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REVIEW

Therapeutic management options for stage III non-small cell lung cancer

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Abstract

Lung cancer is the leading cause of cancer death worldwide. Majority of newly diagnosed lung cancers are non-small cell lung cancer (NSCLC), of which up to half are considered locally advanced at the time of diagnosis. Patients with locally advanced stage III NSCLC consists of a heterogeneous population, making management for these patients complex. Surgery has long been the preferred local treatment for patients with resectable disease. For select patients, multi-modality therapy involving systemic and radiation therapies in addition to surgery improves treatment outcomes compared to surgery alone. For patients with unresectable disease, concurrent chemoradiation is the preferred treatment. More recently, research into different chemotherapy agents, targeted therapies, radiation fractionation schedules, intensity-modulated radiotherapy, and proton therapy have shown promise to improve treatment outcomes and quality of life. The array of treatment approaches for locally advanced NSCLC is large and constantly evolving. An updated review of past and current literature for the roles of surgery, chemotherapeutic agents, radiation therapy, and targeted therapy for stage III NSCLC patients are presented.

Key words: Non-small cell lung cancer; Chemo-radiotherapy; Multi-modality; Targeted therapy; Dose-escalation

Core tip: Locally advanced non-small cell lung cancer consists of a heterogeneous population making management challenging. Multiple strategies are being developed to maximize survival and disease control. The role of surgery is being re-evaluated given new insight into the efficacy chemotherapy and radiation. Multi-modality therapy is playing an increasingly important role for both resectable and unresectable stage III patients. Chemoradiation plays a large role in the management of inoperable or unresectable patients. Third generation

chemotherapy and other targeted therapies are being incorporated into chemoradiation. Radiation dose-escalation, alternative fractionation schedules, intensity-modulated radiotherapy, and proton therapy are evaluated to improve outcomes from chemoradiation.

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INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States and worldwide. In 2016, approximately 224390 Americans are estimated to be newly diagnosed with lung cancer, and 158080 will die from this disease[1]. About 80% of lung cancer cases are non-small cell lung cancer (NSCLC), of which up to half are locally advanced at the time of diagnosis[2]. According to guidelines, locally advanced NSCLC is often defined as the 7th edition AJCC staging classification stage III NSCLC[3,4].

Stage IIIA and IIIB are two subsets within this classification, and the distinction is made because prognosis, treatment options, and long-term outcomes differ from one another. Furthermore, stage IIIA disease must be differentiated as resectable or unresectable at time of diagnosis. Stage IIIA (T1-3 N2, T3-T4 N1, T4 N0) disease involves hilar or mediastinal lymph nodes limited to the ipsilateral mediastinum, and a subset of these patients are amenable to surgery[3,4]. However, Stage IIIB (T1-4 N3, or T4 N2) involves lymph node metastasis in the contralateral thorax or supraclavicular fossa and/or an unresectable primary tumor, making patients with this disease not ideal candidates for surgical resection[3,4]. With such a heterogeneous population, a multi-modality approach involving surgery, radiation, and systemic agents is most commonly employed. A standard treatment option for unresectable or inoperable stage IIIA and stage IIIB disease is concurrent chemoradiation, while management of IIIA is more complex and controversial[5]. Treatment options for IIIA disease includes surgery with neoadjuvant or adjuvant chemotherapy, radiation, or both; as well as definitive chemoradiation[3,5,6]. Long-term outcomes are poor, with baseline 5-year overall survival (OS) of 15%-35% for stage IIIA and 5%-10% for stage IIIB[7]. The appropriate combination, timing, and sequence of individual treatment components in order to improve outcomes are under active research for both disease subsets. The aim of this review is to provide an overview of current and future treatment options for the management of locally advanced NSCLC.

MANAGEMENT OPTIONS FOR RESECTABLE STAGE IIIA NSCLC

Surgery

Up to 30%-50% of stage III NSCLC are locally advanced and inoperable at time of diagnosis[2,8]. Accurate pre-operative staging, particularly of mediastinal lymph nodes, is imperative as it dictates further management. Lymph node evaluation techniques include endobronchial ultrasound, endoscopic ultrasound-guided biopsy, cervical mediastinoscopy, or transthoracic needle aspiration. Positron emission tomography/computed tomography (PET/CT) scans have improved the accuracy of lymph node staging by improved detection of subclinical micro- and macro-metastases[9]. For patients who are deemed to have resectable disease, surgery plays an important role in their treatment. Generally, those with limited mediastinal lymphadenopathy are considered potentially more favorable candidates for resection than those with multistation or bulky mediastinal involvement, as it is associated with a higher rate of micro-metastasis. However, there are no specific guidelines to determine to what extent lung tumors should be considered "resectable"[6]. In fact, data have shown that a substantial proportion of stage IIIA-N2 patients who were considered resectable ultimately had an R1, 2 resection[10].

Pre- and post-operative chemotherapy

While surgery is an important aspect in the management for resectable stage IIIA patients, surgery alone continues to have poor outcomes, and as many as 30%-70% of resected patients experience recurrence or death[11,12]. The addition of post-operative chemotherapy has been extensively studied, and shown to improve treatment outcomes in patients with locally advanced disease[13-15]. In an analysis by the NSCLC Meta-analysis Collaborative Group[13] in which a meta-analyses totaling 34 trials and 8447 patients were evaluated, adjuvant chemotherapy was shown to have an absolute 5-year overall survival benefit of 4%, increasing OS rate from 60% to 64%, for patients with stage I-III disease. More specifically, a 5% absolute improvement in 5-year survival for stage III disease was observed, increasing 5-year OS rates from 30% to 35%. Other recent studies[14,15] have shown similar results, in which post-operative chemotherapy increased median survival from 45 mo from surgery alone to 54 mo[14]. These studies also demonstrated adjuvant chemotherapy increased 5-year progression free survival (PFS) by approximately 5%[14,15]. Because post-operative chemotherapy has been shown to significantly improve treatment outcomes, it is the standard of care for resectable locally advanced disease[3].

While surgical resection followed by chemotherapy is commonly employed, induction chemotherapy followed by surgical resection has also been studied[7,16-19]. Indu-

ction chemotherapy has the potential to eradicate micrometastases prior to resection, reduce tumor size, and increase the likelihood of resection. However, a concern with induction chemotherapy would be to delay a potentially curative surgery due to disease progression or declining health of the patient. The same NSCLC Meta-analysis Collaborative Group recently summarized the findings of 15 randomized controlled trials totaling 2385 patients on the effects of administering chemotherapy prior to surgical resection for patients with stage IB-IIIA disease[16]. In this analysis, pre-operative chemotherapy increased 5-year survival from 20% to 25%. Similar to adjuvant chemotherapy, induction chemotherapy also reduced relative risk of death by 13%. Five-year PFS improved from 30% to 36% with induction chemotherapy, and the time to distant recurrence also improved by 10% at 5-year. Results from older studies have shown that induction chemotherapy improved median survival from 11 mo to anywhere between 22 to 64 mo[17-19]. The NSCLC Meta-analysis Collaborative Group did not note a difference in complete resection rates between surgery *vs* preoperative chemotherapy with surgery, suggesting that the delay for induction chemotherapy does not significantly reduce chances of a potentially curative resection[16].

There does not seem to be a difference in survival or recurrence between adjuvant and induction chemotherapy. In a phase III trial, Felip *et al*[20] randomized 624 stage IA to IIIA patients to surgery alone, three cycles of preoperative carboplatin-paclitaxel followed by surgery, or surgery followed by three cycles of adjuvant carboplatin-paclitaxel. There was no difference in 5-year OS or PFS rates between induction and adjuvant chemotherapy regimens compared to surgery alone, though there was a non-significant trend towards longer PFS in the preoperative arm. Given that pre- and post-operative chemotherapy yields similar outcomes, induction chemotherapy could be reserved for patients with larger, more advanced tumors or those unable to tolerate chemotherapy while recovering after surgery[16]. Adjuvant chemotherapy could be utilized for patients with better prognosis and earlier disease stages[16].

Post-operative radiotherapy

Despite having complete resection and adjuvant chemotherapy, up to 40% of resectable stage IIIA patients experience local tumor recurrence[21,22]. In order to improve local tumor control and survival, post-operative radiotherapy (PORT) has long been utilized to intensify local therapy. Yet the ideal candidate for PORT has been controversial with conflicting results from different trials and series. Historical randomized control trials demonstrated that PORT significantly reduced local recurrence without any impact on overall survival[23-25]. One trial demonstrated a detrimental effect of PORT on survival compared to surgery alone, in which 5-year OS rates were 30% and 43% respectively[23]. The PORT Meta-analysis[26] demonstrated that PORT had an adverse effect on survival by increasing the relative risk of death by 21%, translating to a 7% reduction in 2-year OS from 55% to 48%. Subgroup analysis indicated a detriment in OS for patients with stage I/II N0-1 due to excess of toxicity from PORT. However, PORT for stage III-N2 disease trended toward, but did not reach, a significant survival benefit, suggesting a need for further investigation. A significant flaw of the PORT Meta-analysis was the inclusion of historical series with patients treatments utilizing antiquated techniques that were potentially more toxic than modern radiation delivery with image guidance, respiratory motion assessment, and higher dose conformality.

A recent retrospective analysis of the SEER database analyzing 7465 stage II-III patients receiving PORT following lobectomy or pneumonectomy demonstrated that PORT significantly increased survival for patients with N2 disease and associated with worse survival for N0-1 disease[27]. Among N2 patients, PORT improved 5-year OS from 20% to 27% (HR = 0.85), while reducing 5-year OS by 10% (HR = 1.2) and 4% (HR = 1.1) among N0 and N1 patients respectively[27]. The survival benefit for N2 disease was not observed until 2.5 years after PORT, while the lack of benefit for N0-1 disease was evident within one year of receiving PORT. A similar population-based series from the National Cancer Database also demonstrated an improvement in median OS from 45 mo with PORT *vs* 41 mo without PORT[28]. These results were consistent with a separate subset analysis from the Adjuvant Navelbine International Trialists Association trial[29]. In this trial 850 patients were randomized to adjuvant cisplatin and vinorelbine or observation following complete resection. The decision to provide PORT was left to the discretion of the participating institutions but was suggested for patients with node-positive disease. PORT was delivered to 232 patients. Median survival (MS) improved after PORT among patients with N2 disease receiving either adjuvant chemotherapy (from 23.8 to 47.4 mo) or observation (from 12.7 to 22.7 mo) following surgical resection. This analysis also confirmed that PORT reduced local recurrence regardless of nodal status. However, patients that received PORT and adjuvant chemotherapy with stage N1 disease experienced worse MS compared with chemotherapy alone (46.6 mo *vs* 96.6 mo) and 5-year OS (40% *vs* 56.3%), respectively. This study suggests that PORT may be influenced by the use of adjuvant therapy and extent of nodal involvement.

Since the PORT Meta-analysis, further prospective trials for PORT have drastically declined. However, this series may not be as relevant today since cobalt-60 sources and older treatment delivery systems were used for patient treatment[27]. Today's technology has significantly improved radiation delivery. There is a need for updated PORT studies using modern techniques since more conformal radiotherapy could improve local control while reducing cardiac and pulmonary toxicities observed in PORT Meta-analysis[30,31]. The LungART trial is a large European Phase III multi-institutional

prospective study of PORT using modern staging and treatment planning among N2 patients who have undergone complete resection. This trial is currently being conducted, and results are highly anticipated[32].

Post-operative radiotherapy and concurrent chemotherapy

The benefits of post-operative concurrent chemoradiation continue to be under debate. The Intergroup 0115 (ECOG 3590, RTOG 9501)[33] was a trial of 488 stage II-IIIA patients randomized to PORT alone or with four cycles of cisplatin and etoposide. A total of 50.4 Gy was delivered in 28 daily fractions to both groups. After median follow-up time of 44 mo, no survival benefit of concurrent chemoradiotherapy was observed. MS was not different in the post-operative chemoradiation group (38 mo) *vs* those in PORT group (39 mo) with a relative likelihood of survival to be 0.93. Intrathoracic disease recurrences within the irradiated field were 12% and 13%, respectively and was not significantly different. Compared to these results, the RTOG 9705 trial[34] found more favorable OS and PFS benefit with the addition of adjuvant chemotherapy to PORT. However, this was a phase II non-randomized study. In this trial, 88 stage II-III NSCLC patients received concurrent radiotherapy at 50.4 Gy in 28 daily fractions, carboplatin, and paclitaxel with a MS of 56.3 mo. The 3-year OS and PFS rates in this study were 61% and 50% respectively, while intrathoracic recurrence rate was similar to that observed in INT 0115 at 15%. To date, there remains no evidence supporting concurrent delivery of adjuvant chemotherapy with PORT.

Neoadjuvant radiation and multi-modality therapy

Thus far, treatment strategies incorporating surgical resection have demonstrated the best local control for operable NSCLC, and outcomes may be improved by managing distant metastases by induction or adjuvant therapy. However, OS and local control remains low. In an attempt to further improve resectability, local regional control, and survival for select patients with potentially resectable disease, combinations involving all three treatment modalities have been studied. An international multi-centered European trial[35] sought to compare the benefits of neoadjuvant chemoradiation or neoadjuvant chemotherapy alone prior to undergoing surgical resection randomized. Patients with stage IIIA-N2 disease were randomized to neoadjuvant regimens of 3 cycles of cisplatin and docetaxel followed by radiation to 44 Gy in 22 fractions over 3 wk or chemotherapy alone. Regimens in both study groups were well tolerated, as 91% of patients completed all three cycles of neoadjuvant chemotherapy and 7% experienced radiation-induced grade 3 or higher dysphagia. The primary endpoint of event-free survival was not significantly different between both groups. Those in the neoadjuvant chemoradiation group had median PFS of 12.8 mo compared to patients in neoadjuvant chemotherapy group with a median PFS of 11.6 mo (HR = 1.1). MS for both groups were 37.1 and 26.2 mo respectively (HR = 1), and also not different from one another. The proportion of patients with pathological complete response or nodal downstaging were 61% and 44% in neoadjuvant chemoradiation and chemotherapy group respectively, which was significantly different. While preoperative chemoradiation did not improve survival, it did significantly increase the proportion of patients with mediastinal downstaging and histopathological response. Such improvement in tumor response could improve local control and even survival for carefully selected patients, and neoadjuvant chemoradiation should be further evaluated.

Given that preoperative chemotherapy improves survival for resectable stage IIIA patients, a phase III trial[36] evaluated whether adding preoperative chemoradiation in addition to induction chemotherapy could improve treatment outcomes. This trial randomized 524 stage IIIA/B (N2/3) patients to receive either induction chemotherapy and chemoradiation (intervention) or induction chemotherapy alone (control) prior to surgical resection and PORT. The toxicity and perioperative morbidity were similar between both arms. Pneumonectomies were performed at a rate of 35% in both arms. Hematological toxicities (10% *vs* 0.5%, $P < 0.0001$) and Grade 3 or higher esophagitis (19% *vs* 4%, $P < 0.0001$) were more frequent in the intervention group, whereas Grade 3 or higher pneumonitis was more common in the control group (1% *vs* 7%, $P = 0.0006$). A significantly higher proportion of patients receiving neoadjuvant chemoradiation (46%) experienced mediastinal downstaging compared to those receiving induction chemotherapy alone (29%) ($P = 0.02$). Sixty percent of patients receiving neoadjuvant radiation achieved > 90% tumor regression compared to 20% of patients among the induction chemotherapy group ($P < 0.0001$). While response rates were significantly improved by chemoradiation, neoadjuvant chemoradiation did not improve the primary endpoint for PFS for the entire cohort. Secondary endpoints for OS, rate of disease progression, or site of first progression were also similar for all patients. Five-year PFS between intervention and control groups were 16% and 14%, respectively (HR = 0.99), and 5-year OS were 21% and 18% (HR = 1) respectively. However, subset analysis did demonstrate improved PFS (HR = 1.58, $P = 0.043$) and OS (HR = 2.07, $P = 0.03$) in patients undergoing a complete resection with successful downstaging of the mediastinum from N2-3 to N0-1 following induction radiation compared to patients with incomplete resections. These data suggest that survival outcomes may improve with mediastinal clearance and downstaging prior to surgery, and neoadjuvant chemoradiation should be considered as a treatment option for patients with potentially resectable stage III disease.

