy strategy based on multimodality approaches. Squamous cell carcinoma (SCC) is the most common histology, with poorer prognosis. In the setting of orbital invasion, an orbital exenteration may be required. However, in case of primary rejection of disfiguring surgery or unresectable disease, proton beam therapy (PBT) should be largely considered, allowing for better sparing of neighboring critical structures and improved outcomes by dose escalation.

CASE SUMMARY

A 62-year-old male presented with a recurrent SCC in the nasal septum abutting frontal skull base and bilateral orbits at 7 mo after primary partial nasal amputation. Because of refusal of face-deforming surgery and considerable adverse effects of conventional radiotherapy, the patient underwent a PBT by hyperfractionated accelerated scheme, resulting in complete response and moderate toxicities. After 2 years, a nasal reconstruction was implemented with satisfactory appearance and recurrence-freedom to date. Another patient with an initially extended sinonasal SCC, invading right orbit and facial soft tissue, declined an orbital exenteration and was treated with a normofractionated PBT to the gross tumor and elective cervical lymphatics. The follow-up showed a continuous tumor remission with reasonable late toxicities, such as cataract and telangiectasia on the right. Despite T4a stage and disapproval of concurrent chemotherapy owing to individual choice, both patients still achieved outstanding treatment outcomes with PBT alone.

CONCLUSION

PBT enabled orbit preservation and excellent tumor control without severe adverse effects on both presented patients with locally advanced sinonasal SCC.

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Core tip: The treatment of sinonasal squamous cell carcinoma is exceedingly challenging owing to complex anatomy, delayed diagnosis, and lack of randomized clinical studies about multimodality approaches. In particular, locally advanced disease with indication of orbital exenteration or other disfiguring surgeries, as well as unresectable gross tumor require modern non-surgical treatment options like proton beam therapy, as presented in this case report, to achieve a long-term tumor control without severe late toxicities, such as blindness and cerebral radiation necrosis.

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INTRODUCTION

Sinonasal malignancies (SNMs) occur very seldom and account for only 3% of all head and neck cancers and 1% of all malignant tumor diseases, with a peak incidence in the 5th to 7th decades and predominance among males. The prodromes, such as nasal congestion and discharge, epistaxis and lacrimation, are often misjudged as rhinosinusitis and consequently neglected by both the patients and physicians. At the presence of late symptoms like facial edema, sensory failures and cranial neuropathy, the patient is first referred to sinonasal endoscopy and imaging[1]. At this time, however, over 50% of the cases are diagnosed in an advanced stage (T3/4), with poor prognostic outcome[2,3]. Female sex, nasal cavity tumor, adenocarcinoma and low clinical stage have been identified as positive predictors[4]. Among the epithelial tumors, the squamous cell carcinoma (SCC) is the most common (80%), followed by adenocarcinoma. Both histological subtypes are etiologically associated with occupational exposure to wood, leather and textile dusts, organic solvents, welding fumes, arsenic, *etc*.[5,6].

For sinonasal (SN) SCC (SNSCC), higher age and tumor stage are adverse prognostic factors, while surgery has been shown to improve survival significantly[3]. Based on the analysis of the United States National Cancer Database, surgical approach represents the therapeutic mainstay of SNSCC, whereas neoadjuvant chemoradiotherapy (CRT) is associated with improved R0 resectability[7]. Although recent retrospective studies have validated superior outcomes by multimodality, the optimal combination and sequence of surgery, radiotherapy (RT) and chemotherapy remain controversial[7-9]. Furthermore, locally advanced SNM with orbital invasion is actually challenging for clinicians due to the complexity of complete gross resection, that largely requires an orbital exenteration and consecutive aesthetic restoration by means of plastic surgery, prosthesis and rehabilitation. Given the correlated burden to the patient's psyche and quality of life, the information about prognosis, multimodal therapy approaches and supportive adjuvant measures should be comprehensively discussed between the patient and attending physicians before the therapeutic decision[10].

CASE PRESENTATION

Case 1

Chief complaints: A 62-year-old German male presented with a relapsed tumor in the nasal septum, extending to dual-sided ethmoidal sinuses and abutting frontal skull base, as well as a suspicious lymph node metastasis in the left parotid gland, in an interval of 7 mo after the primary surgery to address a nasal SCC.

History of present illness: The tumor recurrence was confirmed by a sampling excision of nasal mucosa in August 2014, which showed moderately differentiated keratinizing SCC. According to the assessment of otorhinolaryngology, a resection was possible in principle but would have been accompanied by enormous physical defect and face distortion due to the requisite removal of bilateral medial canthi and glabella. The patient rejected the surgery and tried to gain information about RT. Consultation with the radiation oncology team of the university hospital close to his home led to recommendation of a combined CRT or proton beam therapy (PBT). The patient preferred the latter, after he became educated about the more vehement toxicities of conventional RT with photons, such as necessity of artificial nutrition owing to pharyngitis, malfunction of sense of smell and taste, deafness of left ear, and blindness in 2-5 years. Consequently, he contacted three particle therapy institutes in Germany but obtained refusal from two for the following reasons: The benefit of particle therapy for SNSCC was not completely clarified and could not be offered out of clinical trials. Besides, the sinonasal airspaces causing uncertainties in the treatment planning was unfavorable for the exact calculation of dose distribution in the target volume. Therefore, a conventional RT *via* modern technique [(*e.g.*, intensity-modulated RT (IMRT)] with concomitant platinum-based chemotherapy was recommended. On the contrary, Rinecker Proton Therapy Center was the only one of the three consulted institutes which accepted the patient for PBT.

History of past illness: The nasal SCC had been initially noted in October 2013 by recurrent epistaxis with swelling and enlargement of the nose and foreign body feeling. Endoscopy demonstrated an exophytic lesion in the nasal septum, reaching to the nasal floor. Imaging examinations, including computed tomography (CT) scan of head, neck and thorax and ultrasound of neck, showed a tumor perforating the anterior nasal septum with infiltration of nasal bridge and destruction of nasal bone, emphasized on the left side, as well as a suspicious Warthin's tumor in the left parotid gland. In January 2014, the patient underwent a partial nasal ablation (Figure 1A) and selective neck dissection (level I-III) on both sides, with postoperative tumor stage determined to be pT2 pN0 G3 R0 cM0.

Personal and family history: The patient was in good general condition and worked at his own gym. Apart from chronic nicotine abuse (at least 50 pack-years), there was no relevant comorbidity known.

Physical examination upon admission: There was an obvious substance defect in the middle nasal portion with tumorous skin thickening all-round after the partial amputation (Figure 1B), so that the original nasal epithesis no longer fit within. The common clinical examination yielded normal findings.

Laboratory examinations: No special laboratory test was arranged.

Imaging examinations: The magnetic resonance imaging (MRI) prior to PBT showed intensive contrast enhancement in the central nasal cavity with soft tissue swelling of nasal bridge until nostrils, measuring approximately 29 mm × 15 mm × 22 mm, abutting the frontal sinus and skull base (Figure 2A-C). The ethmoidal air cells were partially involved by tumor infiltration as well as mucosal swelling. Apart from at least one strong enhancing nodule of 8 mm × 11 mm diameter at the lower pole of left parotid gland, no pathological cervical lymph node was detected. To complete the restaging examination, the patient underwent additional positron emission tomography with 2-deoxy-2-fluorine-18-fluoro-D-glucose (^{18}F-FDG-PET/CT). Both suspicious tumor recurrences in the nasal bridge and left parotid gland exhibited a maximum standardized uptake value [SUV(max)] of 5.2 and 2.7 in each (Figure 3A-C). Although the parotid lesion was initially interpreted as Warthin's tumor, which is FDG-avid on principle, this finding was assessed by our specialist of nuclear medicine and radiology as highly suspicious of intraparotid lymph node metastasis.

Final diagnosis: Recurrent periorbital SNSCC, tumor stage rpT4a rpN1 G2 cM0.

Treatment: At express request due to continuation of working during the treatment, the patient was treated with hyperfractionated accelerated scheme within 37 d, from October to December 2014. The informed consent was obtained prior to the initiation of the treatment. The PBT was delivered in 44 fractions and single dose of 1.50 Gy [relative biological effectiveness (RBE)], twice a day with minimum interim of 4 h, at a total dose of 66.00 Gy (RBE) to the tumor recurrences in the nasal bridge and left parotid gland. Simultaneously, the left cervical lymphatic drainage, including nodi

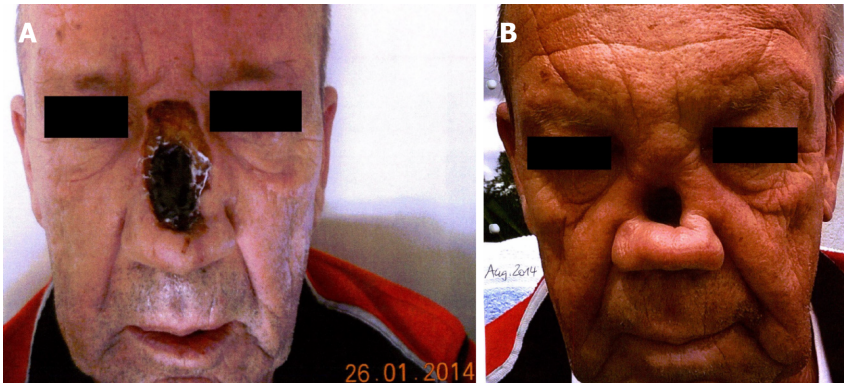

Figure 1 Patient's images prior to proton beam therapy. A: Partial nasal ablation in January 2014 at the initial diagnosis of a nasal squamous cell carcinoma; B: Distinct thickening in the nasal bridge, indicating local recurrence in August 2014.