Randomized phase III trials have not yet successfully demonstrated a survival advantage of induction chemotherapy or chemoradiation prior to surgery over

definitive chemoradiation. EORTC 08941[37] reported comparable MS and 5-year OS for stage IIIA-N2 initially unresectable patients receiving induction platinum-based chemotherapy and randomized to either surgery or radiation therapy. Disease was considered unresectable if there was any N2 disease for non-squamous histology or lymph node spread beyond levels 4R or levels 5/6 for right or left squamous primaries, respectively. Treatment-related mortality was greater perioperatively (4%) compared to one death (0.6%) following radiation pneumonitis. This study suggested that surgical resection may not improve treatment outcomes compared to definitive radiotherapy. Within the context that radiotherapy leads to lower morbidity and mortality compared to surgery, definitive chemoradiation is a reasonable treatment option for patients with stage IIIA-N2 disease. However, several criticisms with this study have been made including that only 50% of patients randomized to the surgery arm received radical resection, and 40% of surgical arm patients received PORT. The chemoradiation regimen used is not an accepted standard, making extrapolation of this trial to current practice challenging. An intergroup trial, INT 0139[38] tested the benefits of trimodality with sequential cisplatin/etoposide with 45 Gy of radiation prior to surgical resection compared to concurrent chemoradiation alone. After a median follow-up of 22.5 mo, 5-year OS and MS were not improved with the induction chemoradiation. Five-year PFS was significantly higher under the intervention arm (22.4%) compared to chemoradiation arm (11.1%) (P = 0.017), which was not observed from EORTC 08941[37]. However, relatively high treatment-related deaths were observed in the trimodality arm (7.9%) compared to definitive chemoradiation arm (2.1%). No benefit of surgery was observed in patients who received pneumonectomies, likely due to an increased rate of death without progression. While induction chemoradiation may have improved 5-year PFS, a survival benefit was not observed. Such results could have been confounded by the higher perioperative mortality observed in the intervention arm, particularly among pneumonectomy patients. A subgroup analysis showed that median survival was significantly improved with induction chemoradiation prior to lobectomies (P = 0.002). In addition, 5-year OS rates were significantly better (P < 0.0001) among those with pathologic stage N0 (41%) and N1-3 (24%) at time of thoracotomy compared with those who did not receive surgery (8%). These subgroup analyses suggest that a survival advantage of trimodality over definitive chemoradiation may be demonstrated in carefully selected candidates.

To minimize perioperative mortality that was observed in INT 0139, surgeons in the RTOG 0229 trial[39] were required to demonstrate expertise in performing surgery following chemoradiation. RTOG 0229 was a multi-institutional phase II trial that followed 57 stage III-N2/3 patients receiving neoadjuvant chemoradiation of carboplatin, paclitaxel, and 50.4 Gy to the mediastinum with 10.8 Gy boost to gross disease followed by surgical resection. An impressive rate of 63% of patients achieved mediastinal disease clearance while residual disease remained in 16% of patients. The primary endpoint of improving mediastinal disease from 50% to 70% with a power of 80% was achieved. One-year OS and PFS were 77% and 52%. Fourteen percent of patients in RTOG 0229 experienced Grade 3 postoperative pulmonary complications. It is important to note that this was not increased compared with other trials of chemoradiation alone. The rate of pneumonectomies was much lower in this trial (5%) compared to INT 0139 (34%). Moreover, rate of perioperative morbidity was 3% (1 patient) which compared favorably to the relatively high rate of morbidity observed in INT 0139 (7.9%). The ability of neoadjuvant chemoradiation to sterilize mediastinal nodal disease was confirmed by this study, and thus should be considered as an option for multi-modality therapy for select patients. Lobectomy should be the preferred surgical management, and surgery should be performed by a thoracic surgeon skilled in this specific approach.

A recent trial[40] studied the outcomes of surgery *vs* definitive chemoradiation boost following both neoadjuvant chemotherapy and chemoradiation. This was a phase III multi-centered randomized control trial for stage IIIA-N2 and select IIIB patients receiving three cycles of cisplatin/paclitaxel as well as induction cisplatin/vinorelbine, and accelerated radiotherapy of 45 Gy in twice daily 1.5 Gy fractions. Patients were reassessed for resectability, and randomized to either receive chemoradiation boost to 65-71 Gy in arm A or surgery in arm B. Grade 3 or higher toxicities were acceptable and balanced between both groups. After median follow-up of 78 mo, 5-year OS was 40% in arm A and 44% in arm B, while 5-year PFS rates were 35% and 32% in arms A and B, respectively. No significant differences were found for either OS or PFS between the two groups, thus making either strategies acceptable for resectable stage IIIA, and select inoperable IIIA or IIIB patients.

Multi-modality management is efficacious for select stage IIIB patients as well. Because induction radiation and chemotherapy improves mediastinal downstaging and pathological response, tumor resectability has proven to increase among stage IIIB patients in several phase II trials[41-45]. 3-year OS rates have approached to 60%[44], and resectability rates increased up to 80%[43]. Table 1 summarizes trials for multi-modality therapy for stage IIIA/B patients.

MANAGEMENT OPTIONS FOR STAGE IIIB AND UNRESECTABLE/INOPERABLE STAGE IIIA NSCLC

Chemoradiation

Definitive chemoradiation remains a standard of care in the management of stage IIIB disease or IIIA patients

Table 1 Prospective trials of multi-modality therapy for resectable stage III non-small-cell lung cancer

Ref.	Phase	Study design	Chemo regimen	RT	Number of patients	Stage	Median f/u (mo)	OS	Median OS (mo)	PFS	Median PFS (mo)	Response rate
Pless *et al*[35] (2015)		Induction chemoRT + surgery *vs* induction chemo + surgery	Cisplatin/ docetaxel	44 Gy in 2 Gy fxns over 3 wk	232	IIIA (N2)	52.4		37.1 *vs* 26.2		12.8 *vs* 11.6 (P = 0.67)	ORR: 61% *vs* 44%
Thomas *et al*[36] (2008)	3	Induction chemo + induction chemoRT + surgery *vs* induction chemo + surgery	Induction: Cisplatin/ etoposide ChemoRT: Carboplatin/ vinorelbine	45 Gy in 1.5 Gy fxns (twice daily)	524	III A/B (N2/3)		5-yr, 21% *vs* 18% (P = 0.97)	15.7 mo *vs* 17.6 mo	5-yr, 16% *vs* 14% (P = 0.87)	9.5 *vs* 10	CR: 60% *vs* 20% (P < 0.0001) Mediastinal downstaging: 46% *vs* 29% (P < 0.02)
EORTC 08941 Van Meerbeeck *et al*[37] (2007)	3	Induction chemo + surgery *vs* chemoRT	Platinum-based	60-62.5 Gy in 1.95-2.05 Gy daily fxns	332	IIIA (N2)	> 72	5-yr, 15.7% *vs* 14% (P = 0.6)	16.4 *vs* 17.5 (P = 0.6)	2-yr, 27% *vs* 24% (P = 0.6)	9 *vs* 11.3 (P = 0.6)	
INT 0139 Albain *et al*[38] (2009)	3	Induction chemoRT + surgery *vs* chemoRT	Cisplatin/ etoposide	45 Gy boost to 61 Gy if definitive chemoRT	396	IIIA (N2)	22.5	5-yr, 27.2% *vs* 20.3% (P = 0.10)	23.6 *vs* 22.2 (P = 0.24)	5-yr, 22.4% *vs* 11.1% (P = 0.017)	12.8 *vs* 10.5 (P = 0.017)	
RTOG 0229 Suntharalingam *et al*[39] (2010)	2	Induction chemoRT + surgery	Carboplatin/ paclitaxel	50.4 Gy + 10.8 Gy to gross disease	60	III A/B (N2/3)		1-yr, 77%	26.6	1-yr, 52%	13.1	Improved mediastinal sterilization 50% to 70% met
ESPATUE Eberhardt *et al*[40] (2015)	3	Induction chemotherapy + induction chemoRT + RT boost *vs* Induction chemotherapy + induction chemoRT + surgery	Induction chemo: Cisplatin/ paclitaxel Induction chemoRT: Cisplatin/ vinorelbine	45 Gy in 1.5 Gy twice daily fxns Definitive chemoRT: Boost to 65-71 Gy	246	III A/B (N2/N3)	78	5-yr, 40% *vs* 44% (P = 0.34)		5-yr PFS, 35% *vs* 32% (P = 0.75)		
Eberhardt *et al*[40] (2015)	3	Induction chemo + induction chemoRT + surgery *vs* induction chemo + definitive chemoRT	Induction: Cisplatin/ paclitaxel ChemoRT: cisplatin/ vinorelbine	45 Gy in 1.5 Gy fxns (twice daily)	246	IIIA (N2), select IIIB (N3)	78	5-yr, 40% *vs* 44%		5-yr, 35% *vs* 32%		

CR: Complete response; ORR: Overall response rate; OS: Overall survival; RT: Radiotherapy; PFS: Progression free survival.

with unresectable or inoperable disease[3]. Radiation provides local therapy for inoperable tumors, and chemotherapy not only reduces or prevents micrometastatic spread of the disease, but also acts as a radiosensitizer to increase the therapeutic index of radiation therapy. Chemotherapy plays a critical role in the management for advanced NSCLC, and when given with radiation, the combination improves survival over supportive care or radiation therapy alone[46-49]. Standard radiation is typically 60-66 Gy in 2Gy daily fractions over 6 wk, as established by RTOG 7301 trial[50], and platinum-based doublet chemotherapy is typically used with standard radiation[3].

Sequential vs concurrent chemoradiation

Concurrent chemoradiation has proven to be superior to sequential chemoradiation, and is now considered standard of care. RTOG 9410[51] was a pivotal trial esta-

blishing the superiority of concurrent chemoradiation. This trial randomized 610 inoperable stage II-III NSCLC patients into one of three groups: Sequential cisplatin/vinblastine and conventionally fractionated radiation to 63 Gy (arm 1), concurrent chemotherapy and radiation to 63 Gy (arm 2), or concurrent chemotherapy with accelerated hyperfractionation of 69.6 Gy in twice daily 1.2 Gy fractions over 6 wk (arm 3). Five-year OS rates among the three groups were 10%, 16%, and 13% respectively, and was significantly higher in the standard chemoradiation arm compared to arm 3 (P = 0.046), but not against arm 1 (P = 0.46). MS was 17 mo in arm 2 while it was 14 mo in arm 1. Furthermore, the response rate in arm 2 was 70% and statistically significantly higher compared to sequential chemoradiation (P < 0.05). While acute Grade 3 or higher non-hematologic toxicity rates, particularly severe acute esophagitis, were higher with concurrent therapy, late toxic effects were ultimately similar in concurrent or sequential therapies.

Since RTOG 9410, the superiority of concurrent over sequential chemoradiation has been confirmed by several other studies, including a meta-analysis evaluating seven randomized controlled trials[52]. Concurrent chemoradiation improved OS by an absolute benefit of 4.5% after 5-years, increasing 5-year OS rate from 10.6% to 15.1% (HR = 0.84)[52]. Moreover, locoregional progression decreased by an absolute rate of 6.1% at 5 years, lowering the rate from 35% to 28.9% after concurrent chemoradiation. While concurrent chemoradiation provides better locoregional control, it does not lower distant disease progression compared to sequential chemoradiation (HR = 1.04). Concurrent chemoradiation, however, is associated with higher rates of Grade 3 or higher esophageal toxicity, and can reach up to 18%. The higher toxicity rates were thought to be clinically acceptable and manageable. Induction or consolidation chemotherapy in addition to chemoradiation was not necessary, as it has not been shown to improve 2-year OS or MS[53-56]. However, it could be considered for patients with bulkier tumors whose gross disease could not be treated with radiation without leading to radiation-induced toxicity[57]. Concurrent chemoradiation is better suitable for patients with minimal co-morbidities, favorable performance statuses, and minimal weight loss[53,58]. Patients who are unable to tolerate concurrent chemoradiation should still receive sequential regimens since it still incurs some benefit over radiotherapy alone by increasing 5-year OS from 5% to 10%[59-62].

Current and future directions with chemotherapy regimens for chemoradiation

Chemoradiation therapy is complex, and the agents needed to achieve the best disease control and survival are unknown. The most commonly used regimens are cisplatin/etoposide or carboplatin/paclitaxel. Cisplatin-based regimens have demonstrated to provide better outcomes compared to carboplatin-based regimens[63-65]. In a phase II randomized trial[63] comparing outcomes from 60 Gy thoracic radiation combined with either cisplatin/etoposide (PE) *vs* carboplatin/paclitaxel (PC), OS was significantly better in the PE arm. Three-year OS was 33.1% in the PE arm, but only 13% in the PC arm (P = 0.04). In a meta-analysis from individual patient data[65], cisplatin achieved significantly higher objective response rate of 30% compared to 24% from carboplatin (P < 0.001) among nine trials using platinum-based agents in first-line treatments. While cisplatin-based chemotherapy was more efficacious, it has also led to increased toxicity, especially Grade 3/4 neutropenia[15,63,65].

An individual patient data meta-analysis[65] also observed patients with non-squamous tumors experienced significantly higher mortality when treated with carboplatin and third-generation chemotherapy (HR = 1.12). However, a small number of studies have reported equivalent outcomes with carboplatin as with cisplatin[66,67]. An analysis of over 1842 patients from Veterans Health Administration data demonstrated PC having similar survival as PE. In fact, PE was associated with more hospitalizations, outpatient visits, acute kidney disease, and esophagitis/mucositis compared to PC[66]. However, the results from this trial should be interpreted with caution since 98% of patients were men, and approximately 50% of tumors was squamous cell histology *vs* approximately 20% adenocarcinoma. This was not representative of true population of stage III NSCLC[51,68,69]. Therefore, carboplatin may be more beneficial for men presenting with squamous NSCLC[70]. Liew *et al*[67] also found PC to have similar survival outcomes *vs* PE, with MS to be 20.7 and 13.7 mo with PC and PE, respectively. Relapse free survival was also comparable, and median PFS was 12 mo with PC *vs* 11.5 mo with PE. PC cause significantly less hematological toxicities compared to PE. Therefore, carboplatin therapy may also be more beneficial for older patients and those with multiple co-morbidities.

Third generation chemotherapy agents are increasingly being incorporated into the management of stage III NSCLC patients (Table 2). Their use has not been shown to improve treatment outcomes compared to "older" generation agents like cisplatin/etoposide. A retrospective review[5] compared PE, PC, and cisplatin/docetaxel (PD), and found that MS from PD was not significantly better compared to PE or PC. Median survivals were 27, 36, and 23 mo respectively. Median PFS were 21, 10, and 15 mo in PE, PC, and PD arms respectively, and was significantly better under PE arm (P = 0.01). PE not only has better treatment outcomes, but also had better objective response rates compared to PD or PC. Additionally, WTOG 0105 trial[71] was a phase III study directly comparing second to third generation regimens in the setting of concurrent chemoradiation for inoperable stage III NSCLC. In this study, patients were randomized to receive MVP, carboplatin/irinotecan, or PC along with 60 Gy of concurrent radiation for 6

Table 2 Chemotherapy agents for non-small-cell lung cancer by generation

Generation	Agents	Effect on survival for stages II-III
First	Methotrexate Cyclophosphamide Vincristine Doxorubicin	No effect
Second	Cisplatin, cisplatin-based combinations Ifosfamide Mitomycin Vindesine Vinblastine Etoposide	Combination with radiation superior to radiation alone Concurrent superior than sequential chemotherapy and radiation
Third	Paclitaxel, paclitaxel-based combinations Docetaxel Gemcitabine Vinorelbine Irinotecan Topotecan	Expected to be superior to second generation agents given with radiation

wk. Five-year OS rates for the three arms were 17.5%, 17.8%, and 19.8% respectively. Thus third generation agents did not significantly improve survival; however, it was also not inferior to second generation agents. While third generation agents may be non-inferior to second generation agents, more treatment interruptions were observed with patients receiving carboplatin/irinotecan compared to other chemotherapy groups. Other studies that have chosen to focus on understanding the efficacy of other single-agent third generation chemotherapy such as vinorelbine have findings that agree with prior phase III trials[72,73]. While third generation agents are equivocal to second generation agents regarding survival and response rates, these agents should still be further investigated, even though they do not add benefit to survival or response rates.

Pemetrexed is a new multi-targeted anti-folate chemotherapy agent commonly used with cisplatin in first-line, second-line, and maintenance therapies for non-squamous NSCLC[55,74,75]. Several phase II studies demonstrated that pemetrexed can be safely administered with either cisplatin or carboplatin, yielding a median survival ranging from 18.7 to 34 mo, and esophageal and pulmonary toxicities reaching no higher than 16% and 23% respectively[76-78]. Better outcomes among non-squamous tumor histologies were observed[76-78]. The PROCLAIM trial[79] was a phase III trial comparing concurrent chemoradiation using cisplatin/pemetrexed *vs* PE among non-squamous NSCLC. Although enrollment ended early due to futility, 598 patients were ultimately randomized. MS were 26.8 mo in the pemetrexed arm and 25 mo in etoposide arm (HR = 0.98), which were similar to those observed in phase II trials. PFS was also not significantly different between pemetrexed over etoposide regimens, but trended in favor of pemetrexed. Median PFS were 11.4 and 9.8 in pemetrexed and etoposide arms respectively (HR = 0.86). Moreover, pemetrexed yielded a mildly higher response rate (35.9%) compared to etoposide (33%). Pemetrexed had significantly lower Grade 3 or higher adverse effects compared to PE (P = 0.01), including neutropenia, febrile neutropenia, and thrombocytopenia.

Targeted therapy

Treatment response varies greatly among individuals, and the heterogeneity of tumor biology is expansive. Few driving mutations that may be exploited by therapy have been discovered. Incorporation of therapies targeted to these driver mutations has not yet been successful and remains under investigation. EGFR and ELM4-ALK mutations are likely candidates for targeted therapy in definitive treatment. EGFR inhibitors include monoclonal antibodies targeting the extracellular domain of EGFR, while tyrosine kinase inhibitors (TKI) target the intracellular domain of EGFR and also act as radiosensitizers.

Early studies with cetuximab have shown some promise. The FLEX trial[80] was an international open-labeled phase III trial that compared the efficacy of cetuximab plus chemotherapy with chemotherapy alone among EGFR-positive NSCLC patients. Patients who were given cetuximab in addition to chemotherapy survived significantly longer than those receiving chemotherapy alone (P = 0.04). MS was 11.3 and 10.1 mo respectively (HR = 0.871). The main toxicity associated with cetuximab was an acne-like rash, and 10% of patients on cetuximab experienced severity of grade 3. The RTOG 0324 phase II trial[81] evaluated whether cetuximab given in conjunction with chemoradiation would provide any benefit for unresectable stage III patients. Through this single-arm trial, MS was 22.7 mo and 2-year OS is 49.3%, which was higher than previous reports at the time[51,56]. With such promising results, RTOG 0617 phase III trial[82] evaluated the use of cetuximab with standard and high-dose chemoradiotherapy. MS among patients receiving cetuximab was 25 mo and 24 mo who did not receive cetuximab (HR = 1.07). Moreover, the addition of cetuximab was associated with significantly higher rate of toxicities (P < 0.0001). Grade 3 or higher toxicity rates were 86% with cetuximab and 70% without. Therefore, the addition of cetuximab to concurrent chemoradiation or consolidation treatment did not provide any survival benefit while increasing treatment-related toxicities.

In contrast, TKIs like gefitinib and erlotinib play a larger role in the management of locally advanced NSCLC. Gefitinib is reserved for patients with disease refractory to standard chemotherapy. When used as a first-line or maintenance agent, it has not shown to improve survival[83-85]. INTACT trials randomized unresectable locally advanced to metastatic, chemotherapy-naïve patients to receiving gefitinib with platinum-doublet chemotherapy or platinum-doublet therapy alone. The addition of gefitinib with chemotherapy as first line

treatment did not improve MS, time to progression, or response rates. In SWOG S0023[85], MS with gefitinib maintenance following concurrent chemoradiation with PE decreased to 23 mo compared to 35 mo from placebo (P = 0.013). The decreased survival is primarily due to disease progression rather than treatment toxicity, as toxic death rate was not different from placebo. It is important to notice that these trials enrolled patients with and without EGFR mutations. Perhaps selectively treating patients only with EGFR mutations with gefitinib may lead to different outcomes.

Erlotinib is often used for patients with locally advanced and metastatic disease. The TRIBUTE study[86] randomized 1059 stage ⅢB and Ⅳ NSCLC to either erlotinib or placebo in combination with six cycles of PC. While there was no benefit with the addition of erlotinib to OS and time to disease progression, there was a survival benefit among patients who never smoked. MS with erlotinib increased to 22 mo compared to 10 mo with just PC alone. In a secondary analysis, patients specifically with EGFR mutations were associated with better response rates (P < 0.05) and a trend toward improved time to disease progression (P = 0.092)[87]. However, OS remained similar with the addition of erlotinib among this subset of patients (P = 0.96).

The IPASS trial[88] was a phase Ⅲ trial randomizing stage ⅢB and Ⅳ pulmonary adenocarcinoma patients in East Asia and who were nonsmokers or light smokers to receive either gefitinib alone or carboplatin/paclitaxel as first line therapy. The primary endpoint for non-inferior PFS was met, and surpassed. Gefitinib resulted in 12-mo PFS rate of 24.9% compared to 6.7% achieved with carboplatin and paclitaxel. For patients specifically with EGFR mutations, PFS survival was significantly longer from gefitinib therapy (P < 0.001). A similar phase Ⅲ trial[89] for European NSCLC patients with EGFR mutations randomized patients to receiving erlotinib alone or standard chemotherapy (cisplatin with either docetaxel or gemcitabine), and demonstrated that erlotinib significantly improved median PFS. Thus, TKIs are now considered first-line therapeutic options for patients harboring EGFR mutations.

Crizotinib is an oral small-molecule tyrosine kinase inhibitor against the product of the EML4-ALK fusion gene. For patients who harbor this mutation, crizotinib can be used as a first-line treatment. As a first line therapy, PROFILE-1014 phase Ⅲ trial[90] demonstrated that locally advanced or metastatic ALK-positive NSCLC patients experience longer progression free survival (10.9 mo) compared to cisplatin/pemetrexed therapy (7 mo) (P < 0.001), and improved overall response rate of 74% *vs* 45%, respectively (P < 0.001). However, 1-year survivals between the two groups were not significantly different. Similar findings were found when crizotinib was used as a second-line agent among patients who received prior platinum-based chemotherapy treatment[91]. Unfortunately, acquired resistance to crizotinib can occur, and manifests after a median period of 7-11 mo[90,91]. In this situation, a more potent agent, ceritinib, can be used to treat ALK-positive NSCLC patients refractory to chemotherapy and crizotinib. ASCEND-2 is a single-arm phase Ⅱ trial that demonstrated a durable response for these patients[92]. The majority of patients enrolled in this study also had brain metastases. Whole body overall response rate was 38.6%, with median duration of response of 9.7 mo and median PFS of 5.7 mo. Similarly, ASCEND-4 and 5 trials are two phase Ⅲ randomized control trials designed to compare progression free survival of ceritinib with or without chemotherapy in chemo-naïve or previously treated patients with stage ⅢB and Ⅳ NSCLC. Based upon their success in patients with metastatic disease, a role for erlotinib and crizotinib are being investigated in the potentially curative setting. RTOG 1306 is a phase Ⅱ in which patients with Stage Ⅲ NSCLC with susceptible mutations are randomized to standard chemoradiation alone or with the addition of erlotinib or crizotinib.

Besides EGFR and EML4-ALK inhibitors, other molecular targets are being explored to use in conjunction with chemoradiation for unresectable stage Ⅲ patients. Bevacizumab is one such anti-angiogenic therapy that could have synergistic effects with radiation[93,94]. Phase Ⅲ trials have shown promising results with higher response rates, and longer OS and PFS. However, the high rate of grade 3 or worse esophagitis including formation of trachea-esophageal fistula makes this agent less likely to be used with chemoradiation[95]. Nivolumab, a PD-1 immune checkpoint inhibitor antibody, is garnering attention. Two recent randomized, international phase Ⅲ trials demonstrated that Nivolumab prolonged 1-year OS, 1-year PFS, and response rates compared to docetaxel for patients whose disease had progressed during or after platinum-doublet chemotherapy for both squamous and non-squamous histologies[96,97]. With such promising results, perhaps immunotherapy will play an increasing role in the management of locally advanced NSCLC patients in the future.

Current and future directions with radiation for chemoradiation

Definitive radiotherapy alone continues to yield poor outcomes for stage Ⅲ patients. MS continues to range from 10 to 26 mo[6,98,99], with a 5-year survival rate of less than 25%[98,100,101]. Such low outcomes are related to the failure to eradicate local disease as well as development of distant metastasis. Several ways to improve local control and survival include dose escalation and altered fractionation schedules.

Increasing dose intensity has been shown to improve local control and survival in early studies. A retrospective analysis[102] of 7 prospective RTOG trials demonstrated that the higher biological effective dose (BED) of radiotherapy was associated with better outcomes in locally advanced NSCLC. Phase Ⅰ and Ⅱ dose escalation studies[103-105] using conformal radiation demonstrated that conformal thoracic radiation up to 74 Gy was fea-

sible and tolerable, and led to encouraging survival and response rates with acceptable toxicity levels. A modified phase I/II trial[103] randomized 62 unresectable stage III NSCLC patients to one of four cohorts where radiation dose was escalated from 60 to 74 Gy. No dose-limiting toxicity was observed from any cohorts, making 74 Gy the maximum tolerated dose (MTD). The most common toxicity was esophagitis, and approximately 8% of patients experienced grade 3/4 esophagitis. Overall response rate was 52%, and MS of 26 mo. Three-year OS rate was 40% and 3-year PFS was 29%. RTOG 0117 trial[104] confirmed that MTD was 74 Gy with 3D-CRT, since doses beyond 74 Gy incurred high pulmonary toxicity levels. Delivering 74 Gy concurrently with PC led to encouraging response rate of 66.6% and 1-year OS of 66.7%. MS was 24.3 mo, surpassing the study's predefined MS benchmark of 18 mo which was chosen to be the best that was achieved by CALGB. Despite such encouraging early results, results from the intergroup phase III RTOG 0617 trial[82] did not recommend use of 74 Gy as OS was significantly worse than the standard dose of 60 Gy. MS was 20.3 mo after delivery of 74 Gy compared to 28.7 mo from standard dose (HR = 1.38, P = 0.004). The rate of severe esophagitis was significantly worse at 21% in high dose group *vs* 7% in standard dose group (P <0.0001). Constraints for heart dose were not mandated, and heart doses were significantly higher among patients receiving high dose radiation, and this likely contributed to a survival detriment in those patients.

Accelerated hyperfractionation (hyperFRT) is a way to deliver a higher dose of radiation over the same time period as one would with conventional fractionation schedules. To do so, a lower dose per fraction is delivered more frequently, typically twice a day. The benefits of hyperFRT schedule were evaluated by various trials, in which early reports were rather mixed. RTOG 8311[106] was a phase I trial of radiation dose escalation. Patients were randomized to receive total doses of total doses of 60.0 Gy, 64.8 Gy, 69.6 Gy, 74.4 Gy or 79.2 Gy delivered in 1.2 Gy twice daily fractions five days a week. Survival did not improve at doses beyond 69.4 Gy. At this dose, MS was 13 mo and 2-year OS was 29%, which was significantly better than lower radiation doses tested (P = 0.02). With an optimal dose set for hyperFRT, the phase III RTOG 8808 trial[107] compared outcomes of conventional fractionation plus induction cisplatin/vinblastine (arm 1), hyperFRT at 69.4 Gy in 1.2 Gy fractions (arm 2), and conventional fractionation RT alone (arm 3). While survival from arm 2 was better compared to arm 3, it was not significantly better than arm 1[107]. Five-year OS rates were 8%, 6%, and 5% respectively, with MS rates of 13.2, 12, and 11.4 mo respectively. RTOG 9410[51] study echoed similar findings as RTOG 8808. This study compared sequential cisplatin/vinblastine and conventional RT (arm 1), concurrent cisplatin/vinblastine and conventional RT (arm 2), and concurrent cisplatin/etoposide with hyperFRT (arm 3). Conventional fractionation was 63 Gy in 1.8 Gy fractions over 7 wk), and hyperFRT delivered 69.6 Gy in 1.2 Gy twice daily fractions. Five-year OS were 10%, 16% and 13% respectively, and significantly better in arm 2 (P = 0.046). MS were 14.6, 17 and 15.6 mo, respectively. Between the two concurrent chemoradiation treatments, overall response rates were similar between arms 2 (70%) and 3 (65%), respectively. Grade 3 or higher toxicities were observed in 45% of patients receiving hyperFRT, though was not significantly different from arm 2. Incorporation of hyperFRT into multi-modality therapy has also been tested. Pöttgen *et al*[108] retrospectively compared outcomes of neoadjuvant chemotherapy and hyperFRT (45 Gy in 1.5 Gy twice daily fractions) *vs* conventional RT (46 Gy in 2 Gy daily fractions). While complete response rates were higher in neoadjuvant concurrent chemotherapy and hyperFRT compared to the control group using conventional RT (P < 0.006), the use of hyperFRT was not associated with improved survival.

Continuous hyperfractionated accelerated radiotherapy (CHART) delivers less than 1.8-2 Gy per fraction in an accelerated course to allow for less normal tissue injury per fraction and inter-fraction normal tissue repair. Despite that total dose of radiation and dose per fraction delivered are lower compared to conventional fractionation schemes, it is hypothesized that delivering greater radiation dose per unit of treatment time outpaces tumor cell repopulation which could improve treatment outcomes[109-111]. Standard CHART delivers 54 Gy in 1.5 Gy fractions three times per day for 12 consecutive days. A randomized control trial[112] comparing the efficacy of CHART to conventional fractionation, which delivered 60 Gy in daily 2Gy fractions, showed that CHART significantly improved 2-year OS by 9%, increasing it from 20% to 29% (HR = 0.76, P = 0.004). This finding translated to a 22% overall reduction in relative risk of death. The largest benefit of CHART was observed within patients with squamous NSCLC, where 2-year survival improved by 14%, increasing the survival rate from 19% to 33%. Adverse effects were higher in patients receiving CHART compared to conventional fractionation schemes within the first three mo of therapy. Severe dysphagia in particular was seen in 19% and 3% of patients, respectively. Overall, acute and late toxicities were not different between groups. CHARTWEL was a modification of CHART in that treatments were not given during weekends. A phase III trial[113] randomized 460 patients to either CHARTWEL or conventional fractionation. Five-year OS were 11% and 7% from CHARTWEL and conventional RT, and were not significantly different from each other. Local control rates were found to improve after CHARTWEL among patients with higher T or N staging (P = 0.006) or after receiving neoadjuvant chemotherapy (P = 0.019). Acute dysphagia and radiation-induced pneumonitis were frequent among CHARTWEL patients. Therefore, unlike CHART, CHARTWEL did not exhibit a survival benefit. Results from CHARTWEL was a proof-of-concept that

delivering lower total dose can be compensated by shorter treatment time, and that time is an important factor for the management of unresectable locally advanced NSCLC patients. The addition of neoadjuvant chemotherapy to CHART did not significantly improve survival or response rates[114,115], but was associated with less toxicity compared to standard fractionated concurrent chemoradiation and therefore could still be an option for locally advanced patients. In a recent small phase I trial[100], escalating total delivered dose from 54 Gy to 64.8 Gy in the setting of CHART was feasible and did not exhibit dose-limiting toxicities. MS was 24 mo across all dose cohorts, and Grade 3 or worse adverse effects were found in 6 of 18 patients. Thus, CHART potentially enhances survival and response outcomes compared to conventional fractionation radiation. Table 3 summarizes key prospective trials evaluating hyperFRT fractionation schedules over conventional fractionation radiotherapy.

A meta-analysis of studies comparing hyperfractionated to conventional radiation[8] determined that hyperFRT ultimately has significant survival benefit despite mixed results from earlier trials. HyperFRT increased 5-year OS by 2.5% (P = 0.009) over conventional fractionation and decreased the risk of death by 12% (P = 0.02). However, hyperFRT did not significantly improve PFS, and was associated with higher toxicities compared to conventionally fractionated radiotherapy. While hyperFRT regimens may be superior to conventionally fractionated radiotherapy, the cost of greater toxicity, particularly severe esophagitis, and logistics of treating patients multiple times per day has prevented its wider adoption in a clinical setting.