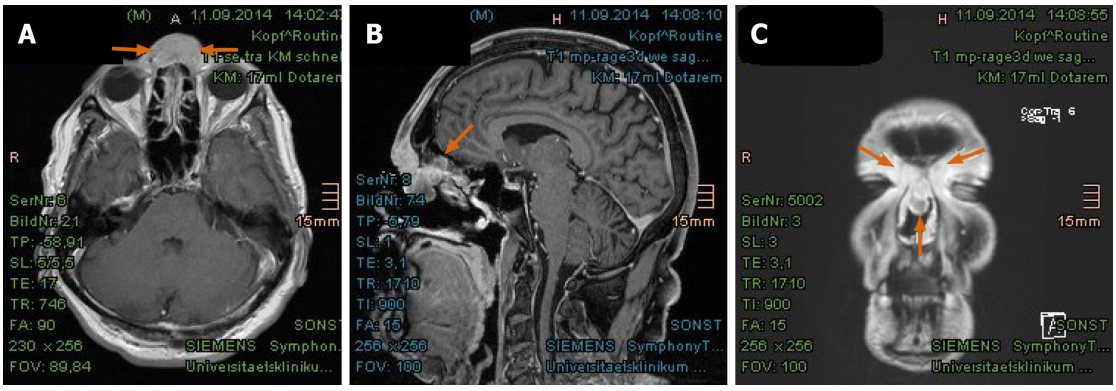

Figure 2 Magnetic resonance imaging from September 2014 revealed tumorous enhancement in the nasal bridge abutting frontal sinus and skull base. A: Axial plane; B: Sagittal plane; C: Coronal plane. Local recurrence marked with arrows.

lymphatici parotidei, submandibulares and jugulares superiores, received 52.80 Gy (RBE) in total, with single dose of 1.20 Gy (RBE). The entire target volume was irradiated from three gantry angles of 30°, 330° and 80° using the pencil beam scanning technique (Figure 4A). After 28 fractions, the safety margin to both eyeballs was reduced because of incipient tumor shrinkage, noticed by weekly-performed low-dose CT scans, as well as for the purpose of better eye sparing (Figure 4B). Since the statutory health insurance refused to reimburse the cost of PBT, the patient deliberately declined a concurrent chemotherapy, in order to demonstrate afterwards that he was exclusively cured by PBT alone.

Outcome and follow-up: Generally, the patient tolerated PBT well and drove 300 km daily between our center and his home. At the beginning of the treatment, he complained of intumescence of the nose, with boring pain in the evening, that was mitigated by anti-edematous medication (dexamethasone 8-16 mg per day) and analgesics. In the further course, he developed increasing radiation dermatitis with superinfection in the middle face, especially at the inner corners of both eyes, corresponding to grade 2-3 by Common Terminology Criteria for Adverse Events (commonly referred to as CTCAE). At the final examination, the skin finding was improved by intensified skin care and disinfectant measures taken immediately after daily irradiation. The patient denied visual impairment and dry eyes as well as dysphagia and changes in taste and smell. Xerostomia only occurred temporarily.

In the first follow-up, at 3 mo after the PBT, MRI scan displayed a significant tumor reduction in the nasal bridge (Figure 5A-C). At this stage, it was normally hard to distinguish between residual tumor and inflammation tissue. Nonetheless, the biopsy from the nasal bridge revealed a chronic granulating mucosal ulcer with no evidence of malignancy. As post-radiogenic changes, the mucosa of the nasal cavity and paranasal sinuses was still distinctly swollen, accompanied by fluid accumulation in the left petrous bone. Subjectively, the patient reported, first, deterioration of moist desquamation after finishing the PBT, which was alleviated by use of a special

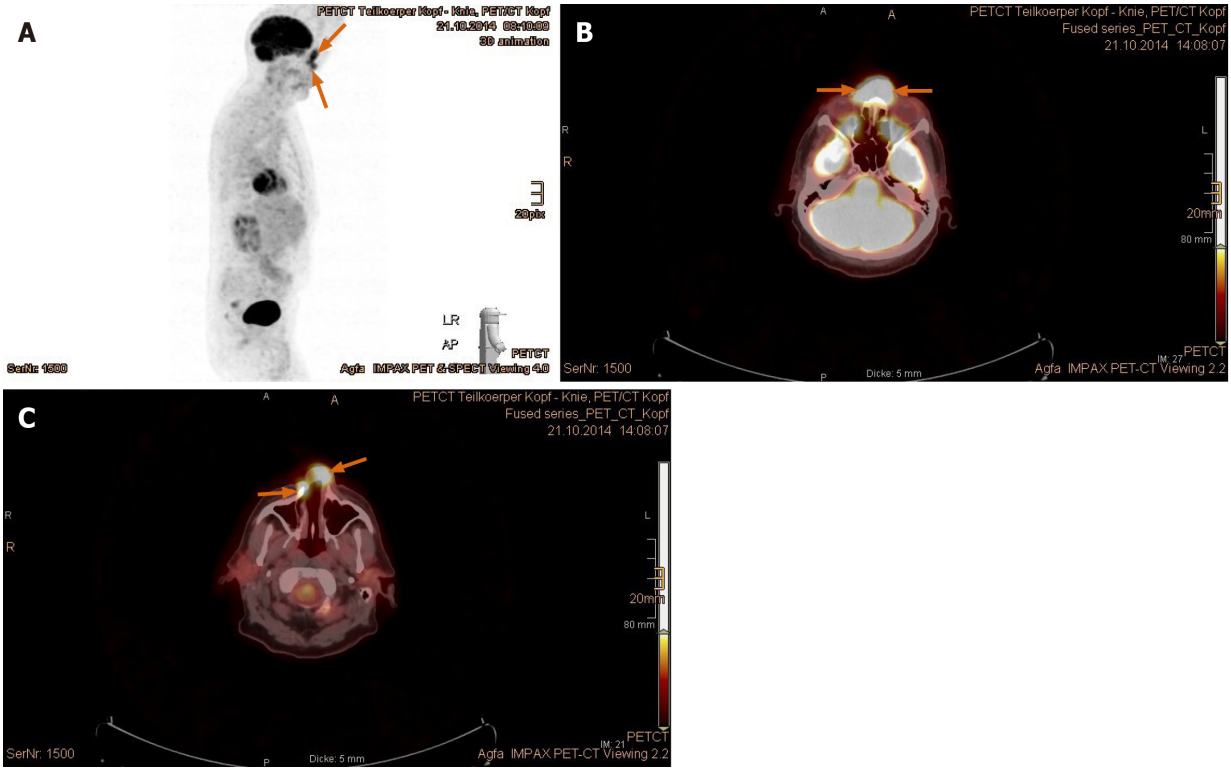

Figure 3 Positron emission tomography with 2-deoxy-2-fluorine-18-fluoro-D-glucose/computed tomography validated tumor recurrence in the nasal bridge and left parotid gland. A: Positron emission tomography overview image; B: Increased uptake in the nasal bridge; C: Fluoro-D-glucose-avid tumor in the nostrils (recurrent tumor marked with arrows).

ointment mixture containing cortisone (prescribed by his dermatologist) after 10 d. Second, he complained of excessive lacrimation, lymphedema of the face and hypacusis on account of post-radiogenic tympanic effusion in the left ear. Other late toxicities, such as visual, olfactory and gustatory disturbances and dry mouth, were absent.

The PET/CT and MRI scans performed at 8 mo after PBT showed a complete tumor remission (Figure 6A-F). The lymphedema in the facial and left retroauricular area regressed by frequent lymphatic drainage massage. Except sustained reinforced shedding of tears, no sensory impairment was present, and the epithesis of nose fit in again. At the 23rd mo after PBT, the patient reported bilateral cataracts, dry eyes, permanent loss of medial eyebrows, eyelashes and nasal hair, and use of lubricating eye drops steadily. Since a tumor recurrence was continuously excluded in the PET/CT and MRI scans (Figure 7A-E), the patient was accepted to undergo a nasal reconstruction in five sessions, carried out between 2016-2017, in cooperation with the otorhinolaryngology and plastic surgery departments[40] (Figure 8A-C). To date, the patient is content with the cosmetic result (Figure 8D) and remains free of tumor recurrence as well as visual and auditory impairment. Despite his objection in view of the successful treatments, the health insurance still declines to refund the expenditure of PBT and reconstruction surgery.

Case 2

Chief complaints: A 59-year-old Polish female was diagnosed with a space-occupying lesion of the right lacrimal sac adjoining nasal cavity and maxillary sinus in the ophthalmology, initially in summer 2017 (Figure 9A).

History of present illness: The patient was referred to the otorhinolaryngology department for the further examinations. Owing to lack of an apparent tumor in the nasal cavity, presence of ulceration and unfavorable curvature of the nasal septum, instead of an endoscopic approach, the histopathology was obtained in January 2018 by an open biopsy through the lower eyelid, submitting moderately differentiated keratinizing SCC. Because the tumor invaded the medial orbit and adjacent paranasal sinuses (Figure 9B and C), an orbital exenteration on the right was defined as the therapy of choice but was rejected by the patient. She then contacted our center for the purpose of organ preservation *via* definitive RT with PBT.

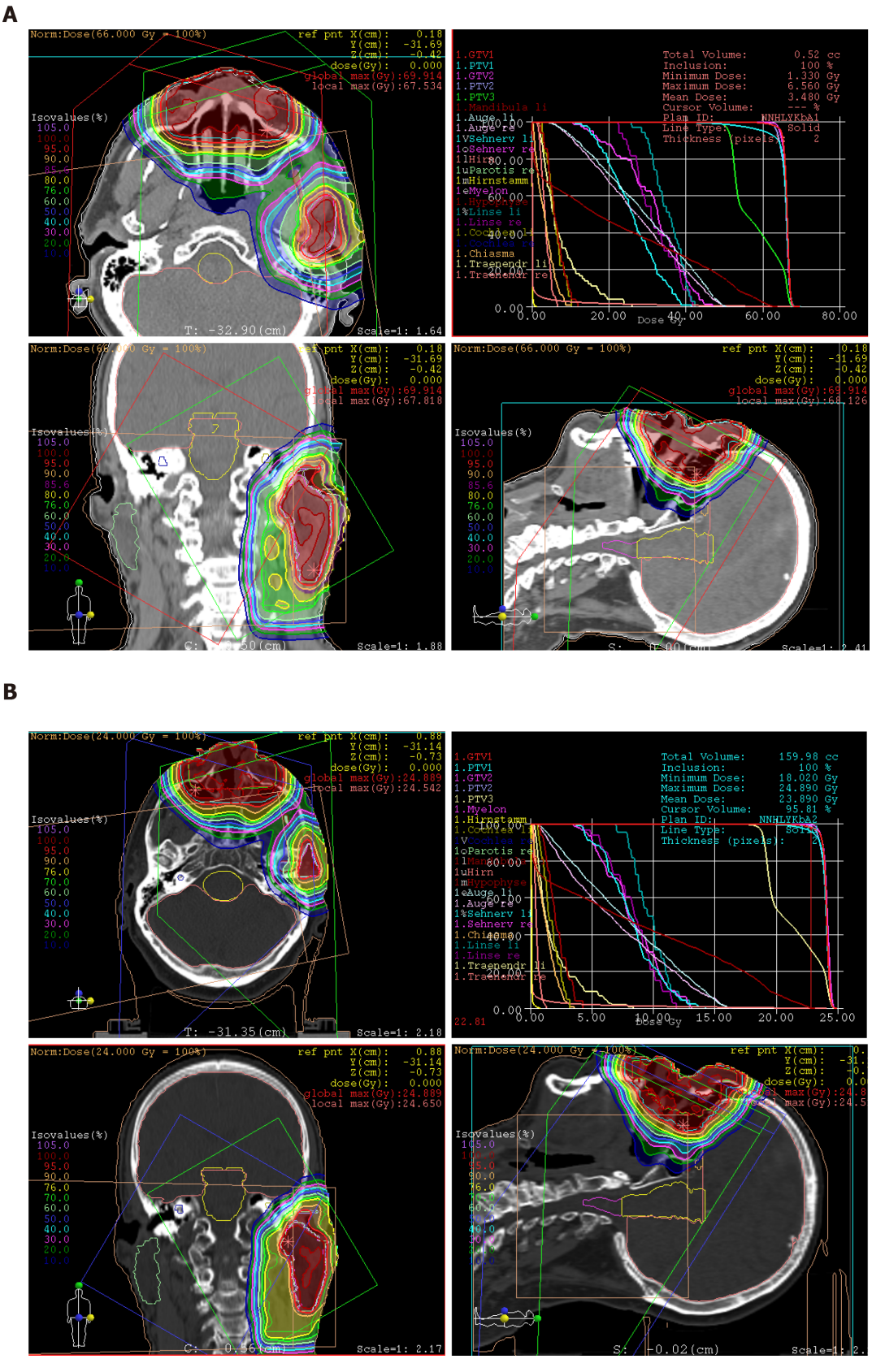

Figure 4 Treatment plans of proton beam therapy with isodose distributions in all three planes and dose-volume-histogram. A: The first section, until the 28th fraction; B: Plan adaptation, with more eye sparing for the remaining 16 fractions.