Hypofractionation (hypoFRT) delivers a higher dose per fraction compared to conventional fractionation schedules. The overall delivered dose is lower than conventional fractionation schemes, but tumor repopulation may be outpaced with greater tumor cell kill per fraction. HypoFRT is potentially able to deliver higher biologically equivalent dose to provide better local control[102,109]. Hypofractionation also offers advantages of less total fractions and less machine time per patient. In a pilot study[116] of 59 stage IIIA/B patients treated with 75 Gy in 28 daily fractions (2.68 Gy/fraction) over 5.5 wk, patients had a MS of 10 mo, and a 3- and 5-year OS of 18% and 4%, respectively. Only three of 59 patients experienced severe late complications from therapy, suggesting that hypoFRT is an acceptable and tolerable regimen. A randomized control trial[117] compared conventional RT (60 Gy in 2 Gy fractions over 6 wk) to hypoFRT (60 Gy in 5Gy weekly fractions for 12 wk). One- and two-year OS were 49% and 23% in the conventional RT arm, and 59% and 29% in hypoFRT arm respectively. These survival rates were not statistically significantly different from each other, but agree with previous reports. Local failure and response rates from hypoFRT were similar to conventional RT as well, thus suggesting hypoFRT is as efficacious as conventional RT but not superior. The EORTC 08972-22973 trial[61] tested the efficacies of sequential gemcitabine/cisplatin *vs* hypoFRT or concurrent cisplatin and hypoFRT therapies. While the trial was underpowered to detect any significant difference, OS and toxicity rates favorably trended towards concurrent arm of the trial. Two-year OS rates for patients treated with sequential chemoradiation is 34% while those in concurrent chemoradiation arm is 39% survival rate. MS for the sequential and concurrent arms are 16.2 and 16.5 mo respectively. The SOCCAR phase II trial[101] also tested sequential *vs* concurrent cisplatin/vinorelbine with hypoFRT. The primary endpoint of this trial was treatment-related mortality, which occurred in 2.9% and 1.7% of patients on concurrent and sequential arms, respectively. The rate of Grade 3 or worse esophagitis was similar between the two arms, as were 2-year OS, median survival, 1-year PFS rates, and median PFS. This trial demonstrated that hypoFRT given with full dose chemotherapy has similar outcomes to previous trials and had a low, acceptable treatment-related mortality rate. Table 4 summarizes key prospective trials evaluating hypoFRT fractionation schedules for NSCLC treatment.

Intensity modulated radiotherapy (IMRT) delivers radiation using inverse computer planning to determine multiple intensity levels across varying beam shapes, which has allowed for improved homogenous and conformal dose distributions for complex target volumes while sparing critical adjacent structures. While there is a hypothetical advantage of reducing toxicity by reducing dose to normal tissue compared to 3D-CRT, there has been no prospective evidence to guide when to use IMRT for select NSCLC patients. There have been concerns with using IMRT which have limited its adoption. It can expose a larger volume of lungs to low-dose radiation, which is often associated with pneumonitis[118]. Additionally, there are uncertainties regarding the delivery of radiation related to multi-leaf collimator movement and respiratory-related tumor motion[119]. These concerns lack convincing evidentiary support. There have been several retrospective institutional studies reporting improvements in overall dosimetry and rates of toxicity with IMRT. Notably, a review of 151 NSCLC patients treated from MD Anderson Cancer Center compared rates of treatment-related pneumonitis among patients treated with 3D-CRT *vs* IMRT[118]. While patients treated with IMRT had more advanced disease, debilitated performance status, and larger median gross tumor volume, rates of Grade 3 or higher treatment-related pneumonitis at 1-year was 8%, compared to 32% observed for patients treated with 3D-CRT (P = 0.002). IMRT also significantly reduced V20 doses compared to 3D-CRT (P < 0.001). RTOG 0617[82] included patients treated with IMRT. Planned secondary analyses for survival outcomes, toxicities, and quality of life from RTOG 0617 trial were done. IMRT had comparable OS and PFS to 3D-CRT[120]. However, IMRT was associated with significantly higher lung V5, while having lower lung V20 (P = 0.08) and heart doses at V5,

Table 3 Prospective trials for hyperfractionated radiation schedules for non-small-cell lung cancer treatment

Ref.	Phase	Study design	Chemo regimen	RT	No. of patients	Stage	Median f/u (mo)	OS	Median OS (mo)	Response rate	Toxicity
RTOG 83-11 Cox *et al*[106] (1990)	1 and 2	Randomized 1 of 5 dose groups: 60, 64.8, 69.6, 74.4, 79.2 Gy	None	Dose delivered in 1.2 Gy twice daily fxns	848	III	N/A	2-yr, 29% (69.6 Gy arm)	13 (69.6 Gy arm)		Risk for severe/ life-threatening pneumonitis- 2.6% (60 Gy), 5.7% (64.8 Gy), 5.7% (69.6 Gy), 8.1% (74.4 Gy)
RTOG 8808/ ECOG 4588 Sause *et al*[107] (2000)	3	Conv. RT + chemo *vs* hyperFRT *vs* conv. RT	Cisplatin/ vinblastin	Conv RT: 60 Gy in 2 Gy daily fxns HyperFRT: 69.6 Gy in 1.2 Gy twice daily fxns	458	II-IIIB, unresectable	> 60	5-yr, 8%, 6%, 5%	13.2, 12, 11.4		6 G4+ RT-related toxic events-4 of them in hyperFRT arm
RTOG 9410 Curran *et al*[51] (2010)	3	Sequential chemoRT (conv., arm 1) *vs* concurrent chemoRT (conv., arm 2) *vs* concurrent chemoRT (hyperFRT, arm 3)	Cisplatin/ vinblastine (arms 1 and 2) Cisplatin/ etoposide (arm 3)	Conv: 63 Gy in 1.8 daily fxns HyperFRT: 69.6 Gy in 1.2 Gy twice daily fxns	610	II-III, inoperable	132	5-yr, 10%, 16%, 13%)	14.6, 17, 15.6	ORR- 61%, 70%, 65%	G3+ acute esophagitis- 4%, 22%, 45% No difference in G5 toxicities
Saunders *et al*[112] (1999)		CHART *vs* conv. RT	None	Conv RT: 60 Gy in 2 Gy daily fxns HyperFRT: 54 Gy in 1.5, 3 x daily fxns, for consecutive days	563	III	> 48	2-yr, 29% *vs* 20% (P = 0.004) 2-yr, 33% *vs* 19% if SCC			Severe dysphagia, 19% *vs* 3%
ARO 97-1 Baumann *et al*[113] (2011)		CHARTWEL *vs* conv. RT	None	Conv RT: 66 Gy in 2 Gy fxns for 6.5 wk CHARTWEL: 60 Gy in 1.5, 3 x daily fxns for 2.5 wk	460	I-IIIB	40.8	2-yr, 31% *vs* 32% 3-yr, 22% *vs* 18% 5-yr, 11% *vs* 7%			Higher incidence of acute dysphagia with CHARTWEL
INCH trial Hatton *et al*[114] (2011)		Induction chemo + CHART *vs* CHART alone	Cisplatin/ vinorelbine	54 Gy in 1.5 Gy fxns (3 x daily) for 12 consecutive days	46	I-III, inoperable	33		25 *vs* 17		G3/4 adverse effects 65% *vs* 57%
ECOG 2597 Belani *et al*[115] (2005)	3	Induction chemo + conv. RT *vs* induction chemo + CHART	Carboplatin/ paclitaxel	Conventional RT: 64 Gy in2 Gy fxns (daily) 57.6 Gy in 1.6 Gy fxns (3 x daily) for 15 d	141	IIIA/B, inoperable	> 36	2-yr, 24% *vs* 44% 3-yr, 14% *vs* 34%	14.9 *vs* 20.3	ORR, 22% *vs* 25%	Acute esophagitis 16% *vs* 25% G3/4 acute pulmonary toxicity observed in conventional RT arm
Hatton *et al*[100] (2016)	1	Randomized 1 of 4 dose groups: 54, 57.6, 61.2, 64.8 Gy	None	Each dose group delivered in 1.8 Gy, 2-6 fxns daily	18	IIIA/B	21	2-yr, 49% (entire cohort)	24 (entire cohort)	ORR, 61% (entire cohort) CR, 28% (entire cohort)	G3/4 adverse effects in 6 of 18 patients No dose-limiting toxicities

SCC: Squamous cell carcinoma; OS: Overall survival; RT: Radiotherapy.

V20, and V40. V20 was ultimately predictive of grade 3 pneumonitis. Rate of Grade 3 or higher pneumonitis was 2 fold lower among patients treated with IMRT (3.5%) *vs* 3D-CRT (7.9%) despite that patients with IMRT

Table 4 Prospective trials for hypofractionation radiation schedules for non-small-cell lung cancer treatment

Ref.	Phase	Study design	Chemo regimen	RT	No. of patients	Stage	Median f/u (mo)	OS	Median OS (mo)	Response rate	Toxicity
RTOG 8312 Graham *et al*[116] (1995)	Pilot	HypoFRT	None	75 Gy in 2.68 fxns daily for 5.5 wk	59	IIIA/B		1-yr, 41% 2-yr, 25% 3-yr, 18% 5-yr, 4%	10		Most common was G1/2 pulmonary fibrosis and pneumonitits
Slawson *et al*[117] (1990)		Conv. RT *vs* HypoFRT		Conv. RT: 60 Gy in 2 Gy fxns (daily) HypoFRT: 60 Gy in 5Gy fxn (weekly)	150	Locally advanced, unresectable	36	1-yr, 49% *vs* 59% 2-yr, 23% *vs* 29%		CR, 17% *vs* 26%	No difference for later reactions
EORTC 08972-22973 Belderbos *et al*[61] (2007)	3	Sequential *vs* concurrent chemo + hypoFRT	Gemcitabine/ cisplatin	66 Gy in 2.75 Gy fxns in 32 d	158	I-IIIB, Inoperable	39	2-yr, 34% *vs* 39% 3-yr, 22% *vs* 34%	16.2 *vs* 16.5		G3 hematological toxicity higher in sequential arm (30% *vs* 6%) Esophagitis more common in concurrent arm (5% *vs* 14%)
SOCCAR Maguire *et al*[101] (2014)	2	Sequential *vs* concurrent chemo + hypoFRT	Cisplatin/ vinorelbine	55 Gy in 2.75 Gy fxns over 4 wk	130	III, inoperable	N/A	2-yr, 46% *vs* 50%	18.3 *vs* 24.3		G3+ esophagitis 8.5% *vs* 8.8% Tx-related mortality, 1.7% *vs* 2.9%
Liu *et al*[126] (2013)		Concurrent chemo + HypoFRT dose escalation	Carboplatin/ vinorelbine	60-75 Gy in 3 Gy fxns for 5 wk	26	IIIA/B, unresectable	11.5	1-yr, 60.9%	13	CR, 26.9% Partial, 53.8% Stable, 19.2% ORR, 80.8%	Acute esophagitis, 88.5% (G3 = 15.4%) Pneumonitits, 42.3% (G3 = 77%)
Lin *et al*[127] (2013)	1	Concurrent chemo + hypoFRT dose escalation	Carboplatin/ vinorelbine	60-72 Gy in 3Gy fxns for 5 wk	13	IIIA/B, unresectable	10			CR, 23.1% Partial, 15.4% Stable, 15.4% ORR, 84.6%	4 instances dose-limiting toxicities, all occurring in 72 Gy arm
Kim *et al*[128] (2013)		Concurrent chemo + hypoFRT IMRT dose escalation	Cisplatin/ vinorelbine	48 Gy in 2.4 Gy fxns with boosts of 16.8 Gy/7, 20 Gy/7, or 22.7 Gy/7	12	II-IIIB, unresectable	22	1-yr, 58.3%	12.7	CR, 75% Partial, 33% Stable, 25%	No G3 acute or late radiation-toxicities

HypoFRT: Hypofractionation; IMRT: Intensity-modulated radiotherapy; CR: Complete response.

had more advanced stage disease and larger PTV to lung ratios compared to those treated with 3D-CRT[120]. Quality of life at 12 mo was significantly higher for patients treated with IMRT than those with 3D-CRT[121]. In an attempt to identify patients who may derive a survival benefit from IMRT over 3D-CRT, Jegadeesh *et al*[119] used the National Cancer Data Base to analyze stage III NSCLC treated with chemoradiation for curative intent. This analysis suggested that patients with T3 and T4 disease are associated with improved median survival (17.2 and 14.6 mo respectively) and 5-year OS (19.9% *vs* 13.4% respectively). T stage and treatment time was significantly associated on multivariate and propensity-matched cohort analysis. With such promising results,

a prospective randomized trial comparing IMRT and 3D-CRT for NSCLC is needed.

Proton therapy for the treatment of NSCLC is under active research. Protons have characteristic energy "Bragg peaks", which limit exit dose into adjacent tissues[122]. This unique feature could reduce the irradiated volume of normal tissues, such as the heart, normal lungs, esophagus, and spinal cord, relative to photon dose distributions. This may limit toxicity to allow improved tolerance of relatively higher doses than photon radiation. Proton therapy from single-institution reports have delivered 74 cobalt gray equivalent (CGE) with concurrent chemotherapy for locally advanced NSCLC. In various small trials and single-institution reports, MS typically ranged from 26.7 to 30.4 mo[99,123,124], which was longer compared to that achieved in RTOG 0117 trial which delivered 74 CGE with conventional photon RT. Local recurrences range from 5.5% to as high as 40%[99,124,125], and development of distant metastases is still difficult to control as up to 45% of patients experience distant progression[123,124]. Toxicity rates were expectedly lower compared to those experienced at 74 Gy with conventional photon RT from RTOG 0117 trial[124]. Results of RTOG 1308, a phase III randomized trial comparing overall survival outcomes after photon *vs* proton chemoradiation for inoperable stage II-IIIB NSCLC patients, is anticipated.

CONCLUSION

Locally advanced stage III NSCLC continues to be a deadly disease, and consists of a heterogeneous patient population. Generally, treatment requires combined modalities that address local disease control, with surgery or radiation, and control of systemic spread with chemotherapy. Several combinations and various sequences of systemic and local therapies have been investigated with similar or conflicting outcomes making determination of the optimal management for these patients challenging. Multiple strategies have been developed in order to maximize survival through improved disease control through treatment intensification; however, disease progression treatment-related toxicities continue to limit survival. For patients with resectable disease, surgery offers highest rates of local control. With new awareness of chemotherapy and radiation, the role of surgery as well as disease staging are being evaluated. Multi-modality therapy is playing an increasingly important role for both resectable and unresectable stage III patients. Concurrent chemoradiation remains the standard of care in the management of inoperable or unresectable patients. In an effort to maintain or improve outcomes with less toxic effects, 3rd generation chemotherapy agents have been studied and incorporated into treatment. Targeted therapy, immunotherapy, and other non-cytotoxic drug therapies are also being investigated, and may play a greater role in the future. While dose escalation with conventional RT has not proven to improve treatment outcomes, alternative fractionation, particularly hypofractionation, may play a larger role in future management. IMRT and proton radiotherapy provides an opportunity to provide higher radiation doses with less toxicity. Future work will be needed to exploit biological tumor differences and integrate advancements in radiation technology.

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REVIEW

Three-dimensional bio-printing: A new frontier in oncology research

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Abstract

Current research in oncology deploys methods that rely principally on two-dimensional (2D) mono-cell cultures and animal models. Although these methodologies have led to significant advancement in the development of novel experimental therapeutic agents with promising anticancer activity in the laboratory, clinicians still struggle to manage cancer in the clinical setting. The disappointing translational success is attributable mainly to poor representation and recreation of the cancer microenvironment present in human neoplasia. Three-dimensional (3D) bio-printed models could help to simulate this micro-environment, with recent bio-printing of live human cells demonstrating that effective *in vitro* replication is achievable. This literature review outlines up-to-date advancements and developments in the use of 3D bio-printed models currently being used in oncology research. These innovative advancements in 3D bio-printing open up a new frontier for oncology research and could herald an era of progressive clinical cancer therapeutics.

Key words: Cancer; Three-dimensional bio-printing; *In vitro*; *In vivo*; Biomaterials

Core tip: This review highlights the recent advancements in three-dimensional (3D) bio-printing in the field of oncology research and how the use of 3D bio-printed models can revolutionise and accelerate the development of new cancer therapeutics for human use.

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INTRODUCTION

Cell culture and animal models are the accepted evaluative methodology in all types of preclinical studies, including oncology research. These models have contributed a lot to the overall understanding of the pathological mechanisms of several diseases including different types of cancers, however, their value in predicting the effectiveness of treatment options and strategies in clinical trials have remained doubtful[1,2]. Apart from the ethical controversies; lead by the animal activist, the main problems with animal models lays in the species differences when compared with human. These species differences often causes misleading interpretation[3]. In fact, clinical trials are mandatory because preclinical studies on cell culture and animal models do not envisage with sufficient confidence the likely outcomes in human studies.