History of past illness: Not specified.

Personal and family history: The patient was in reduced general condition, being wheelchair-bound (Karnofsky Performance Score 60) by rheumatoid arthritis and on

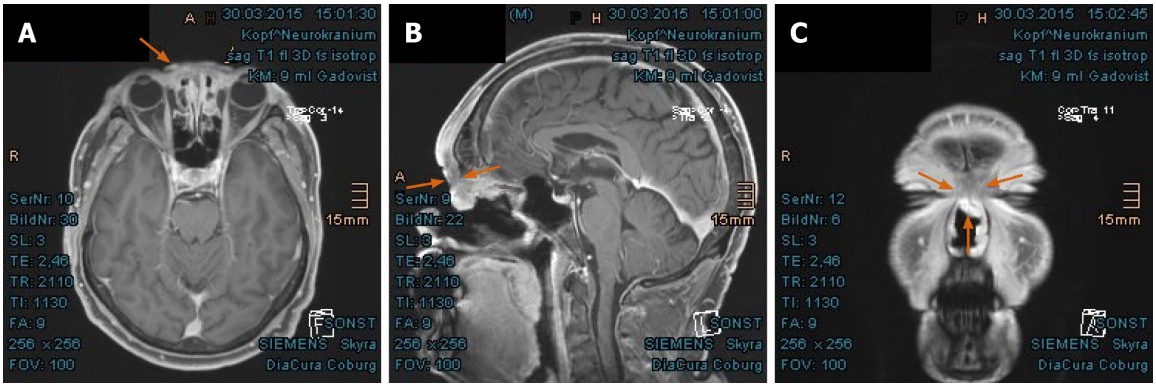

Figure 5 Significant shrinkage of recurrent tumor in the nasal bridge with mucosal swelling in the first follow-up at 3 mo after proton beam therapy. A: Unequivocal reduction of tumor thickening in the nasal bridge, presented in axial plane; B: Pronounced tumor regression abutting the frontal skull base, presented in sagittal plane; C: Coronal presentation of shrinking tumor in the nasal bridge. Local recurrence marked with arrows.

long-term treatment with methotrexate.

Physical examination upon admission: The patient presented with a bean-like flushed elevation inferiorly to the medial canthus of the right eye (Figure 10). The cervical lymph nodes were not as enlarged as to be palpable. No suspicious findings of tumor spread were apparent in the common clinical examination.

Laboratory examinations: No special laboratory test was arranged.

Imaging examinations: The MRI scan in February 2018 demonstrated nodular progress of the naso-orbital tumor up to 3.7 cm × 2.5 cm × 2.5 cm with bony destruction of the right infero-medial eye socket, possible invasion of the muscle cone and shift of the right eye to apico-lateral (Figure 11A and B). Furthermore, the tumor penetrated the neighboring ethmoidal sinus and nasal cavity with involvement of turbinates as well as the antero-medial recess of right maxillary sinus and facial soft tissue in the naso-labial fold and zygomatic area (Figure 11C-E). The ^{18}F-FDG-PET/CT revealed an increased uptake [SUV(max): 12.0] of the multi-compartment SCC in splanchnocranium without definite evidence of metastasis (Figure 12A and B).

Final diagnosis: Periorbital SNSCC, tumor stage cT4a cN0 G2 cM0.

Treatment: The patient has undergone a normofractionated PBT in 33 fractions, from February to April 2018. The informed consent was obtained prior to the initiation of the treatment. The primary tumor manifestation was treated with single dose of 2.10 Gy (RBE) at a total dose of 69.30 Gy (RBE), while the right lymphatic basins including buccofacial, parotideal, retropharyngeal, submandibular and suprajugulary nodal stations received 59.40 Gy (RBE) overall with a single dose of 1.80 Gy (RBE). Under immobilization with head, neck and shoulder mask and vacuum cushion (BlueBAG™; Medical Intelligence, Schwabmünchen, Germany), the target was irradiated from two gantry angles of 5° and 300° using the pencil beam scanning technique (Figure 13). Because of lack in remarkable change of tumor size (according to weekly-performed low-dose CT scans), adaptation of the treatment plan was not required. Given the aforementioned comorbidity, simultaneous chemotherapy was dismissed by the patient.

Outcome and follow-up: During the treatment, the patient developed moderate dysphagia, odynophagia, nasal congestions, and conjunctivitis. The greatest effect was painful radiation dermatitis (CTCAE grade 2-3) on the right cheek, extending from the right orbit to the lips. In the first follow-up at 3 mo after PBT, the patient reported significant amelioration of pharyngitis, dermatitis, and swelling of the nasal mucosa. The motility disorders of eye muscle regressed as well. In the first MRI scan in June 2018, the tumor mass was found to have dwindled considerably (Figure 14A-D). The consequent control at 8 mo and 14 mo showed complete tumor remission (Figures 15A-D, and 16A-D). As late toxicities, telangiectasia on the right infraorbital fold and cataract of the right eye were indicated at 18 mo after the PBT (Figure 17). The first was corrected by laser skin treatment, while a cataract surgery is still pending as of the writing of this report. Apart from nasal mucosa dryness, repeated conjunctivitis and

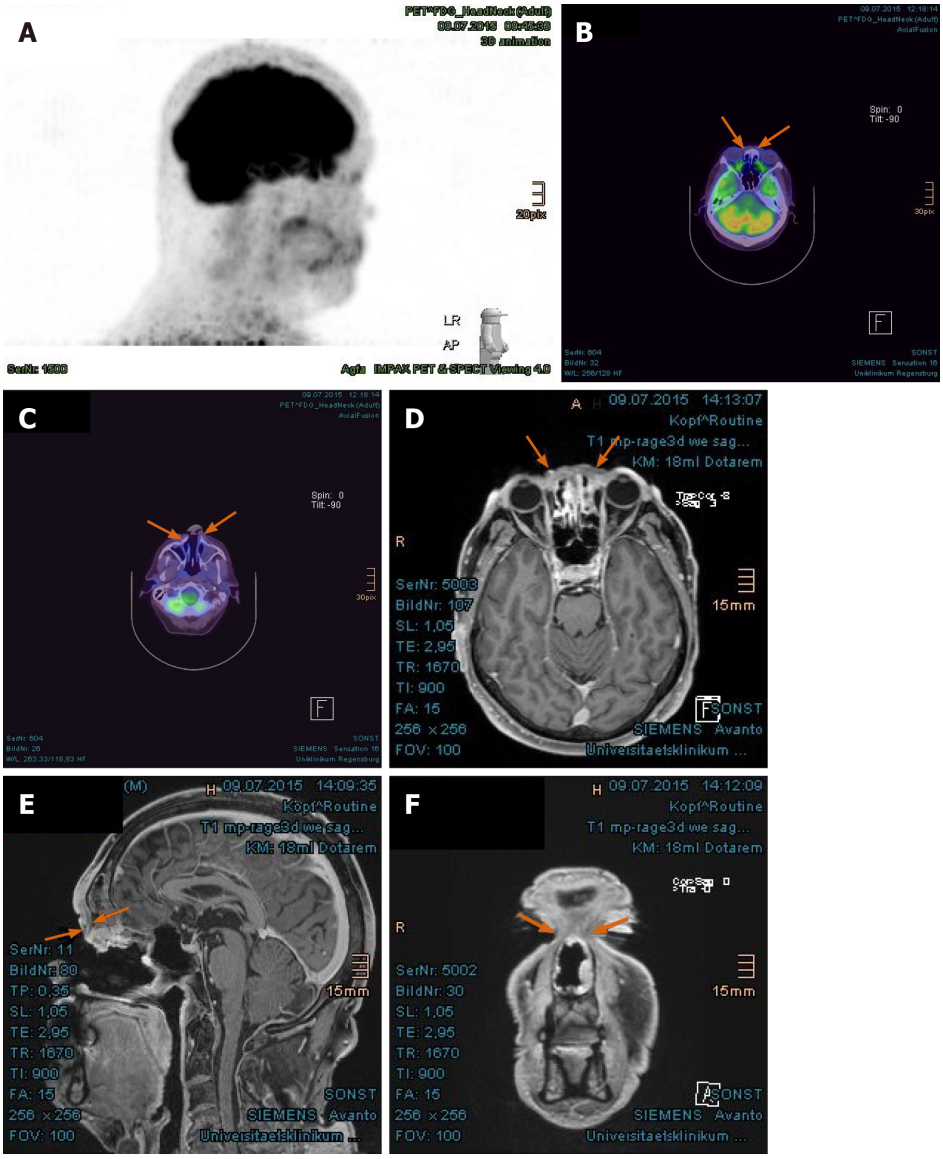

Figure 6 Complete tumor remission demonstrated in the positron emission tomography with 2-deoxy-2--fluorine-18-fluoro-D-glucose/computed tomography and magnetic resonance imaging at 8 mo after proton beam therapy. A: No pathologically increased activity in the positron emission tomography, overview image; B: Absence of increased fluoro-D-glucose avidity in the nasal bridge (arrow-marked in positron emission tomography/computed tomography); C: Absence of metabolically active tumor in the nostrils; D: Corresponding area in the nasal bridge in magnetic resonance imaging, presented in axial plane; E: Corresponding area in the frontal skull base, presented in sagittal plane; F: Coronal presentation of tumor remission in the nasal bridge. Previously enhancing tumors marked with arrows.

right nasolacrimal duct obstruction, the patient is continuously free of tumor recurrence and radiation-related symptoms to date.

DISCUSSION

SNSCC is not only related to occupational exposures, as mentioned above; in a population-based case-control study, tobacco smoking emerged as a strong risk factor for nasal cancer, with 60% increased risk in ever-smokers and an increment of 6% annually[11]. Smoking also favored malignant transformation and relapse of sinonasal inverted papilloma after surgical resection[12,13]. Similar to pharyngeal and cervical SCC, the impact of human papillomavirus (HPV) on the carcinogenesis and prognosis of SNSCC was investigated progressively. HPV positivity is more common in SCC of nasal cavity and nonkeratinizing SNSCC, yielding improved overall survival[14,15]. With reference to this, both cases presented herein were not attributed to professional exposures, as the HPV status remained unknown because of missing testing at the

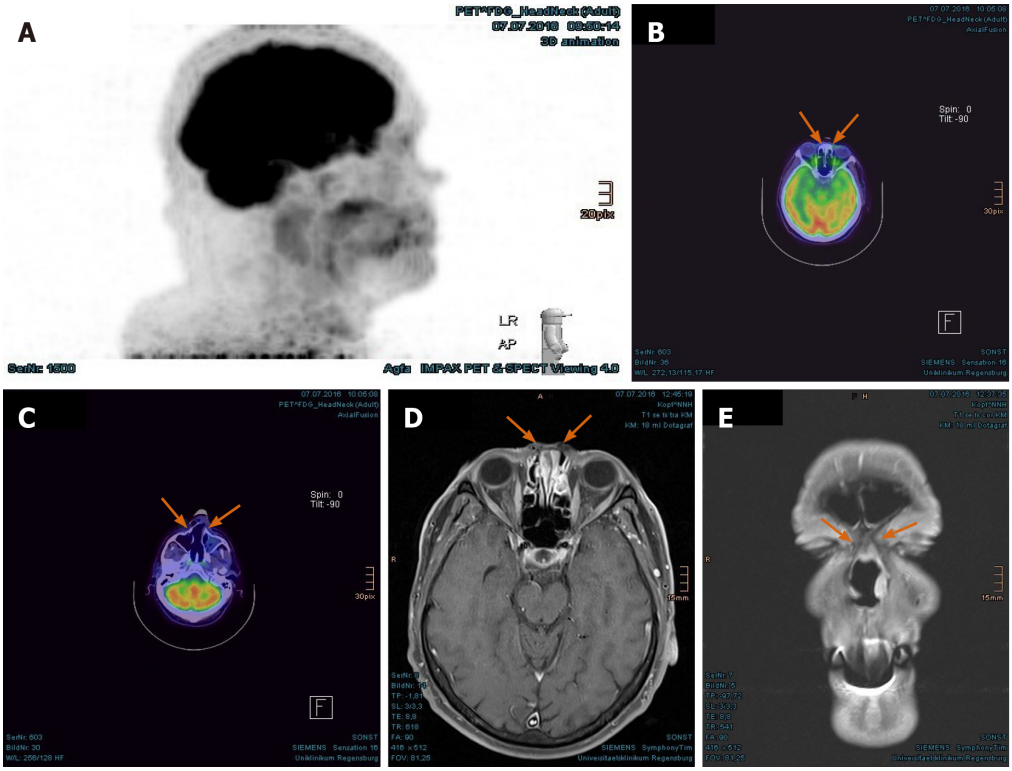

Figure 7 Persisting tumor remission evidenced in the positron emission tomography with 2-deoxy-2--fluorine-18-fluoro-D-glucose/computed tomography and magnetic resonance imaging at 20 mo after proton beam therapy. A: No pathologically increased activity in the positron emission tomography, overview image; B: Absence of increased fluoro-D-glucose avidity in the nasal bridge (arrow-marked in positron emission tomography/computed tomography); C: Absence of metabolically active tumor in the nostrils; D: Flat surface above the nasal bridge in the magnetic resonance imaging; E: No evidence of tumor enhancement in the nasal bridge, presented in coronal plane.

time of tumor diagnosis. Nevertheless, the patient with recurrent nasal SCC showed long-standing smoking habits, and the other with right periorbital SCC was under long-term immunosuppression with methotrexate, that is significantly associated with various malignancies[16].

SNSCC is typically earmarked by bony destruction of the adjoining sinus walls and oftentimes accompanied by invasion of the orbital wall, infratemporal fossa, skull base and contralateral site, owing to delayed diagnosis. Besides, complex anatomy and diverse normal variants of the sinonasal tract aggravate the identification of tumor origin and extension[17]. In case of tumor invasion of orbit, orbital exenteration - one of the most face-deforming operations - with removal of all the orbital contents, including eyelid and periosteum, is indicated to achieve better survival outcomes[18,19]. Flaps, such as temporoparietal, galeal, free gracilis and free vastus lateralis musculocutaneous flap, are available for the reconstruction of defects; however, they should be employed with special diligence, due to the known comorbidities and postoperative complications[20,21]. In the recent publications, there is a trend of eye-sparing surgery without previously assumed survival disadvantages, especially in combination with adjuvant RT[22-25].

In the past few decades, there have been progressions in endoscopic endonasal surgery, microvascular reconstruction, RT, and systemic therapy. Even though surgery, with or without subsequent RT or CRT, remains the standard regime in most of the cases, Cracchiolo *et al*[26] pointed out that the choice of therapeutic strategy was influenced by multiple tumor and non-tumor factors, stating apparent deviation from the National Comprehensive Cancer Network guidelines for the treatment of SNSCC. When utilizing a primary surgical approach, constant tumor factors and variable treatment factors, preeminently negative margin resection, were associated with improved survival. Additionally, patients with advanced tumor stage and positive margin resection profited significantly from adjuvant RT or CRT.

Concerning the regional metastases of SNM, levels I, II and III, and retropharyngeal lymphatic basins are frequently involved. Notwithstanding, the accurate assessment of elective nodal treatment in clinically N0 neck is fastidious, with an estimated risk for occult disease of 10%-20% or more. Notably, in tumor stage III-IV of SNSCC, elective neck irradiation should be intended in absence of selective neck dissection[27]. In a

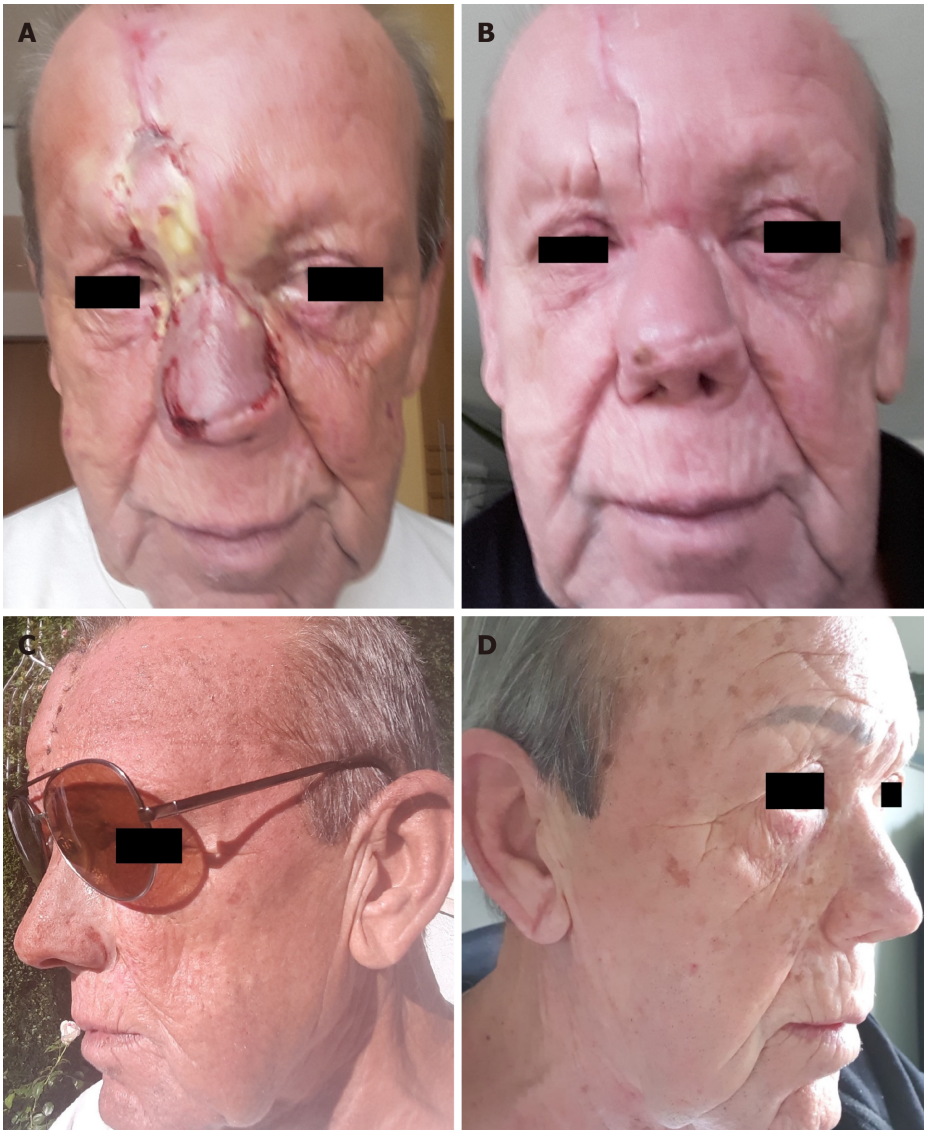

Figure 8 "Case 1" patient's images after a nasal reconstruction in five sessions between 2016-2017. A: Taken in November 2016; B: Taken in February 2017; C: Taken after the last surgery in May 2017; D: Current image in March 2020.

retrospective review, Peck *et al*[28] identified the histologic types of SNM as the most impacting factors in predicting regional metastases, whereas the invasion of adjacent structures like dura, infratemporal fossa, palate and facial soft tissue was associated with increased occurrence of regional metastases. Taking this into account, we had decided to perform an elective nodal irradiation of the ipsilateral neck for both patients, surrendering an effective locoregional control and adequate tolerability.