In oncology research, due to the ethical concerns associated with human experimentation, animal models and cell culture studies have become an important source of information. However, the average rate of successful clinical translation from animal models to clinical trials are not very encouraging; at present not more than 8%[4]. Animal models have the restricted ability to mimic the complex process of human cell proliferation and pathophysiology conditions. In oncology research, studies on cell culture and animal models are critical instruments in determining the efficacy, pharmacodynamics and mechanism of action of novel anti-cancer drugs. It should be remembered that heterogeneity of the tumour cells leads to the huge diversity with a high degree of genetic instability and phenotypic variation.

Prior to plunge into the trial of a promising anticancer drug, pharmaceutical companies and institutional investigators conduct wide pre-clinical experimental studies. *In vitro* and *in vivo* studies preliminary covers safety, efficacy, toxicity and pharmacokinetic profiles of the candidate molecules. Early *in vivo* testing aims specifically to provide initial safety and efficacy data to supports investigators claims about compound under investigation. To justify further development, preclinical experiments add sufficient confidence to the research data. This is important because as per the Food and Drug Administration guidelines, successful animal need/preclinical testing have to be completed before humans are exposed to the potential therapeutic entity[5].

Apart from possible misleading *in vitro* results, relating to inaccuracies in potency, efficacy, toxicity, genotoxicity and carcinogenicity, the financial cost of clinical research also plays a decisive role in the development and establishment of the successful therapeutics. Given that three-dimensional (3D) bio-printed structures could produce better models of the *in vivo* microenvironment, there is the significant potential for cost reductions in pre-clinical research. The 3D bio-printed tissues and organs have the capacity to provide viable substitutes to cell cultures and animal models. The 3D printing of solid objects is already guiding major innovations in diverse areas, such as education, manufacturing, engineering, art, pharmaceuticals and medicine[6]. Recent innovation in 3D printing and material science have enabled construction of complex 3D functional living constructs (tissues and organs)[6]. Without worrying about the rejection, 3D bio-printing has already been used for the generation and transplantation of several important tissues including, bones, skin, heart tissue, *etc*. Other lucrative applications include developing more reliable 3D bio-printed tissue models for pharmaceutical and drug discovery research. Accurate reproduction of the tissue or an organ is a significant feature of the 3D bio-printing which ultimately could lead to the standardisation of therapeutic testing[7]. This is possible to achieve by reproducing all the functional components of the tissues and organs, such as mimicking the exact branching patterns of the tinniest capillary in a complex organ like the heart, kidney, liver and lungs, or manufacturing the biomaterials to take care of the natural physiology.

PRECLINICAL *IN VITRO* MODELS AND THERAPEUTIC DEVELOPMENT

New drug development programmes generally take about 12 years to get an experimental lead compound to the patient bedside. The average cost involved in this process can be as high as exceeding $1.2 billion dollars[8,9]. The drug development process is highly risky in terms of economic gain; evident by an overall average attrition rate of approximately 90%, which means that only 10% of clinical trial compounds could finally reach to the market[10]. Consequently, scientists are now putting greater efforts in reducing the cost of the drug development process. Computer aided drug design[11], *in silico* pharmacokinetics[12] and toxicity testing[13] are few of the newer methodologies available, which could reduce the initial cost of the drug development process.

Accurate preclinical determination of efficacy and toxicity would lower the failure rate of new molecules during the important stage of clinical evaluation. Drug testing on 3D bio-printed human organs could eliminate the possibility of drawing uncertain conclusions from preclinical animal and cell culture studies. Conflicting conclusions from preclinical animal models and human experiments usually surface during the final stage of the clinical trials, when most of the resources have already been invested in the research and development process. Several promising lead candidates have faced failures in clinical trials after successful animal testing[14-19]. Preventing these problems in the first place would improve the cost and time involved in bringing a new drug to the market. To accurately predict the unwanted parameters of the drug candidates in clinical trials, various classical, existing and emerging technologies (models) are available. This comprehensive list includes

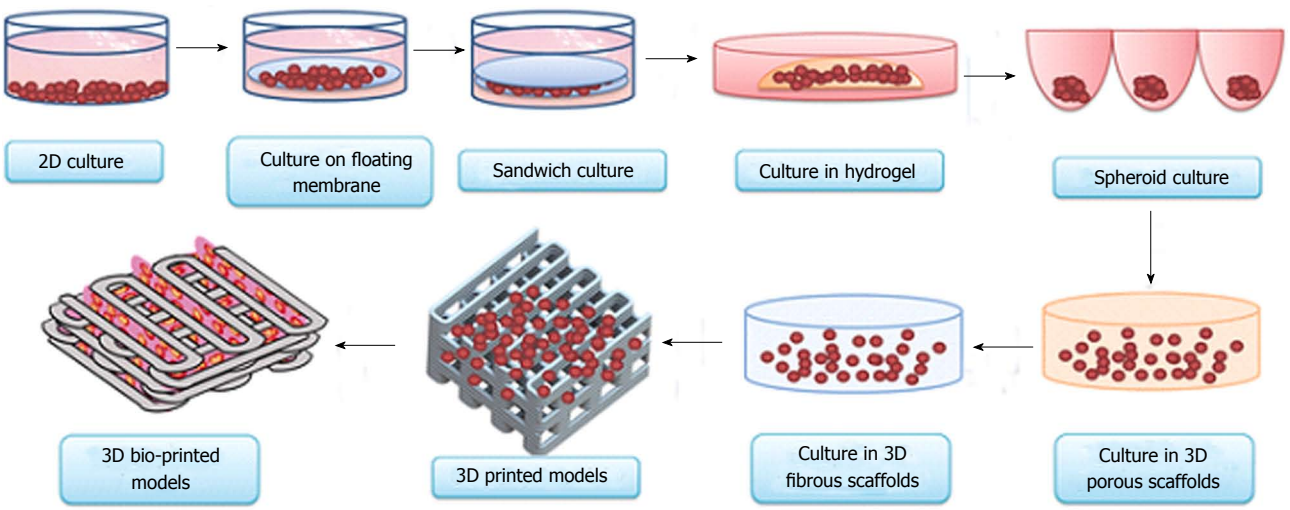

Figure 1 Evolution of cell-culture models from simple two-dimensional to complex three-dimensional bio-printed models. Currently, 3D bio-printing is the most sophisticated technique used to make tissue/organ constructs[65]. 3D: Three-dimensional.

traditional 2D tissue culture[20], classical whole rodent models[21], humanised mouse models[22], 3D culture models[23], co-culture systems[24] and 3D tissue models[25] (Figure 1).

Traditional 2D cell culture systems which employ cell lines in a single layer, themselves contain abundant genetic mutations. 2D cell culture systems also lack the important natural microenvironment present in the tissues and organ from which they were originally seeded[26]. Traditional culture performed with primary cells do not offer 3D microenvironmental characters similar to that of its origin[27]. Classical cell culture systems not only lack the influential tissue microenvironment and gradient but may also include the rapid loss of important proteins and its functions and gene expression profiles. To get a better representation of tissue complexity, microenvironment and whole-body physiological impact, studies on the animal model have become the backbone of preclinical studies. However, as discussed earlier, basic molecular, physiological and pathophysiological differences between the species lead to the likelihood of erroneous conclusions being drawn about an under trial candidate. Erroneous conclusions are the leading cause of failures in clinical trials.

Co-culture systems, 3D culture models, 3D tissue models and humanised mouse models which could mimic the host microenvironment are available for preclinical studies. To some extent, these methodologies allow drug testing in human-like systems, eliminating the species differences and, thereby, increasing acceptability in clinical trials. Developing pharmacological assays based on configuring multiple cells into a 3D-orientated structure could provide more realistic data. The 3D cell culture and models could mimics native tissue architecture more closely and hence could address drug development concerns in a more actual ambience than traditional 2D culture models.

Humanised mice model is another approach to testing drugs in more human-like conditions. This type of the animal models include mice bearing tumours derived from humans, known as xenografts or mice in which the endogenous liver has been compromised and repopulated with human liver cells[22]. Xenografts are important and proving useful in anticancer drug development. Xenografts often enable the assessment of drug efficacy, safety and toxicity in the context of tumour phenotypic and genotypic heterogeneity. Similarly, mice with humanised liver offer the ability to assess drug pharmacokinetics and metabolism preclinically *in vivo*. Humanised liver is an important tool to understand drug excretion and toxicity[28]. One important thing to remember about all humanised models is their chimeric nature. They are a single human tissue or cell type planted within the animal body, which may lead them to behave differently from their native environment. This may propagate false interpretation due to inter-species variations. For example, the stromal and vascular components of xenograft models largely come from an animal in origin[29]. Similarly, mice with humanised livers contain human hepatocytes, however, the other cell types found in the liver and all of the interrelated organ systems are of mouse[30] which ultimately could affect the liver functions. Hence, such models cannot be considered as the perfect model for human systems modelling. However, as stated earlier, humanised mouse models are a popular model in the study of human cancer. They provide an understanding of factors involved in pathology, physiology, metastasis and invasion.

In xenograft models, human tumour cells are transplanted into a different species, either into the organ type in which the tumour originated or under the skin. Human tumour cells are commonly transplanted into the mice which are severely immunocompromised. The weak immune system of such mice accepts foreign human cells readily. For example, the xenograft (foreign

cells or organ) will be readily accepted by athymic nude mice (lacking T cells producing thymus), severe combined immunodeficiency mice strains, or other immunocompromised mice[31,32]. Therapeutic agents can be studied in these immunocompromised mice as it readily allows the growth of human tumour within itself. The size of the tumour is generally depends on upon the number of cells originally transplanted, however, growth occurs over 1 to 8 wk to give more natural humanised environment. Genetically engineered mouse (GEM) model is another type of widely used animal model used for studying human cancer.

GEM mouse model allows the investigator to study the genes which are speculated to be the reason of the malignancy. Such genes are deleted, silenced or sometimes overexpressed and the animal is observed for the molecular and phenotypical changes over the period of time to study the therapeutic response. GEM provides an opportunity to study the therapeutic response *in vivo.* Xenograft models and immunocompromised athymic nude mice have been in used for several decades to increase our understanding of pathophysiological and genetic factors involved in uncontrolled cell proliferation and metastasis. Recent information about the role of the microenvironment on the tumour progression, growth and resistance towards the drugs has made GEM and primary human xenografts in humanised mouse models a primary choice for the experiments. However, because of the species difference, xenografts of human cell lines in mice to test drug responses do not always necessarily correlate with the actual pathophysiological condition in patients[29].

The importance of the tumour microenvironment on tumour growth not only leads to the general acceptance of the humanised mouse models and GEM for the development of the cancer therapeutics but also paved ways for the development of 3D printed tissues and organs in oncology research. The 3D culture and co-culture systems already exist and recent refinement increases their availability for therapeutic research. Certain drawbacks, such as low cell density, and use of artificial matrices and scaffolds add a non-human or non-native aspect to the system, which could affect the final outcome. However, more recent approaches that generate 3D culture systems, such as 3D bio-printing, could help nullify the non-human aspect.

3D BIO-PRINTING

The 3D bio-printed tissues and organs could be designed to mimic the exact cellular density of target tissues and organs, with proper consideration given for cellular component, extracellular matrix and three-dimensional spatial components. Since complex tissues are not constructed exclusively from a single cell type, 2D mono-cell culture models are of debatable value[33]. However, 3D bio-printers deposit more than one cell type, co-culturing them in one single spatial arrangement making them a closer match for the natural architectural arrangement. With the recent advancement in bio-printing, it is now feasible to combine the most important elements of spatial patterning to generate 3D *in vitro* tissue/organ systems that could mimic the key cellular and extracellular functional machinery, including innervation[33]. The 3D printers use various types of cells in the form of bio-inks, which technically have enhanced the speed of 3D printing of organs and tissues. The 3D organ scaffold generated with the help of computed tomography or another imaging technology and the solid surface made up of the biocompatible materials is used as the substrate to generate the 3D tissues and organs. Bio-inks are made up of cells suspended in a biocompatible gel-like material then deposited on the substrate using 3D printers which work on the principal like mechanical extrusion[33]. During and after deposition on the substrate the bio-ink is gelled by polymeric inter-linking with the help of photo or thermal activation. Because of the involvement of the high energy, care is always taken to leave the cells intact and functional. Hydrogels not only play an important role in physically restraining the suspended cells and in the maintenance of the cell viability but also can be personalised and tailored according to the biocompatible material or dimensions[33].

The development of aqueous-based systems enabled direct printing of bio-inks into 3D scaffolds[34]. Sequential deposition of the living cells, biocompatible extracellular and materials with spatial control over the 3D architectural parameters is the heart of the 3D bio-printing and 3D bio-printed organs. The 3D bio-printing works on the several established principals based on bio-mimicry, autonomous self-assembly and mini-tissue building blocks[6].

Technological advancement in imaging and digital design technology has positively impacted the 3D bio-printing by reproducing and visualising the very complex, heterogeneous architecture of complex tissues and organs. Non-invasive imaging techniques, like computed tomography, magnetic resonance imaging, computer-aided design and computer-aided manufacturing tools and mathematical modelling, are used to collect and digitise the complex tomographic and architectural information of the tissues/organs. The 3D digital images of complex organs are then used to print tissues and organs using techniques like inkjet[35-38], micro-extrusion[39-41] and laser-assisted printing (Figure 2)[42-44].

The 3D printing technologies first became prominent in non-biological applications, such as the deposition of ceramics, metals and thermoplastic polymers in heavy and light industries. Organic solvents, high temperatures and cross-linking agents (*e.g.,* photo-activation) used in 3D printing poses immediate compatibility problems for delicate living cells, thermal liable biological (*e.g.,* proteins) and biocompatible materials[6,45,46]. Among several, one of the main and dare challenges in the

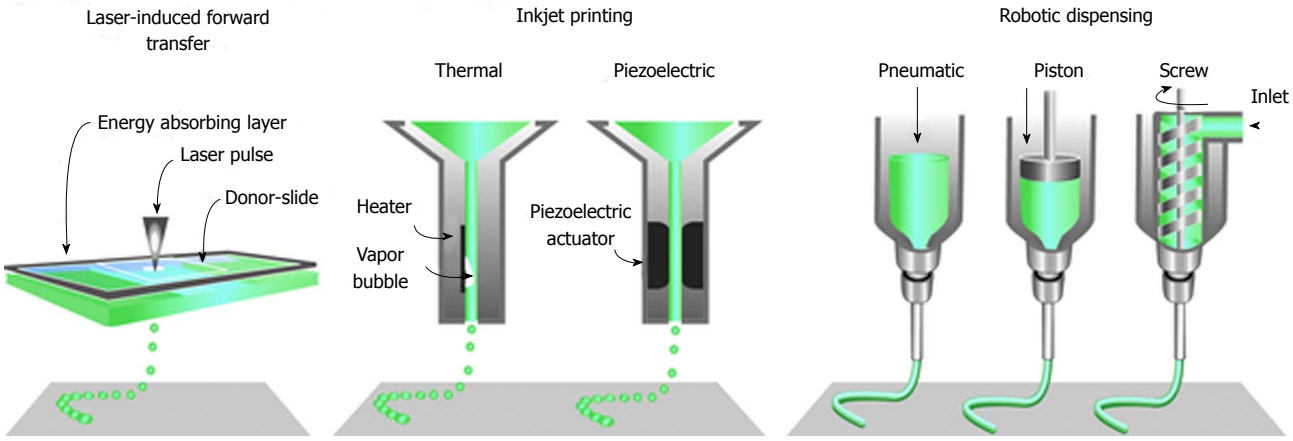

Figure 2 The common approaches currently used to bio-print tissue, are laser-assisted, inkjet-based and extrusion-based robotic dispensing techniques[110].

3D bio-printing of tissues and organs is to develop the compatible materials that not only should go well with the several other biological materials and the harsh printing process but should also provide the required mechanical and functional properties to the 3D bio-printed constructs. Materials currently used in the field of regenerative medicine are based on either natural polymers (*e.g.,* alginate, gelatin, collagen, chitosan, fibrin and hyaluronic acid *etc.*) or synthetic molecules (*e.g.,* polyethylene glycol). Some of the major advantages of the natural polymers in 3D bio-printing are its similarity to the human extracellular matrix, non-toxic nature and inherent bioactivity. Whereas the typical advantage of the synthetic polymers is that they can be personalised and tailored to the specific application and can also be obtained in the most purified form. But like other synthetic molecules, synthetic polymers not only possess the risk of the poor bio-acceptability but could also lead to the toxicity because of the toxic degradation. Other challenges could be the loss of the mechanical strength over the period of time and immunogenicity. Despite this, synthetic hydrogels polymers owing to its hydrophilic, absorbent and manageable physical and chemical properties are an attractive alternative in 3D bio-printing. The correct functioning of the 3D fabricated tissue or organ does not only depend on upon the accurate deposition of the cells but the choice of the cells is also crucial. Other criteria need to be satisfied is that the cell chosen for 3D bio-printing should have the capability to proliferate of its own. Precise control of cell proliferation (*in vitro* and *in vivo*) ensures the functionality of the construct. In addition to the primary cell of interest (*e.g.,* hepatocytes in liver construct), most tissues also contain other cell types that are involved in supportive, structural or barrier functions (selective transport) (*e.g.,* liver also contains sinusoidal endothelial cells and phagocytic Kupffer cells) and may also be involved in vascularization or may play role in stem cell maintenance and differentiation.