Because of the rare occurrence and heterogeneous histologic subtypes and primary sites of SNM, there have been no randomized clinical trials to compare the various treatment modalities. In principle, early stage tumor is adequate to be managed with surgery alone, while locally advanced disease requires multimodality approaches. For patients who refuse up-front surgery, a RT-based approach is a legitimate option as well. In view of rapid growth and aggressive local spread of SNSCC to the neighboring organs at risk, such as optic nerves, eye globes, orbitofrontal and temporopolar cortex, as presented in our case report, sufficient local control (LC) is crucial for improved survival. Among SNMs, SCC incidentally seems to submit lower survival rates in comparison to other histologies[29,30]. Novel development of RT technique, above all PBT and carbon ion therapy (CIT), should be generally considered to ameliorate treatment outcomes, to prevent long-term radiation-induced toxicities, and to facilitate organ preservation. Although photon irradiation stays the RT paradigm, more and more particle therapy institutions, mainly in the United States and Japan on account of generous availability, have delivered convincing results in the treatment of SNM[29-34]. In their multi-institutional Proton Collaborative Group registry

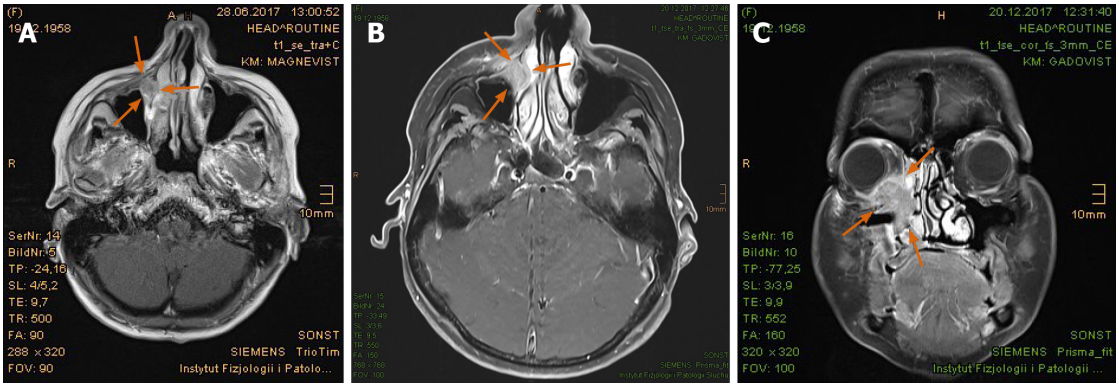

Figure 9 Continuously growing tumor lesion in the right lacrimal sac invading the adjacent orbit and sinonasal spaces, presented as magnetic resonance imaging. A: Initial tumor extent in the antero-medial recess of the right maxillary sinus in June 2017; B: Size progression in December 2017, presented in axial plane; C: Distinct tumor invasion of the right orbit and neighboring sinonasal spaces, presented in coronal plane. Tumor marked with arrows.

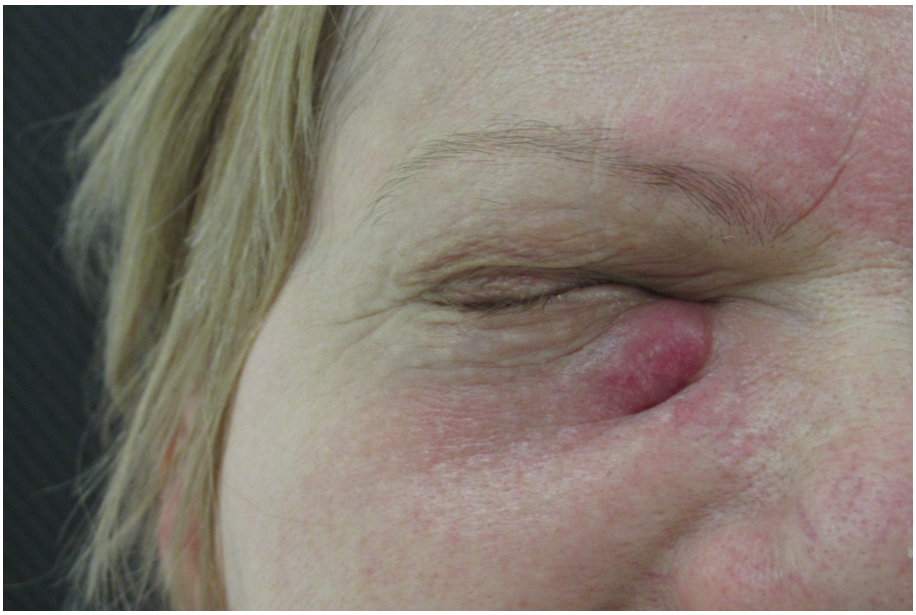

Figure 10 "Case 2" patient's image showing a tumorous nodule beneath the medial canthus of the right eye.

study, Yu *et al*[30] reported promising outcomes of 69 patients with SNM treated with PBT predominantly, which was provided as de novo RT or reirradiation in curative intention. Late ≥ grade 3 toxicities, such as vision loss and symptomatic brain necrosis, were not notified.

As recapitulation, the advantages of charged particle therapy are known to be comparatively low entrance dose and minimum exit dose, according to the physical feature of PBT and CIT, the so-called Bragg peak, as well as higher RBE and linear energy transfer than photons, which is utterly relevant for treating radioresistant tumor histologies. Based on the privileged physical and biological characteristics, the sophisticated amendment of dose distribution may provide superior conformality of target coverage with feasible dose escalation. Particularly, locally advanced, unresectable gross tumors may benefit from higher dose regimes. Toyomasu *et al*[33] reported 3-year/5-year overall survival and LC rates of 56.2%/41.6% and 54.0%/50.4% in the largest retrospective study of SNSCC treated with particle therapy alone. Of the patients, over one-third had unresectable disease, while almost half of the entire cohort obtained 65.0 Gy (RBE) in 26 fractions. Another study dealing with dose-intensified, hyperfractionated PBT to SNM with or without concurrent chemotherapy[29] also showed magnificent 3-year LC rates (of 90% for gross total resection and PBT, 61% for primary PBT, and 59% for patients with gross residual disease). Analogous to our patient in "Case 1", these patients obtained 1.20 Gy (RBE) twice daily, to a median total dose of 73.80 Gy (RBE). The incidence of ≥ grade 3 late toxicities was 24%, and in

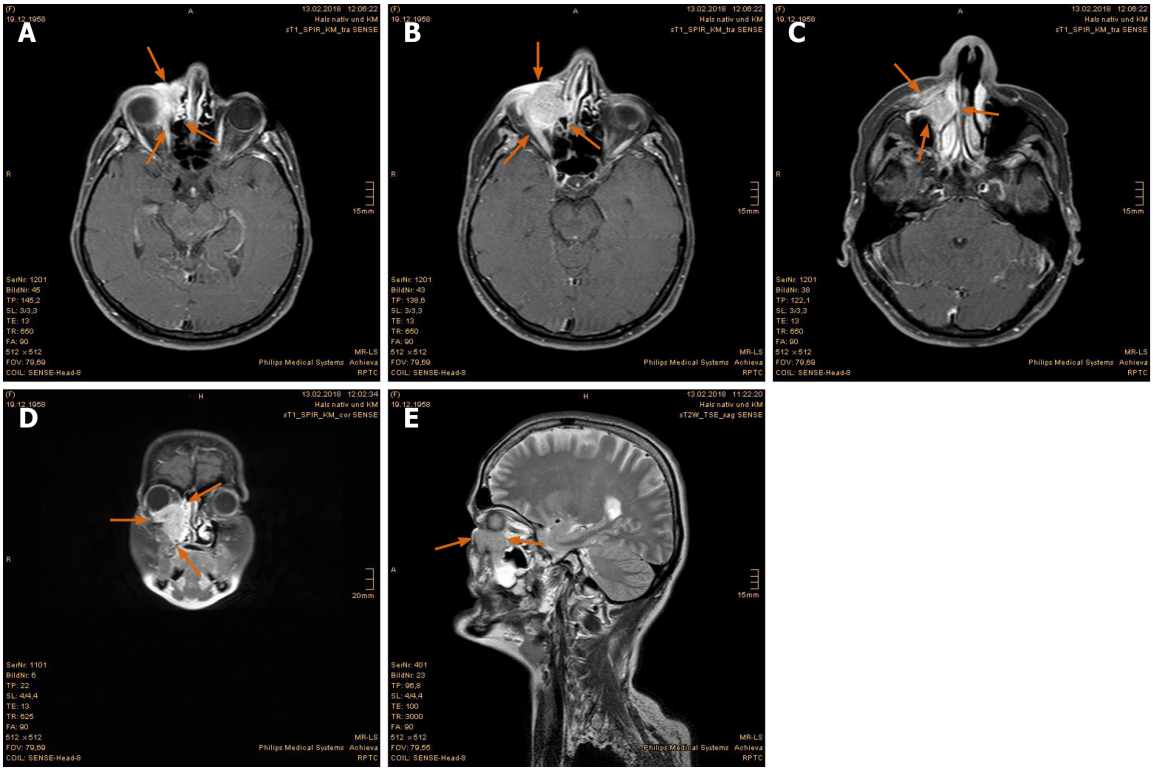

Figure 11 Magnetic resonance imaging prior to proton beam therapy. A: Naso-orbital nodular enhancement with lacking delimitation to the right eyeball; B: Tumor displacement of the right eye; C: Growing tumor invasion of the right maxillary sinus, turbinates and adjoining facial soft tissue; D: Coronal presentation of the right periorbital sinonasal cancer; E: Sagittal presentation of the sinonasal cancer with shift of the right eyeball upwards. Tumor marked with arrows.

another study with CIT of SNM, the high-grade late toxicities occurred in 17% of the cohort[34].

On the other hand, the utility of dose escalation in the former investigations using photons and neutrons was equivocal. Hoppe *et al*[35] demonstrated improved progression-free survival and overall survival in patients receiving RT dose ≥ 65 Gy, while other studies exhibited poorer survival outcomes as this dose limit was surpassed[36,37]. That might be ascribed to increment of potentially life-threatening dose-related toxicities, like radiation necrosis of temporal lobe and blindness. However, the utilization of pencil beam scanning technique allowing for intensity modulated proton therapy (IMPT, used on our patients presented) may reduce overall toxicities, largely by sparing of adjoining normal tissues, and increase LC, by delivering higher dose to the target[38-39, 41-42]. In the setting of extended ipsilateral orbital invasion as reported in "Case 2", moderate excess of maximum dose to the right optic nerve [65.30 Gy (RBE)] and right eyeball [70.11 Gy (RBE)] was deliberately permitted due to decreased integral dose of the critical structures by means of IMPT. Herein, the mean doses for the right optic nerve and eyeball were 60.06 Gy (RBE) and 52.36 Gy (RBE) respectively. At a follow-up period of 2 years, severe ocular toxicity was not observed.

Furthermore, unlike CIT, with confined irradiation field size, and aforementioned publications, mostly on the ground of obsolete passive scattering PBT, IMPT using active scanning technique facilitates the implementation of an elective neck irradiation simultaneously at uncertain nodal metastases. Even for manifest nodal disease as our patient in "Case 1", PBT can be affiliated with an inferior demand of opioids and a reduced rate of gastrostomy tube dependence. In comparison of acute toxicities between PBT and IMRT for nasopharyngeal and sinonasal cancers with comprehensive head and neck irradiation, the mean doses to the oral cavity, esophagus, larynx and parotid glands was significantly lower when utilizing PBT, corresponding to a retrospective study of McDonald *et al*[43]. To estimate the potential benefit for PBT over IMRT in terms of dose reduction in organs at risk, normal tissue complication probability models may support treatment selection for head and neck cancer patients[39,42].