Presently, 3D bio-printing involves the deposition of multiple primary cell types into patterns that accurately represent the native tissue. In the case of the auto-rearrangement and self-assembly to the 3D construct, printing involves the bio-ink of the stem cells that can proliferate and differentiate into the required cell types. Maintenance and exact mimicking of the physiological function of cells in 3D construct are important and hence the criteria applied for selecting the cells plays the decisive role in proper functioning[47].

Rejection by the host immune system is the challenge in the tissue and organ transplant. This issue can be sort out by using the autologous cells for 3D bio-printing of organs and tissues. Autologous cell source involves biopsies, generation and differentiation of autologous stem cells or induced pluripotent stem cells. Although autologous cells are the very reliable source, it's of no use in case if the patient is already ill, cells are infected or have metabolic or hereditary disorders. In such cases, especially in the case of genetic disorder, 3D construct is not useful for the transplant but could be useful in case of therapeutic development (*e.g.,* genetic mutation in cancer cells will be useful to construct 3D bio-printed tumour model). In the case of the metabolic disorder, autologous cells may not be able to produce the normally desired function in bio-printed organs.

Prolong functionality of any 3D bio-printed tissues and organs are the key to the success. However, cells types like heart, liver and immune cells are not only difficult to isolate from the source but is also difficult to culture in a lab because of their limited lifespan[48]. Self-renovating, ability to differentiate into any cell type and capability to generate multi-functional tissue-specific cell phenotypes is the solution for such problems. Embryonic stem cells and induced pluripotent stem cells have all these characters and hence are the promising cell types for 3D bio-printed organs and tissues[49]. The 3D bio-printed organs require the self-renovating or self-replenishing character to maintain the functionality, in this regard pluripotent stem cells ability to multiply several times highlight its potential in 3D bio-fabricated

construct. Other types of stem cells, such as stem cells from bone marrow[50-52] and fat[53] or perinatal stem cells from amniotic fluid[54] or placenta[55], have limited multi-potent differentiation ability. These cell types but are considered safer for 3D bio-printed construct. These cells also satisfy the criteria of the autologous cell types and hence have the potential application in regenerative medicine. Mesenchymal stromal cells (MSC) are also a good cell source but its Isolation is difficult. However, the establishment of the new protocols for isolation, expansion and differentiation now make them the reliable and promising source for bio-fabricated constructs. Clinically required amount of MSC has been effectively generated *in vitro* and have found application in clinical trials and regenerative medicine[50-52]. Future development in biotechnology and cell-culture techniques is likely to be useful to exploit other stem cell populations for bio-printing and regenerative medicine; this is not just a hypothesis but a potential possibility.

3D PRINTING IN PRE-CLINICAL TESTING AND THERAPEUTIC DEVELOPMENT OF ANTI-CANCER DRUGS

Therapeutic drug development and therapy optimisation experiments in genetically modified mouse, 2D cell culture, 3D co-culture and xenografts of human tumour cells into nude mice are the important tool and have immensely contributed in the oncology research[31,56,57]. Physiologically, tumour microenvironment is extremely complex in which genetically mutant and phenotypically proliferative cancerous cells not only interact with each other but also reciprocally interact with the stromal and immune system microenvironment[58]. Modelling the heterogeneous complexity of a typical tumour using 3D bio-printed tissues and organs for preclinical testing could be an innovative and novel approach for the pre-clinical testing and therapeutic development of anti-cancer drugs.

Determination of the efficacy, toxicity, pharmacodynamics, pharmacokinetics and mechanism of action are the critical studies towards the development of efficient anti-cancer therapeutics. Cell culture and animal studies have played important roles in this process. Tumour cells and host microenvironment interaction leads to the recruitment of the components essential for the inflammatory and immune signalling. This recruitment of the signalling components is preceded by the fibroblasts and endothelial cells activation. The microenvironment of the host tumour is modified to select and adapts the genetic and phenotypic characters of the tumour cells. In fact, the modified microenvironment of the host organ in cancer pathology ultimately helps in the growth of the tumour cells. This reciprocal interaction between tumour cells and the microenvironment is actually essential for tricking the immune system, proliferation and metastasis[59]. Host microenvironment not only subjected to the different environmental stimuli but if looked from the population perspective it is genetically and phenotypically so diverse that the same tumour will grow and behave differently in different physiological condition (different patient). Simulation of such huge diversity (thousands of genes) in 2D cell culture and in animal models to test the toxicity and efficacy of drug candidate is the mammoth task. Essentially, it is impossible to extrapolate the results obtained from single or two test models to the numerous tumour variants in a broad genetically heterogeneous population.

Cell cultures derived from the human tumour cell line only offers the advantage of the biology to the primary tumour but it cannot simulate or mimic the complexity involved in the interaction between the proliferating tumour cells and microenvironment. Xenografts in immunocompromised mice interact with the surrounding cell types which are different from the native cell types and hence grafted tumour cells could behave differently in mice. Overall, the xenografts mice models have added limited value to the 2D cell culture. Similarly, lack of working the immune system and insufficient interactions between the human tumour cells and human stromal cells do not essentially represent the human tumour microenvironment.

Organovo is now an early-stage but established medical research company, which designs and develops functional 3D human tissues and organs for medical and pharmaceutical research and therapeutic development. The main focus of this innovative company is to speed up the preclinical and clinical drug testing by bio-printing human tissues and organs which mimics the human organ *in vitro*. The 3D bio-printed constructs enable the researcher to develop treatments and therapeutics faster, at very low cost and without risk to the living subjects. To assist the drug development process, Organovo now associated itself with biopharmaceutical and pharmaceutical companies and renounced academic medical research centres to design, build, standardised and validate more human-like *in vitro* tissues for disease simulation and drug, efficacy and toxicity testing.

The 3D bio-printed tissues and organs printed form human/autologous cells theoretically provides similar microenvironment as that of tissues and organs inside the body. Individual cells of the 3D construct experience the similar microenvironment as that of the tissues of the body. This provides an opportunity to the researcher to carry out the drug testing experiments *in vitro* in living tissues and organs. This also eliminates the possibility of the testing of drugs in living human subject; thereby bridging the gap between preclinical experiments and clinical trials.

Organovo's bio-printed tissues are created from human cells. Bio-printed construct recreates various biological aspects *in vitro*, *e.g.,* microenvironment and biology, reciprocal interactions between cells and micro-environmental factors and simulation of original tissue extracellular matrix including extracellular electrolytes. Organovo's exVive3D™ bio-printed human tissues may

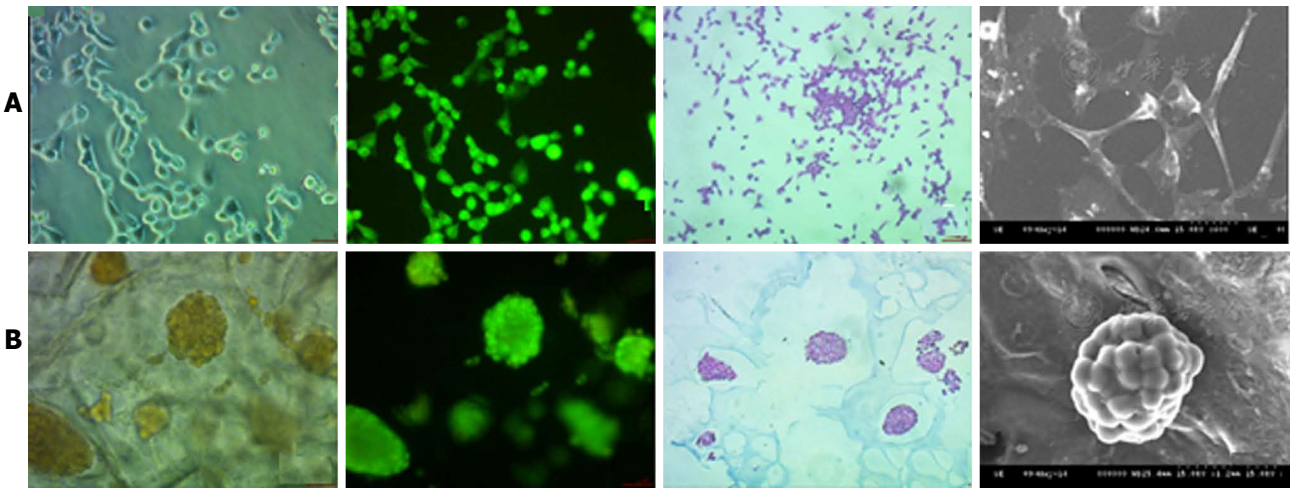

Figure 3 Nonsmall-cell lung cancer 95D cell morphology under two-dimensional and three-dimensional culture conditions. The 2D cultured cells (A) are tiled, polygonal, of long spindle shape and display more pseudopodia. In contrast, 3D morphology culture groups (B) are a combination of round and oval shapes, display intercellular tight aggregation and adhesion. Furthermore, there is evidence of multiple sizes of cells distributed in different scaffold pores[60]. 2D: Two-dimensional; 3D: Three-dimensional.

reduce the failure risks and costs involved in the drug and therapeutic development process. Drug testing experiments *in vitro* 3D printed human tissues enable to secure human tissue-specific data prior to initiating the clinical trials in humans.

The liver is the primary site for the metabolism of many endogenous (*e.g.,* hormones) and exogenous (*e.g.,* xenobiotics) substances. Organovo's exVive3D liver is a bio-printed human liver model composed primary of hepatocytes, hepatic stellate cells and endothelial cells. Organovo's exVive3D liver tissue secretes important proteins like fibrinogen, albumin and transferrin proportional to levels in whole liver. Levels of ATP and lactate dehydrogenase secreted are also in the normal range when compared with the whole liver. This liver model could be a very important tool to study the route of metabolism of various exogenous and endogenous substances.

The realistic implications of 3D printing technology in drug discovery and development process involves the optimisation of the preclinical and clinical research methodologies. The research gap present between the lead molecule optimisation, preclinical studies and clinical research could be filled by the 3D construct of human tissues. Moreover, 3D constructs can reduce the failure risk and cost associated with the final stages of the drug discovery and development process. The 3D bio-printed models, unlike traditional cell culture models, could be standardised and validate for answering the complex questions related to the human cancer biology at molecular and tissue levels.

Today's 3D bio-printed human research data is not sufficient enough to replace the classical cell culture and animal models. However, the recent pragmatic shift towards the 3D bio-printed tissues and organs may be sufficient enough to generate enough evidence to prove its usefulness in drug discovery process. Sooner or later the researcher will be confident enough to make a call with a high level of confidence. The Early conclusion at the preclinical stage could be possible with the advancement in the 3D bio-printed technology; thereby reducing the risk associated with final-stage clinical trials.

Early prediction of the risk associated with the drug discovery process could be reduced with the help of 3D printed tissues, *e.g.,* Mou *et al*[60] used non-small cell lung cancer 95D cells to co-culture with a 3D bio-printed scaffold to construct a lung cancer model *in vitro*. This study of Mou *et al*[60] was focused on the relative comparison of the biological functions of lung cancer cells under the 2D and 3D environmental conditions. The 3D scaffold was constructed using the natural products like agarose and alginate and 3D printing technique was utilised to deposit the cell cultures on the scaffold. 95D cells types were used to co-cultured with this scaffold. The most important observation of this research tells us about the spindle and polygonal morphology of the cell cultured in 2D wells, whereas those cells which were grown in the 3D culture aggregated into spheroids and was able to migrate and invade the surrounding area of the scaffold (Figure 3).

Cell metabolic activity assay showed that the multiplication rates of the 3D-cultured cells for 2-6 d were significantly lower when compared with the 2D-cultured cells. On the other hand, those cells which were cultured for a longer time (8-9 d) were significantly higher than that of the 2D-cultured cells, demonstrating the proliferative activity of the cancer cells grown in 2D cultures for 8-9 d was inhibited. It is also observed that the cells grown on 3D scaffolds maintained a high rate of proliferation over the longer period of time. At the end, it was concluded that not only the cell mor-

phology and proliferation rate was different but also the associated protein expression was different. The growth of the lung cancer cells in 3D culture was also found to be different from the 2D cultured cells. We can also conclude that the agarose-alginate 3D scaffold can better simulate the microenvironment of lung cancer *in vivo* and in future this 3D construct may be established as a promising model for research in lung cancer.

Bone were constructed using human mesenchymal stem cells which were co-printing with acrylated peptides and acrylated poly (ethylene glycol). Inkjet bio-printing technique was used to make this construct[61]. Bone marrow stem cells with hydrogels like alginate, agarose, Matrigel®, and Lutrol® F127 were dispensed together using 3D bio-printer[62]. The printed bone marrow stem cells in combination with hydrogels were found to be functional and viable in the 3D construct. A mechanically stronger 3D bio-printed construct containing two different cell types has also been fabricated for osteochondral tissue regeneration[63].

Adipose-derived stem cells have the versatile ability to differentiate along with multiple lineage pathways. These cells could be isolated from human adipose tissue and could play the crucial role in regenerative medicine. Yao *et al*[64] used adipose-derived stem cells along with hydrogel (gelatin-alginate) to bio-print 3D construct in cubical shape. This work has significantly contributed to the idea of 3D construct of adipose tissue with functional vessels for efficient blood flow. Development of blood vessels inside the 3D printed adipose tissue means the better simulation to study complex biological phenomenon's *in vitro*, *e.g.,* differentiation of stem cell, cell signalling and interaction *etc*. One important finding of this study is that adipose stem cells not only proliferated of its own but were also found to differentiate within the 3D construct. When basic fibroblast growth factor was added, cells present in the 3D scaffold converted into endothelial cells and the cells rooted in the hydrogel separated into adipose-like cells. The constructs were found to remained intact for around 60 d[65].

Lee *et al*[66] used cells like keratinocytes, fibroblasts and collagen to develop the skin construct *in vitro*. Keratinocytes represented and converted to epidermis layer, fibroblasts into dermis layer and collagen epitomised the extracellular matrix of the skin (Figure 4). Histological, biochemical, light and fluorescence microscopic examinations have proved that the 3D printed skin was not only morphologically but was also found to be biologically similar to the natural skin[66,67]. Koch *et al*[68] on the other hand utilised laser-induced forward transfer (LIFT) for the development of 3D skin. Koch *et al*[68] used skin cells like fibroblasts and keratinocytes to represent the cells of dermis and epidermis layers of skin respectively and also used human mesenchymal stem cells for differentiation into other useful cells. All these cells were used in the form of bio-ink and were then deposited using laser-induced forward transfer method.

Vascular system transports oxygen, nutrients and toxic residue to-and-fro from the cell and hence considered as the very important component of the complex organ system. In regenerative medicine, development of the *in vitro* vascular structures could help us to bio-print the bigger and hugely complex organ[69]. Skardal *et al*[70] was the first to cross-linked tetrahedral polyethylene glycol tetracrylates with hyaluronan hydrogels to generate the 3D bio-constructed vascular system. Skardal *et al*[70] utilised bio-printers which work in the principle of extrusion (Figure 5). Recently Kolesky *et al*[71] also developed the complex vascular scaffold using gel-based cellular suspensions, sacrificial and fugitive gel and casting cavity filled with a GelMA gel.

Miller *et al*[72] first time used bio-printed complex vascular structure using carbohydrate glass. Carbohydrate glass was used as a sacrificial substrate/template for the cell adhesion. The sacrifice of the carbohydrate glass after cell deposition lead to the formation of the cylindrical vessels. Carbohydrate glass wall was lined with endothelial cells and the blood was forced through it under high pulsated pressure. After sacrifice of the carbohydrate glass wall, the hollow channel network left behind was populated with human umbilical vein endothelial cells to attach themselves to the wall of hollow channels. As compared with the other methods discussed earlier, Miller *et al*[72] approach is not only simple and gives greater control over the network geometry but is also well-suited with the different types of natural and synthetic extracellular materials, different variety of cells and various cross-linking methods. Miller *et al*[72] also proved that the vascular system was able to tolerate the metabolic function of rat hepatocytes in 3D engineered constructs[72]. Norotte *et al*[73] on the other hand, developed a method for preparation of the scaffold-free vascular tissue l. Norotte *et al*[73] utilised fibroblasts and smooth muscle cells with agarose as the supporting gel.

To study the inflammation in the intestinal mucosa Leonard *et al*[74] developed a complex *in vitro* model. Leonard *et al*[74] have utilised enterocyte cell line, immunocompetent macrophages and dendritic cells to construct 3D-fabricated intestinal mucosa model. This 3D printed intestinal mucosa model was then stimulated with the help of lipopolysaccharides from *Escherichia coli* and *Salmonella typhimurium*, interleukin-1β, and interferon-γ. Stimulation helped to develop the natural pathophysiological changes which occur in the intestine during inflammation. Different cell lines like Caco-2, HT-29 and T84, were used to develop the 3D constructs and were stimulated with the same pro-inflammatory molecules. It was observed that the Caco-2 cells were highly responsive towards the pro-inflammatory interleukin-1β molecules (Figure 6).

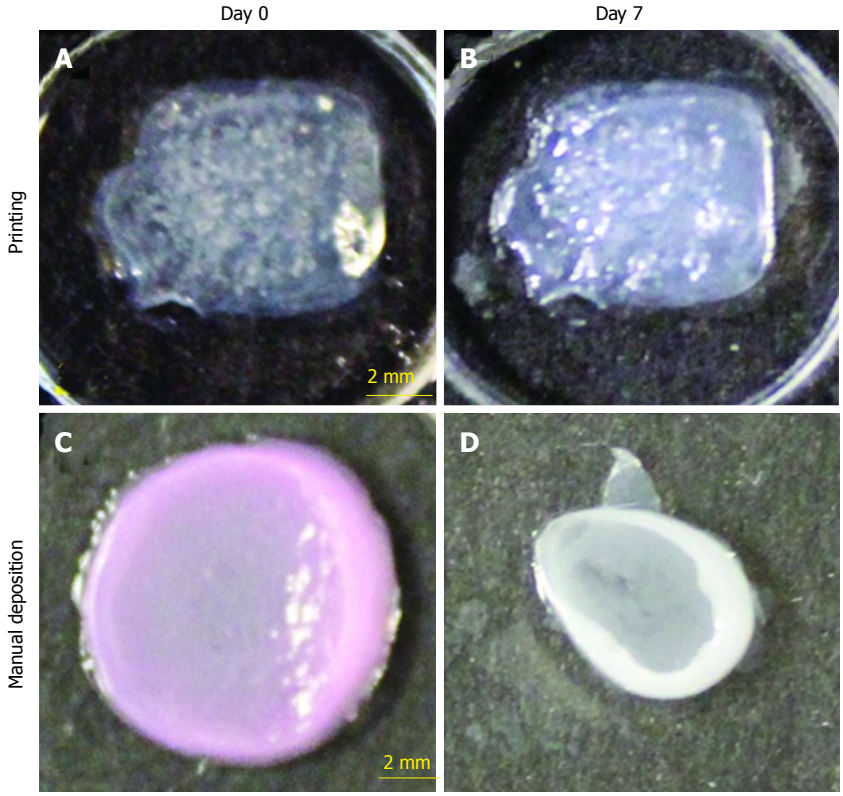

Figure 4 Shape and form of printed skin tissue. A comparison of skin tissues fabricated *via* 3D bio-printing and manual deposition indicates that printed skin samples (A, B) retain their form (dimensions) and shape, whereas manually deposited structures (C, D) shrink and form concave shapes (buckle) under submerged culture condition after 7 d.

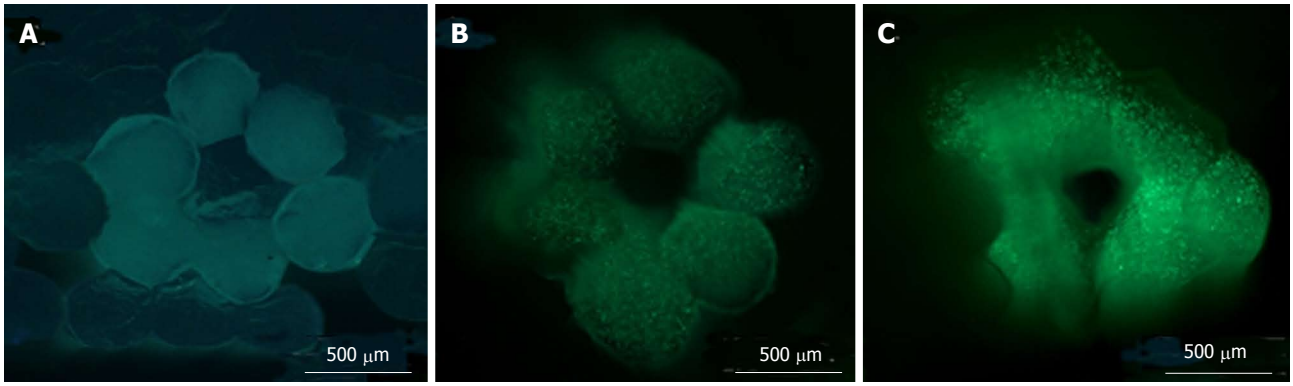

Figure 5 Cross-sectional images of three-dimensional bio-printed tissue (NIH 3T3 cells) containing an encapsulated fluorescent HA-BODIPY tracer for increased visualisation. Cross-sectional views of the bio-printed vascular constructs were taken (A) immediately after printing; (B) at 14 d; and (C) at 28 d of culture using LIVE/DEAD staining to highlight viable and dead cells. Green fluorescence indicates calcein AM-stained live cells[70].

The above-mentioned examples of 3D bio-printed tissues and organs could fasten the therapeutics development process and would facilitate the *in vitro* study of cancer pathophysiology. Recent advancement in the stem cell technology (Induced pluripotent stem cell) will hugely supplement the research in 3D bio-printing. Induced pluripotent stem cell has the unique character of dedifferentiated and then redifferentiated into tissues of choice[75]. Induced pluripotent stem cell technology has the very important role to play in 3D bioprinting and in solid organ transplantation. In the future, patient specific 3D tumour model also has the ability to revolutionised the field of personalised treatment.

ADVANTAGES OF 3D PRINTED TUMOUR MODELS - A COMPARISON WITH 2D PLANAR MONO-CULTURE AND 3D CO-CULTURE MODELS

The most efficient way of learning about the tumour progression and anticancer drug evaluation is by regulated and structured clinical trials on humans. However, direct evaluation of pathophysiological process in cancer

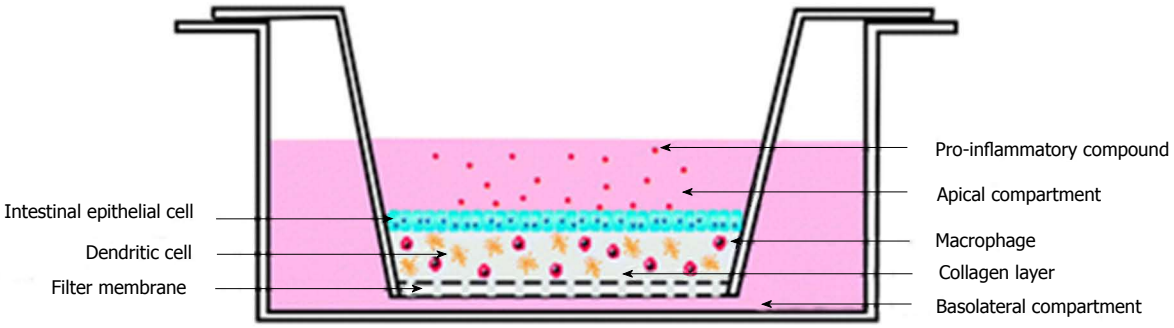

Figure 6 Experimental setup of three-dimensional co-culture comprising of intestinal epithelial cells, macrophages and dendritic cells[74].

development and anticancer activity of drugs is highly unethical because of the safety concerns. To overcome ethical challenges, preclinical studies on tumour models are highly appreciated. Several preclinical tumour models like cell culture, xenograft, mouse model and 3D tissue culture are developed which are thought to resemble with the natural tumours in terms of pathophysiological processes involved[76-78]. Evidence are now available which proves that the tumour microenvironment is the key regulator of the several stages involved in the pathophysiology of cancer progression. Tumour microenvironment is particularly very important in terms of the development of resistance, inventions of the distance organs and escape from the immune surveillance

This recent development not only challenged the past concept which mostly focused on the tumour cells but also impacted the research strategies of future. In future, the medical interventions in clinical oncology will also involve the therapeutics targeting the microenvironments. A systematic and methodological study of the tumour microenvironment, with the help of 3D bio-printed tumour models, would promote evaluation and selection of candidate agents from preclinical trials[79]. This would not only fasten the drug development process but would also save the resources.

A factor that plays an important role in the advanced malignancies is inappropriate activation of the supportive tissue called stroma. In most of the malignancy cases, stroma loses its connective and structural role. The various types of stromal cells are pericytes, smooth muscle cells, adipocytes immune cells, endothelial cells, fibroblasts, *etc.* Tumour microenvironment also found to contain various growth factors, many hormones, several structural and functional proteins, enzymes, cytokines and small cytokines of which most works as a primary and secondary signalling molecules and ligands for the receptors. The presence of all these functionalities in microenvironment could widely affect not only the pharmacokinetics but also the pharmacodynamics of the anticancer drugs. Thus the therapeutic outcome is widely regulated by the normal or abnormal expression of these extracellular proteins. It is now well recognised that protein and gene function varies strangely when studied them *in vivo* and *in vitro*. Studying the effect of these genetic alterations on drug response in either original or damaged neoplastic microenvironment is very critical for the fruitful drug development, translational anticancer regimes, and optimisation of therapies. These and several other factors are vital for the development of malignancies and are very difficult to re-orchestrate in 2D and co-culture models[80,81].

The genetically activated stroma of sarcomas and carcinomas is not only composed of cancer associated fibroblasts and myofibroblasts but can be identified due to altered matrix components, change in the proteins synthesis associated with repair machinery and reprogrammed breakdown process[80,81]. Except for the supportive function, stromal cells also play the important role in the physical and biological protection of microenvironment protection. This functionality actually limits the effective delivery of the therapeutic drugs to the cancer cells. Altered components of the tumour microenvironment, including the synthesis of the proteins involved in the repair mechanism, allows the unrestricted growth of the tumour cells. Tumour cells in favourable environment successfully evade the apoptosis signals triggered by cytotoxicity and develop various resistance strategies to select the malignant phenotypes.

Correlation of the survival rate and capability of stroma to overpower the carcinogenesis is already established[82]. However, once distorted to a tumour-associated neighbour because of the stimuli like inflammation, infection, mutation, *etc.,* the stromal protective function can be altered to stimulate the proliferation[83-85]. Under the altered condition, stromal cells start to evolve with the cancer cells and begin synthesis of growth factors, cytokines, chemokines, *etc.,* which fast-track the disease progression[86]. In addition to this, many *in vitro* studies have proved the complex role of the tumour microenvironment in cancer development. Experiments with genetically modified stroma proved the importance of the tumour microenvironment in disease progression[87,88].

Infection, immune-associated signalling and inflammation have been found to be associated with several cancer types. For example, liver carcinoma which is the leading cause of death in patients with liver cirrhosis and

increased the risk of colorectal cancer in the patients with increased inflammation is credited to unresolved inflammatory signalling[89]. Similarly viruses, bacteria and parasites are also the leading cause of the variety of cancers. A higher incident of multiple cancers like gastrointestinal tract, lung, reproductive and skin cancers has been found in female immunosuppressed organ transplant recipients[90]. Retrospective analysis revealed a higher incidence of AIDS-associated cancers (*e.g.,* Kaposi's sarcoma, Cervical cancer, Non-Hodgkin lymphoma), and non-AIDS-related cancers (*e.g.,* tongue, skin, lung, CNS and multiple myelomas) in HIV-infected patients[91]. Various enzymatic proteins, like matrix metalloproteinase, in particular, matrix metalloproteinase-2 and matrix metalloproteinase-9 have a role in the tumour progression. For example, matrix metalloproteinase-2 and matrix metalloproteinase-9 allow cancer cells to breach through the extracellular matrix of the tumour microenvironment and are closely related to cancer metastasis. The activity of the various matrix metalloproteinase is found to increase with the development of cervical cancer[92] and can be studied efficiently in 3D bio-printed tumour models[93].

Development of the resistance towards the therapeutic intervention is the foremost challenge in clinical oncology. In addition to fuelling the tumour growth, the altered tumour microenvironment modifies treatment responses by affecting cell sensitivity towards anticancer agents. Decreased cell sensitivity towards anticancer drugs gives rise to the drug resistance. The drug resistance facilitated by the alteration tumour microenvironment is not limited to classical agents like chemotherapies. Instead, it covers various therapeutic materials, including targeted agents and targeted drug delivery systems[94]. The role of tumour microenvironment in the protection of acute myeloid leukaemia or chronic lymphocytic leukaemia cells from pharmaceutical agents like anthracyclines, alkylating agents, imatinib and nucleoside analogues has been recently evaluated. The defending role of tumour microenvironment is detected in the protection of the mutant Janus kinase 2 cells from Janus kinase inhibitors. Tumour microenvironment role is also observed in protecting solid tumours from erlotinib and cetuximab. Similarly, recent findings described the protection of melanoma against RAF inhibitors, like vemurafenib[95-97]. Tumour microenvironment assisted resistance is found to be directed through several cell lineages and alteration in the stromal components (*e.g.,* fibroblasts, endothelial cells, *etc.*)[94,98].

Tumour microenvironment assisted protection of tumour cells applies to multiple therapeutic strategies and varies with the inter-individual differences. For example, in the treatment of melanoma by mitogen-activated protein kinase pathway inhibitors, tumour-associated macrophages multiplies and release cytokine-like tumour necrosis factor-α as a crucial growth factor that provides resistance to the targeted therapy through the microphthalmia transcription factor[99]. Similarly, certain cancer endothelial cells secrets interleukin-6 and tissue inhibitor of metalloproteinases-1 as the survival factors. Both of the factors were found to be significantly involved in the resistance of lymphoma when the Eμ-Myc mice model of Burkitt's lymphoma treated with anticancer antibiotic doxorubicin. This could be reversed or good chemotherapeutic efficacy could be achieved by the inhibition of these survival factors or by stimulating the p38 mitogen-activated protein kinase pathway[100]. Another noted example of tumour microenvironment-exerted protection of cancer cells is the chemoresistance caused by the amplification of the CXCL1/2-S100A8/9 loop by antineoplastic agents used in breast cancer treatment[101].

The examples illustrated above demonstrate various pathways by which therapies or targeted agents can be affected by the changes in the tumour microenvironment. Tumour microenvironment not only contains the tumour cells but also contains the several other cells, *e.g.,* immune cells, lymphatics cells fibroblasts, pericytes, *etc*. This composition of microenvironment essentially affects the therapeutic outcome[102]. The 2D monolayered and 3D coculture cellular models lack illustrated characteristics of natural 3D tissues *in vivo*[103]. 2D monolayered culture has the increased drug diffusion properties which do not match with the natural tumour character. A lot of drugs have their site of action inside the cells and hence their penetration is very important for effectiveness. This character of cell culture models explains the importance of three-dimensional arrangements for the proper success of the therapy.

To overcome the drawback of the cell culture models various alternative animal models were developed, *e.g.,* genetically altered and immunocompromised mice models. Animal models have contributed enormously to the present understanding of cancer, however, they could not reflect the actual pathophysiology involve in disease progression because of the species differences[104].

To overcome the hurdles of simulating the exact complex tumour microenvironment in cell culture, 3D printing technology was adapted to produce the 3D bio-printed tissues and organs. Similarly, 3D printing technology could be easily utilised to produce the 3D tumour models which subsequently could be utilised to study the cancer biology and anticancer drugs[105,106]. Various techniques, such as cell-seeding 3D scaffolds, hydrogel embedding, multicellular spheroids, cell patterning and microfluidic chips have been explored for the construction of 3D tumour models *in vitro*[76].

Several advances in 3D printing technology and stem cell research offers unique opportunity for the construction of complex organs and tumours. The 3D printed organs and tumour models essentially simulate the exact physiological and pathophysiological microenvironments. The exact recreation of the tumour microenvironment facilitates the better understanding

of the disease[107,108].