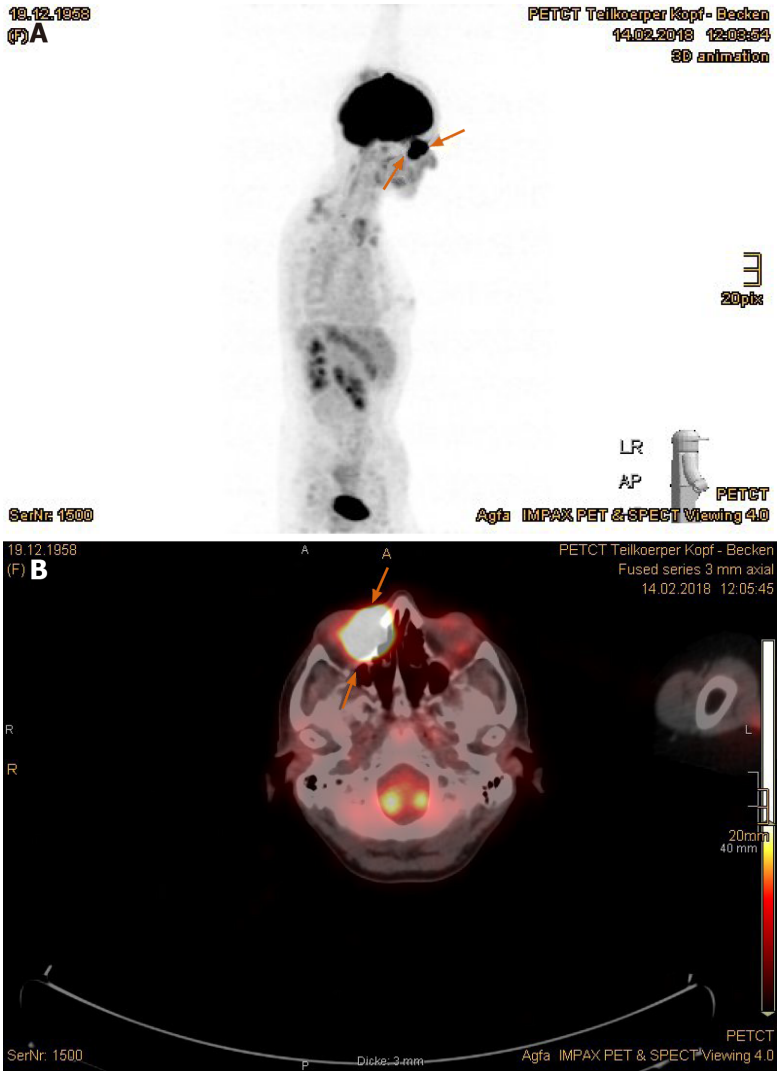

Figure 12 Positron emission tomography with 2-deoxy-2--fluorine-18-fluoro-D-glucose/computed tomography exhibited remarkably increased uptake in the right periorbital sinonasal squamous cell carcinoma. A: Positron emission tomography overview image; B: Axial plane (tumor marked with arrows).

CONCLUSION

Both cases with locally advanced periorbital SNSCC treated with PBT alone demonstrate excellent results in view of tumor control and quality of life at a follow-up period of 5 years and 2 years. In general, the therapy regimes of SNM should be managed individually according to histology, tumor stage, prior treatments, personal risk factors, and patient preference. Both multimodality and non-surgical approaches are overdue to be reviewed profoundly in prospective randomized trials. Still, given the dosimetric advantages of PBT, especially in reducing the ocular and brain toxicities for unresectable gross disease, it is somehow unethical to withhold IMPT from the patients on account of random allocation of study design, limited availability of IMPT, lack in clinical experience, and insurance status. A model-based approach on normal tissue complication probability may relieve the selection of suitable patients with clinically significant benefit from PBT.

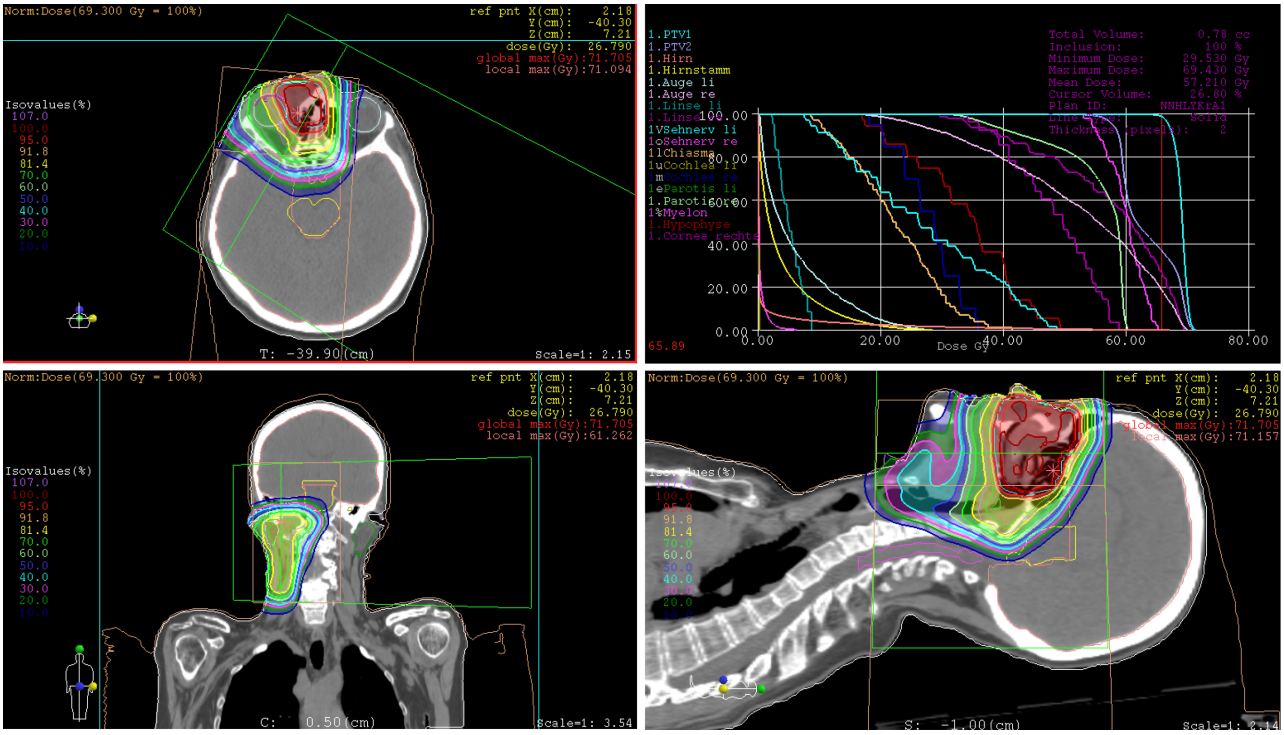

Figure 13 Treatment plan of proton beam therapy with isodose distributions in all three planes and dose-volume-histogram.

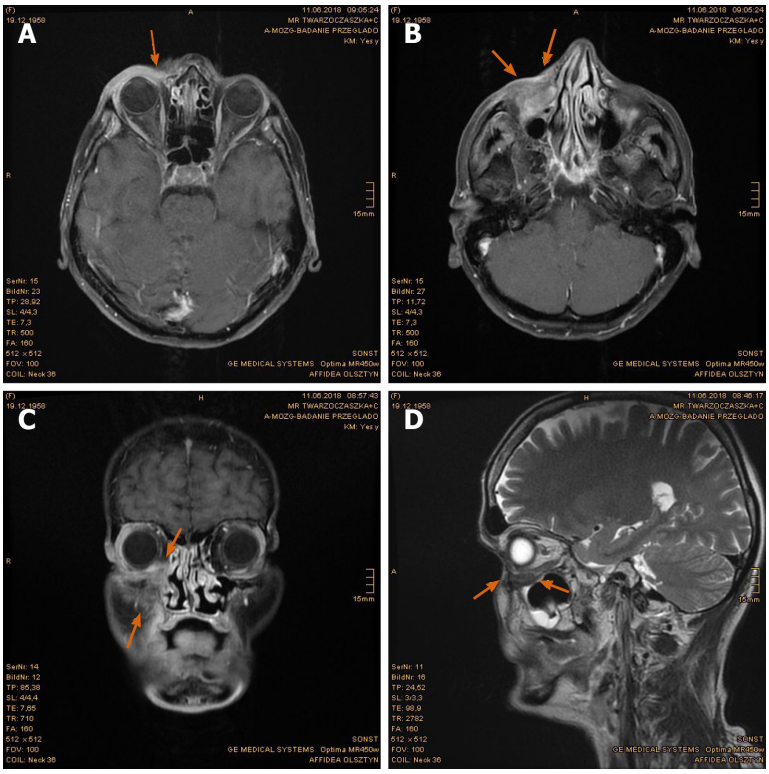

Figure 14 Pronounced tumor reduction in the first follow-up magnetic resonance imaging scan. A: Dwindling of enhancing tumor nodules at the right naso-orbital corner; B: Decreased enhancement in the soft tissue of the naso-labial fold and zygomatic area; C: Significant regression of the right periorbital sinonasal cancer, presented in coronal plane; D: Restored delimitation of the right orbital floor, presented in sagittal plane. Former tumor extent marked with arrows.

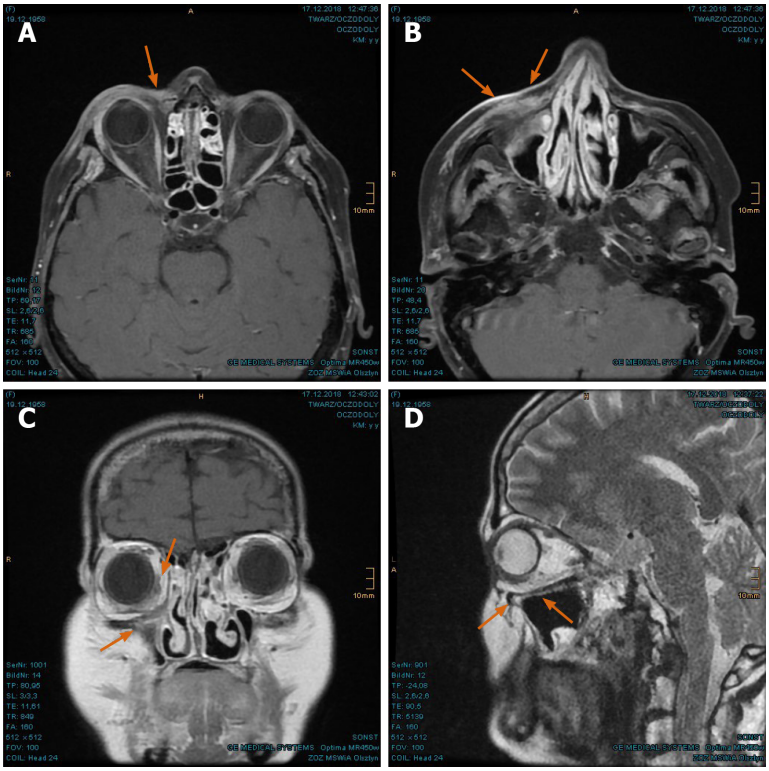

Figure 15 Complete tumor remission verified in the magnetic resonance imaging at 8 mo after proton beam therapy. A: Further regression of the tumorous enhancement in the right lacrimal sac marked with arrows; B: Postradiogenic changes of the right facial soft tissue with no evidence of residual tumor; C: Coronal presentation of the fully regressed right periorbital sinonasal cancer; D: Clearly defined orbital floor with normal position of the right eye.

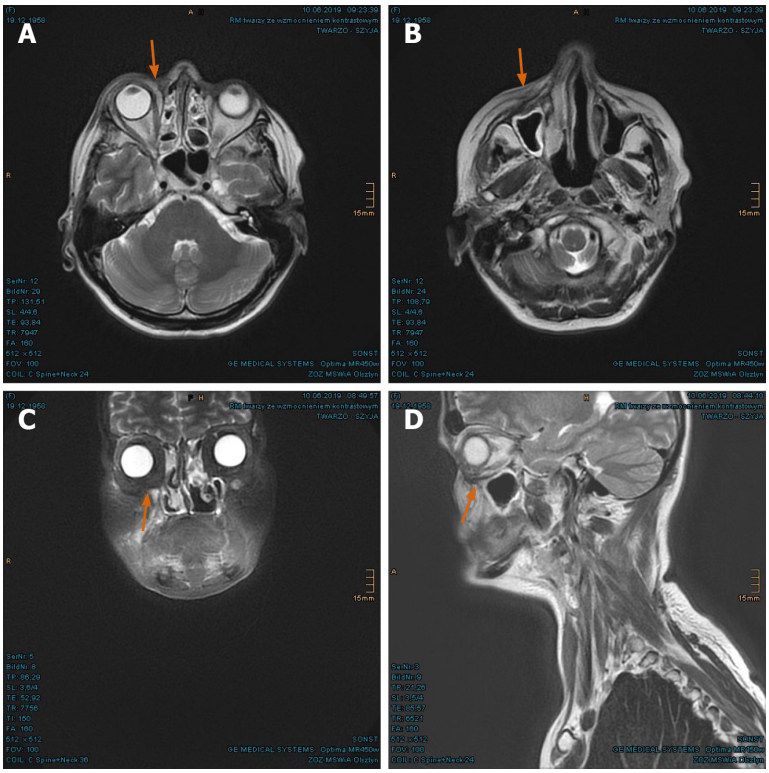

Figure 16 Sustained full remission in the magnetic resonance imaging at 14 mo after proton beam therapy. A: Right naso-orbital corner; B: Maxillary sinus and naso-labial fold; C and D: In coronal and sagittal plane, respectively.

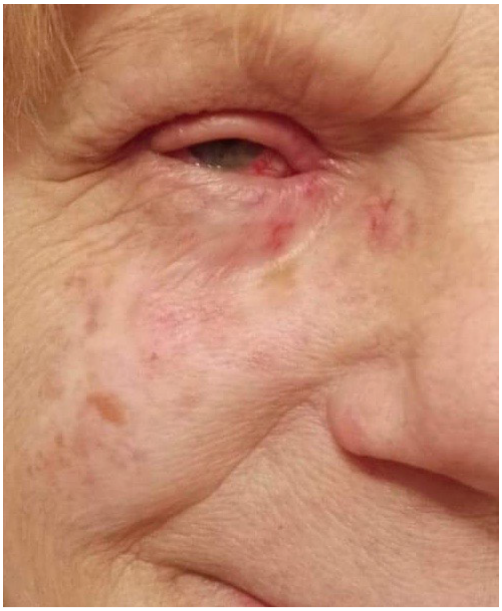

Figure 17 "Case 2" patient's current image at 2 years after proton beam therapy.

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CASE REPORT

Intravascular lymphoma with hypopituitarism: A case report

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Abstract

BACKGROUND

Intravascular lymphoma (IVL) is a rare subtype of lymphoma involving the growth of lymphoma cells within the vessel lumina without lymphadenopathy. Because of various modes of presentation and its rarity, IVL is often diagnosed postmortem. Herein, we report a case of intravascular B-cell lymphoma with hypopituitarism, an extremely rare complication, that was successfully treated with chemotherapy.

CASE SUMMARY

An 80-year-old Japanese woman presented with a 7-mo history of a tingling sensation in the lower limbs. She also presented with various other symptoms such as pancytopenia, high fever daily, and unconsciousness with hypoglycemia. Although the doctor who previously treated her diagnosed hypoglycemia as being due to hypopituitarism, the cause of the other symptoms remained uncertain despite a 7-mo evaluation period. We performed bone marrow aspiration to evaluate pancytopenia and found that she had hemophagocytic lymphohistiocytosis (HLH). On the basis of a random skin biopsy for assessing the cause of HLH, she was diagnosed with intravascular B-cell lymphoma. HLH and hypopituitarism were considered secondary to IVL. All her clinical findings matched the presentations of IVL. She was immediately treated with chemotherapy and achieved complete response. She was relapse free two years after treatment.

CONCLUSION

IVL should be included in the differential diagnosis of hypopituitarism, which although life-threatening, is treatable through prompt diagnosis and appropriate chemotherapy.

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Core tip: When encountering cases showing a variety of symptoms that cannot be reasonably explained, general physicians should consider intravascular lymphoma (IVL) and its useful diagnostic tool, *i.e.*, random skin biopsy. Additionally, the mechanisms underlying lymphoma-associated hypopituitarism associated with IVL have not yet been elucidated, thereby necessitating further case studies and laboratory-based research.

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INTRODUCTION

Intravascular lymphoma (IVL) is a rare subtype of lymphoma, characterized by the selective proliferation of lymphoma cells within the lumina of small-to-medium-sized blood vessels[1]. A B-cell immunophenotype is most commonly observed, although cases with T-cell receptor rearrangements have also been reported[2]. Although various organs can be affected by lymphoma cells, hypopituitarism is an extremely rare complication, and limited cases have been reported[3]. We herein report a case of hypopituitarism due to IVL, which was successfully treated with chemotherapy.

CASE PRESENTATION

Chief complaints

An 80-year-old Japanese woman with no significant comorbidities noticed a bilateral tingling sensation on the anterior surface of her lower limbs.

History of present illness

The sensation was localized below her thighs; however, she felt neither numbness nor weakness, and could walk and ride a bicycle without any problems. Her symptoms persisted for about one month, which resulted in a visit to a local outpatient clinic. Laboratory studies performed in the clinic showed pancytopenia. Although the patient was examined at another general hospital, the cause of her condition was unknown. Subsequently, she experienced a high fever (a temperature of 38°C - 39°C) daily. However, she did not have any other accompanying symptoms, such as chills, pain, and appetite loss, and her general state was relatively normal. Despite a more detailed evaluation, the patient's diagnosis was inconclusive, and she was observed as an outpatient. Five months after her first visit, she was taken to the emergency department of the hospital at which she was previously treated, because of sudden loss of consciousness. Laboratory studies showed that she had lost consciousness because of hypoglycemia, and further evaluation revealed that hypoglycemia was one of the signs of hypopituitarism. She received glucocorticoid and replacement therapy. Whereas treatment resulted in blood glucose level control, the tingling sensation gradually progressed. Although seven months had elapsed since her first visit, the causes of her symptoms remained unknown. Therefore, she was admitted to the Department of Hematology of our hospital for further assessment.

History of past illness

Her past medical history was noncontributory.

Personal and family history

Her family history was noncontributory.

Physical examination upon admission

On physical examination, we observed the presence of a tingling sensation on the anterior regions of the lower limbs, under the thighs, and on both palms of the patient. Decreased vibratory sensation on both sides of the lower extremities was also noticed. Hepatosplenomegaly, lymph node enlargement, and skin eruption were not observed.

Laboratory examinations

Blood test results were as follows: White blood cell count, 300 cells/μL; Hemoglobin level, 9.0 g/dL; and Platelet count, 113000 platelets/μL, lactate dehydrogenase, 971 IU/L; Ferritin, 710 ng/mL; Soluble interleulin-2 receptor alpha, 3412 U/mL. No evidence of infection or solid tumor was observed on serum examination, culture, and imaging. Brain magnetic resonance imaging (MRI), however, showed enlargement of the pituitary gland (Figure 1A and B). It did not appear to be a tumor as the entire pituitary gland was almost equally contrasted; no other abnormalities were present. We performed stimulating hormone tests, as the patient had been diagnosed with hypopituitarism in the previous hospital. The patient received an injection of four kinds of hormones [growth hormone (GH)-releasing hormone, corticotropin-releasing hormone, luteinizing hormone (LH)-releasing hormone, and thyrotropin-releasing hormone], and we monitored the levels of seven kinds of hormones [GH, LH, follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), cortisol, adrenocorticotropic hormone (ACTH), and prolactin] produced in response. Low levels of six of the seven hormones were observed, with the exception of prolactin, which confirmed the diagnosis of hypopituitarism.

Imaging examinations

Contrast-enhanced computed tomography scans of the abdomen and pelvis showed a hepatic cyst and mild splenomegaly. There was no evidence of tumor and lymphadenopathy could not be seen. Bone marrow aspiration and biopsy, performed to evaluate pancytopenia, revealed the presence of hemophagocytic lymphohistiocytosis (HLH) (Figure 2A and B). There was no evidence of tumor involvement. She met five of eight criteria of HLH (fever ≥ 38.5°C, splenomegaly, peripheral blood cytopenia, hemophagocytosis in bone marrow, ferritin > 500 ng/mL, elevated soluble IL-2 receptor alpha two standard deviations above age-adjusted laboratory-specific norms)[4]. We suspected lymphoma as a cause of HLH, as no evidence of infection or solid tumors were present. One of the most common causes of adult HLH is lymphoma. However, as the patient did not show lymphadenopathy, a random skin biopsy was performed, which showed that the tumor cells had proliferated predominantly within the small vessels of the dermis with no infiltration outside the vessels (Figure 3). Immunohistochemical staining revealed positivity for the B-cell markers CD20 and CD79a in the absence of staining for T-cell markers. These characteristics were consistent with those of intravascular large B-cell lymphoma.

FINAL DIAGNOSIS

The patient was diagnosed with IVL, as well as HLH and hypopituitarism secondary to IVL.

TREATMENT

The patient was immediately treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone. After the first chemotherapy cycle, the tingling sensation partially improved and the symptoms in the palms disappeared. Bone marrow aspiration performed after the first chemotherapy cycle demonstrated the absence of hemophagocytosis, and the bone marrow image was normal, suggesting that HLH had improved with chemotherapy. She completed six cycles of chemotherapy. After the third cycle, head MRI showed that the pituitary gland had returned to its normal size (Figure 1C and D), and it remained at that size after the six cycles. Although the patient required hormone replacement therapy after chemotherapy, her LH, FSH, TSH, free T4, ACTH, and cortisol levels, which were all lower than normal, and prolactin levels, which were higher than normal, returned to normal levels and remained so after completion of the final chemotherapy cycle (Table 1). She did not develop any signs and symptoms associated with

Table 1 Hormonal levels before and during/after chemotherapy

Hormone	Normal range	Before chemotherapy	During/after chemotherapy		
			After the first cycle of chemotherapy	After six cycles of chemotherapy	After 1 yr of chemotherapy
Thyroid-stimulating hormone	0.38-4.31 μIU/mL	0.04	0.28	0.52	0.50
Free T3	2.17-3.34 pg/mL	0.71	1.17	2.39	2.00
Free T4	0.82-1.63 ng/dL	0.63	1.22	1.22	1.74
Growth hormone	0.13-9.88 ng/mL	0.68	0.33	0.35	0.24
Prolactin	3.1-15.4 ng/mL	24.20	25.60	16.80	
Adrenocorticotropic hormone	7.2-63.3 pg/mL	5.00	10.70	1.90	< 1.5
Luteinizing hormone	5.72-64.31 mIU/mL	< 0.1	< 0.1	7.70	8.90
Follicle-stimulating hormone	0-157.79 mIU/mL	0.50	0.70	19.60	25.10
Cortisol 8 a.m.	4.5-21.1 μg/dL	3.40	5.60	38.70	20.10

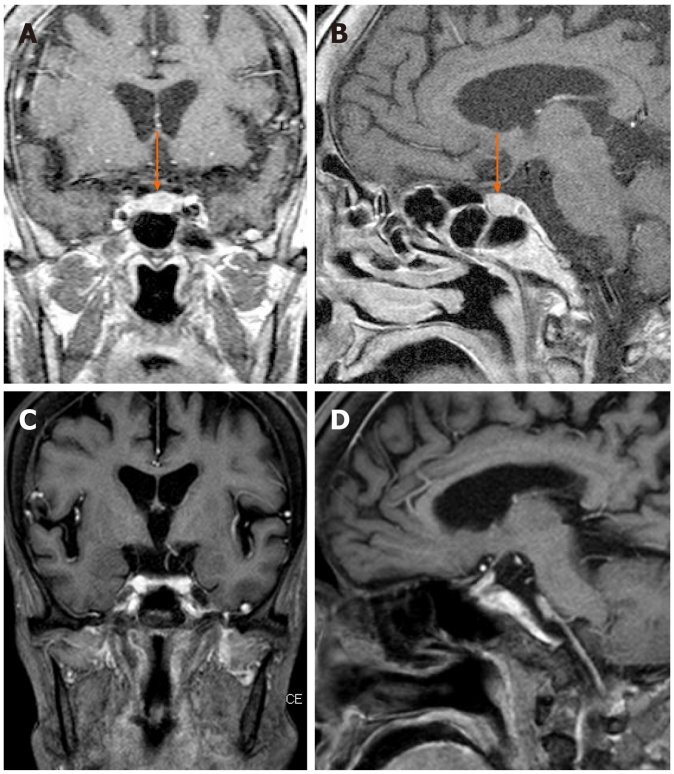

Figure 1 Magnetic resonance imaging: T1-weighted imaging. A and B: At presentation: coronary (A) and sagittal (B) view magnetic resonance images (MRI) showing an enlarged pituitary gland; C and D: Three months later (after the third cycle of chemotherapy): Coronary (C) and sagittal (D) view MRI showing substantial mass reduction.

hypopituitarism, such as hypoglycemia, after the second cycle of chemotherapy, and her peripheral blood cell count remained normal.

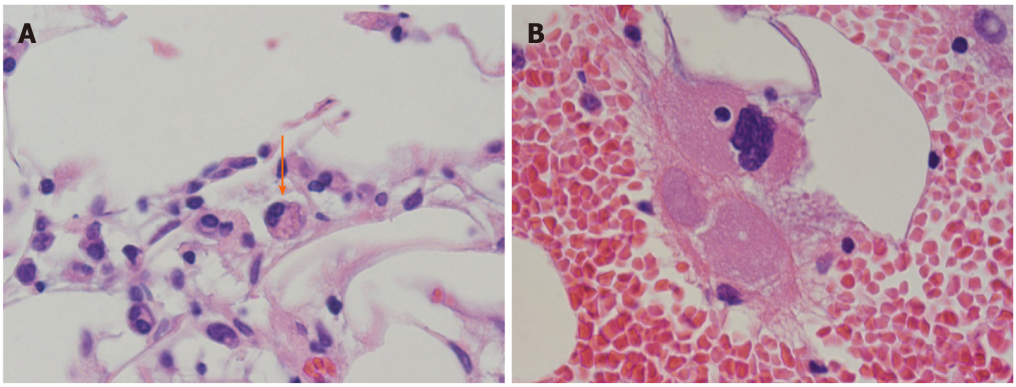

Figure 2 Bone marrow aspiration showing hemophagocytosis (designated by an arrow). A and B: Wright and Giemsa staining at ×400 (A) and ×1000 (B) magnification.

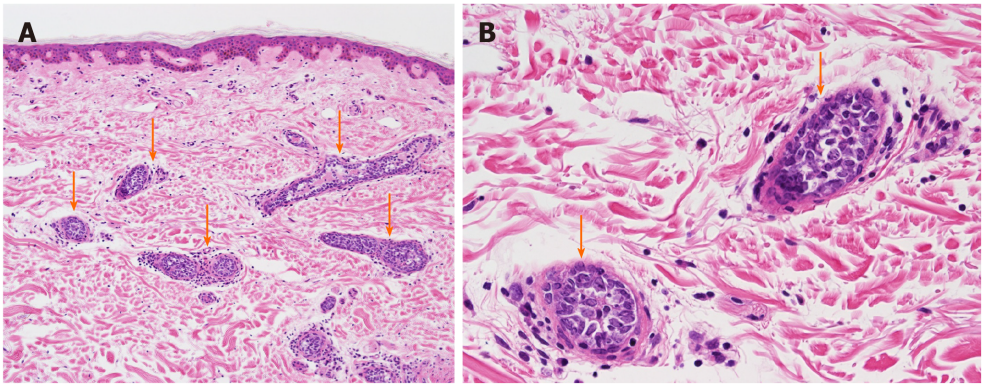

Figure 3 Hematoxylin and eosin. A and B: Histopathology of intravascular lymphoma by random skin biopsy: the arrows indicate lymphoma cells seen within the lumen of small vessels: hematoxylin and eosin stain at ×400 (A) and ×1000 (B) magnification.

OUTCOME AND FOLLOW-UP

A complete response was achieved, and the patient was relapse free two years after treatment. Although she still required minimal hormone replacement therapy, 5 mg/d of hydrocortisone, she does not show any signs of hormone deficiency.

DISCUSSION

Antemortem diagnosis of IVL is often challenging because it affects various organs, resulting in highly variable and unpredictable presentations, although the lymph nodes are typically spared[1]. For example, the presence of a tingling sensation, which was the first complaint in this case, is a common symptom of IVL. The proliferation of tumor cells leads to multiple ischemic necrosis of the central nervous system, nerve roots, cranial nerves, and peripheral nerves[5,6]. This case highlights the importance of a random skin biopsy in diagnosing IVL. IVL is diagnosed by the identification of large lymphoma cells within small-to-medium blood vessels[7]. IVL has been reported following biopsies of various organs, such as the bone marrow, liver, and/or spleen[7]. However, with a random skin biopsy, sufficient specimens can be obtained easily with minimal invasion, and like the bone marrow trephine biopsy, it is highly sensitive for IVL diagnosis[7,8]. Sitthinamsuwan *et al*[8] demonstrate that random skin biopsy is reliable method for diagnosis of IVL especially in patients with unusual neurological symptoms with co-existing hematologic abnormalities and without lymphadenopathy, such as our case.

In this case, we observed hypopituitarism, a rare complication of IVL. Endocrinopathy is a rare presentation of IVL. Hypopituitarism associated with IVL B-cell lymphoma has been described in fewer than 20 reports[3]. The pituitary gland is a hypervascular organ, and hypopituitarism may be caused by vascular occlusion by the

lymphoid tumor cells in the hypothalamus or pituitary gland[3,9]. It is unclear why selective growth of tumor cells occurs[3,9]. Although we did not perform a pituitary biopsy, we diagnosed lymphoma infiltration of the pituitary gland based on poor evidence of other causes of hypopituitarism and pituitary gland enlargement; both conditions improved after chemotherapy. Although most patients of IVL with central nerves system involvement need intrathecal chemotherapy, most patients with pituitary involvement have responded to chemotherapy alone, as this case showed[10].

Although the common clinical or hormonal course after treatment for pituitary involvement with IVL remains uncertain because of the limited number of reported cases, early chemotherapy may be effective. Sawada *et al*[3] described the case of a patient with hypopituitarism associated with IVL and the endocrinological course that followed. In this case, although the patient showed symptoms of panhypopituitarism before treatment, the levels of LH, FSH, TSH, ACTH, and prolactin returned to normal. She did not receive hormonal supplementation before and after chemotherapy. Sawada *et al*[3] stated that hematological therapy at an earlier disease stage may contribute to better endocrinological prognoses, and that the amelioration of pituitary infarction by chemotherapy improves pituitary function when the damage is not irreversible, enabling avoidance of hormone replacement therapy. Our case supports their theory, as the function of the pituitary gland in our patient also partially improved after chemotherapy. The infarction-related damage was not irreversible.

CONCLUSION

In conclusion, we observed a case of IVL with hypopituitarism as a rare complication, which was diagnosed antemortem and successfully treated. Although early chemotherapy is effective, the clinical diagnosis of IVL is challenging. On encountering cases with a variety of symptoms that cannot be explained reasonably, general physicians should consider IVL. Additionally, the mechanisms of lymphoma-associated hypopituitarism associated with IVL have not yet been elucidated, necessitating further case studies and laboratory-based research.

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