Till date, very few reports have been published describing the 3D printed tumour models. Zhao *et al*[93] demonstrated the use of HeLa cells in gelatin/alginate/fibrinogen hydrogels to bio-print the 3D *in vitro* models of cervical tumours. When compared with 2D cell culture model, 3D printed tumour model have shown 90% proliferation rate. Zhao *et al*[93] also observed the increased expression of matrix metalloprotease protein and chemoresistance in 3D printed tumour models when compared with 2D cell culture model. Work of Zhao *et al*[93] is just one example of the advancement of 3D bio-printed tumour model, with further advancement in 3D printing technology, *a revolution* in the field of cancer research is on the corner.

CONCLUSION

The 3D bio-printing of tissue and organ models is a developing field in which several ground-breaking results have been obtained over the past few years. The 3D-bioprinted tissue constructs are being prepared not only for the solid organ transplantation but also for use in drug discovery process. Fabrication of the realistic tissues, organs and tumour models with the help of the various 3D bio-printing techniques is now possible. Extrapolation of the results obtained from the cell culture and animal models are not trustworthy because of the species differences. This challenge of species difference could be overcome by printing the 3D tissues and organs from the human cells. The 3D printed tumour model fabricated from the human tumour cell lines will definitely revolutionise the oncology research. The 3D printing is the very precise which could be demonstrated by its (inkjet printer) use in transfecting genes into cells[109,110]. In coming days, 3D bio-printed tissues and organs will find its way in the pharmacological and toxicological testing of the molecules under drug development process. Bio-printing has the potential to change the way the drug enters the clinical trials after preclinical studies. The 3D printing not only has the capability to improve the attrition rate of the clinical trials but will also reduce the cost and time required in the drug discovery process. This is possible because of the speedy identification of the efficient candidate molecule. Use of 3D bio-printed models will eliminate the need of animal models and hence the data obtained in the preclinical studies will be more trustworthy.

Most published results are the early prediction and only a few studies methodologically explored the developmental method parameters. Standardisation and optimisation of the printing process parameters are essential for the successful adaption of the 3D printed tissues and organs to use them in drug development process. This is possible to achieve to by establishing the relationship between structural and functional parameters. Moreover, modern fabrication schemes rely on mathematical modelling and computer simulations for optimising the process design and making predictions[107,109]. Therefore the performance of the tissue constructs could be predicted virtually using computer simulations before actually printing the construct.

Stem cells already have revolutionised the field of regenerative medicine and have very important role to play in the construction of 3D tissue, organs and tumour models. Stem cells (*e.g.,* induced pluripotent stem cells) offer greater possibility for fabricating complex constructs because of their ability to differentiate in various another kind of cells, as highlighted by various research groups[107,109,111,112]. However, some issues need to be fixed before stem cells can be used for 3D bio-printing. This issue includes optimisation of the cellular microenvironment to combine the advantages of cell attachment, cell stimulation and mechanical stability to mimic the *in vivo* environment to the highest degree.

Printed 3D models match closely with the natural organs and when compared with the cell culture models. Novel 3D cell printing technology may help to develop the tumour models *in vitro* which will be more useful in studying cancer cell biology. Although, 3D bio-printing techniques are still in their infancy, they offer potential to overcome many challenges associated with the production of complex tissues and organs. This technique is a promising tool for replacing current and often misleading results obtained from cell culture and animal based screening of pharmaceuticals. Interdisciplinary research and collaboration of the researcher from the various field are required to overcome the hurdles before 3D bio-printed concept accepted by the institutional and pharmaceutical researchers. To be successful, we will have to sort-out the progressive challenges of 3D bio-printing, including cell sources and biocompatible material requirements, proper vascularization and autonomous maturation and continuous functionality of the construct.

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REVIEW

From targeting the tumor to targeting the immune system: Transversal challenges in oncology with the inhibition of the PD-1/PD-L1 axis

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Abstract

After that the era of chemotherapy in the treatment of solid tumors have been overcome by the "translational era", with the innovation introduced by targeted therapies, medical oncology is currently looking at the dawn of a new "immunotherapy era" with the advent of immune checkpoint inhibitors (CKI) antibodies. The onset of PD-1/PD-L1 targeted therapy has demonstrated the importance of this axis in the immune escape across almost all human cancers. The new CKI allowed to significantly prolong survival and to generate durable response, demonstrating remarkable efficacy in a wide range of cancer types. The aim of this article is to review the most up to date literature about the clinical effectiveness of CKI antibodies targeting PD-1/PD-L1 axis for the treatment of advanced solid tumors and to explore transversal challenges in the immune checkpoint blockade.

Key words: Immune checkpoint inhibitors; PD-1; PD-L1; Checkpoint inhibitors; Cancer treatment; Immune checkpoint blockade; Anti-PD-1 antibodies; Anti-PD-L1 antibodies

Core tip: The onset of PD-1/PD-L1 targeted therapy in oncology has demonstrated the importance of this axis in the immune escape across almost all human cancers. A sort of revolution has been happening with the investigation of the new immune checkpoint inhibitors in the field of anticancer therapy. The aim of this article is to review the most up to date literature about the clinical effectiveness of the antibodies targeting PD-1/PD-L1 axis for the treatment of advanced solid tumors and to explore transversal challenges in the immune checkpoint blockade.

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INTRODUCTION

After that the era of chemotherapy in the treatment of solid tumors have been overcome by the "translational era", with the innovation introduced by targeted therapies, medical oncology is currently looking at the dawn of a new "immunotherapy era" with the advent of immune checkpoint inhibitors (CKI) antibodies.

The strategy to maintain physiologic self-tolerance and to restore latent anti-tumor immunity is currently going through the whole oncology, gradually revolutionizing the standard of treatment of the most chemo-resistant tumors such as melanoma, lung and renal cancer. From the first class of antibodies against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), like ipilimumab and tremelimumab, burdened by significant autoimmune toxicity, the scenario is currently evolving in favor of the antibodies against programmed cell death protein 1 (PD-1) and its ligand PD-L1, in both cases inhibiting the PD-1/PD-L1 axis[1].

The monoclonal antibodies nivolumab and pembrolizumab (anti-PD-1), atezolizumab, durvalumab and avelumab (anti-PD-L1), have been tested against multiple cancer types in the last years and are currently under investigation in several phase Ⅱ and phase Ⅲ clinical trials. Further similar antibodies are currently undergoing phase Ⅰ experiences, in order to compete with the first arrivals on the clinical scenario[2-4]. All the antibodies cited in the text are reported in Table 1.

In all cases, the mechanism targets the inhibitory signal that contributes to the balance between co-stimulatory and inhibitory pathways in the regulation of T-cell response, starting from the antigen recognition by T-cell receptor. In fact, in contrast to other antibodies currently used for cancer therapy, CKI do not target tumor cells directly, but instead they target lymphocyte receptors or their ligands, with the aim to enhance endogenous antitumor response[5].

PD-1 belongs to the inhibitory B7-family molecules; it is upregulated and expressed by activated T-cells (but also B-cells, T regulatory and natural killer cells) and engaged through its ligands PD-L1 and PD-L2, expressed by the antigen presenting cells (APC) and by non-hematopoietic stem cells, aside from tumor cells. The role of PD-1 consists in the inhibition of the effector T-cells activity in peripheral tissues during the inflammatory response to infection and in the regulation and limitation of autoimmunity[6]. Within the tumor microenvironment, this endogenous mechanism favors immune resistance[7]. The major PD-1 ligand expressed on solid tumors cells is PD-L1, whose most important signal for induction is interferon-γ (IFN-γ), produced by T helper 1 (Th1) cells[8]. Most types of solid tumors have been demonstrated to express high levels of PD-L1 (melanoma, ovarian, lung cancer and genitourinary tumors among others), and more recently the importance of PD-L1 expression on the immune cells infiltrating the tumor also emerged, in particular on tumor-infiltrating lymphocytes (TILs). Nevertheless, the evidence about the prognostic and predictive role of these elements have not yet been clarified and it seems to be different basing on tumor type[5].

Table 1 Immune-checkpoint inhibitors antibodies with their targets

CKI	Mechanism of action
Nivolumab	Anti-PD-1
Pembrolizumab	Anti-PD-1
Atezolizumab	Anti-PD-L1
Durvalumab	Anti-PD-L1
Avelumab	Anti-PD-L1
BMS936559	Anti-PD-L1
Pidilizumab	Anti-PD-1

CKI: Checkpoint inhibitors.

Despite these unresolved issues, the findings described above provided the rationale for the capacity of the blockade of PD-1/PD-L1 axis to enhance intratumoral immune responses in a transversal way across different tumor types, firstly encouraged by preclinical evidence and then largely satisfied by the early results of several recent clinical studies.

RESEARCH

The aim of this article is to review the most up to date literature about CKI antibodies targeting PD-1/PD-L1 axis for the treatment of advanced solid tumors, particularly considering phase Ⅲ randomized trials, starting from the first performed trials on the issue. Published papers were obtained from the Medline database. The search was implemented by reviewing the most important international scientific meetings abstract databases. In addition, indirect data on the topic were achieved by reading the most recent publications related to the use of CKI in different types of solid tumors.

The ongoing trials were reached on the official website www.clincaltrials.gov, considering only randomized phase Ⅲ studies.

RESEARCH RESULTS

Melanoma

Treatment of advanced melanoma has been radically changed by the advent of CKI. After that the anti-CTLA4 antibody ipilimumab in the last years had become the backbone of this malignant tumor treatment, where traditional chemotherapy harvested very little success, the introduction of the anti-PD-1 antibodies nivolumab

Table 2 Phase Ⅲ randomized clinical trials currently ongoing with PD-1/PD-L1 axis blockade in adjuvant setting for solid tumors

Trial name/NCT	Cancer type	Immune checkpoint inhibitor	Arms	Primary endpoint	Expected primary completion date	No. of patients
KEYNOTE-054[20]	Melanoma	Pembrolizumab	Pembrolizumab *vs* placebo	RFS	2018	900
NCT02506153[21]	Melanoma	Pembrolizumab	Pembrolizumab *vs* high dose recombinant interferon-α-2B or ipilimumab	OS	2020	1378
KEYNOTE-091 (PEARLS)[22]	NSCLC	Pembrolizumab	Pembrolizumab *vs* placebo	DFS	2021	1380
IMvigor010[23]	Bladder cancer	Atezolizumab	Atezolizumab *vs* observation	DFS	2021	440
IMpower010[24]	NSCLC	Atezolizumab	Atezolizumab *vs* BSC after adjuvant CT[1]	DFS	2020	1127
NCT02768558[25]	NSCLC (locally advanced)	Nivolumab	Nivolumab *vs* placebo (after CT[1]-RT)	OS	2022	660
ANVIL[26]	NSCLC	Nivolumab	Nivolumab *vs* observation	DFS	2018	714
CheckMate 238[27]	Melanoma	Nivolumab	Nivolumab + placebo *vs* ipilimumab + placebo	RFS	2018	800
CheckMate 274[28]	Urothelial cancers	Nivolumab	Nivolumab *vs* placebo	DFS	2020	640
CheckMate 577[29]	Esophageal or gastroesophageal junction cancer (locally advanced)	Nivolumab	Nivolumab *vs* placebo (after CT[1]-RT and surgery)	DFS	2019	760
PACIFIC[30]	NSCLC (locally advanced)	Durvalumab	Durvalumab *vs* placebo (after CT[1]-RT)	OS	2017	702
NCT02273375[31]	NSCLC	Durvalumab	Durvalumab *vs* placebo	DFS	2025	1100

[1]According to the standard of care and basing on the choice of the investigator. RFS: Recurrence free survival; NSCLC: Non-small cell lung cancer; DFS: Disease free survival; CT: Chemotherapy; OS: Overall survival; RT: Radiotherapy.

and pembrolizumab further improved the therapeutic armamentarium for melanoma.

The first published phase Ⅲ randomized study about PD-1/PD-L1 axis inhibition in this disease demonstrated, at the beginning of 2015, the advantage of nivolumab over chemotherapy with dacarbazine both in terms of overall survival (OS) and of progression free survival (PFS) among previously untreated patients with metastatic melanoma without *BRAF* mutation. Median PFS of 5.1 mo in the nivolumab group was more than doubled when compared to dacarbazine treated patients, with 2.2 mo [hazard ratio (HR) = 0.43, 95%CI: 0.34-0.56, $P < 0.001$]. OS was not reached in the nivolumab group, instead being 10.8 mo in the group treated with chemotherapy (HR = 0.42, 99%CI: 0.25-0.73, $P < 0.001$)[9].

An analogous comparison was made in patients who progressed after anti-CTLA4 treatment in the phase Ⅲ randomized study CheckMate 037, reporting a response rate (RR) of 32% for nivolumab *vs* 11% with chemotherapy according to investigator's choice. These findings have resulted in the inclusion of nivolumab in the new treatment options for a cancer with high unmet need[10].

In parallel, pembrolizumab was compared with ipilimumab as the new standard of care for first line treatment of advanced melanoma in a phase Ⅲ randomized trial, demonstrating to prolong PFS and OS with less toxicity respect to the CTLA4 inhibitor[11].

Nevertheless, the new frontier for untreated melanoma is currently represented by the combination of anti-CTLA4 and anti-PD-L1 antibodies: Larkin *et al*[12] demonstrated that the association of nivolumab and ipilimumab resulted in a significantly longer PFS than ipilimumab alone, despite 55% of treatment-related adverse events (AEs) of grade 3 or 4 (G3-4) *vs* 16% in the nivolumab group and 27% in the ipilimumab group. This three arms phase Ⅲ randomized trial closed the matter of first line ipilimumab alone, otherwise confirming good effectiveness for nivolumab monotherapy in this setting[12].

Further phase Ⅲ-Ⅳ trials are currently ongoing to test different dosing schedules of CKI[13], others to verify their efficacy in particular subgroups of patients like those with brain metastases[14], or to establish the correct duration of anti-PD-1 therapy in metastatic melanoma, especially in the case of long responders[15]. Again, more others are investigating alternative combinations[16,17] or treatment sequences, like ipilimumab plus nivolumab followed or preceded by dabrafenib and trametinib in *BRAF* mutated patients[18].

Moreover, after the Food and Drug Administration approval of ipilimumab for the adjuvant setting for melanoma[19], as discussed below, the PD-1 and PD-L1 inhibitors are currently under investigation for the adjuvant and neoadjuvant setting also in different tumor types in several clinical trials, which results are eagerly awaited, given the lower toxicity expected from this "second generation" of CKI (Table 2)[20-31].

Lung cancer

Lung cancer immunotherapy have an historical back-

ground, but it has not shown significant survival benefit until the recent advent of CKI.

Conversely to anti-CTLA4 antibodies, which demonstrated a certain efficacy only when combined with chemotherapy, the inhibition of PD-1/PD-L1 axis clearly works as single strategy in non-small cell lung cancer (NSCLC)[32].

The first step through immunotherapy for lung cancer in clinical practice was the approval of CKI monotherapy with nivolumab (and more recently with atezolizumab) for NSCLC patients pretreated with first line chemotherapy, on the basis of the first published randomized trials[33-35].

Anti-PD1 antibodies are going to radically revolutionize lung cancer treatment regardless of the histology, especially after the recently published results of KEYNOTE 024 trial[36], providing the outstanding evidence of pembrolizumab superiority compared to chemotherapy as first line treatment for NSCLC, in terms of PFS (10.3 mo *vs* 6 mo, $P < 0.001$), OS (80% *vs* 72% at 6 mo, $P = 0.005$), RR (45% *vs* 28%) and safety among patients bearing strong PD-L1 expression on tumor cells (at least 50% was required for enrollment). This latter evidence, despite concerned to the 30% of overall NSCLC population, will provide the rationale to radically change the therapeutic paradigm for NSCLC, shifting CKI treatment option to first line in a great subgroup of patients. The selection of patients basing on a single biomarker, despite potentially harmful, has been demonstrated to be effective in this case, as proven by the recently announced failure of the analogue phase Ⅲ trial with nivolumab, whose patients were enrolled independently from PD-L1 status[37].

Several phase Ⅲ studies are currently still ongoing in order to investigate further CKI antibodies in all treatment lines, in different treatment regimens and with alternative combinations targeting PD-1/PD-L1 axis in advanced NSCLC (Table 3)[37-96].

Also adjuvant paradigm has been pursued in lung cancer: Table 2 summarizes all the ongoing phase Ⅲ studies in this field.

Squamous cell lung cancer: Squamous cell histology had the first indication for CKI therapy, basing on the outstanding results of CheckMate 017 trial comparing nivolumab *vs* docetaxel in advanced squamous NSCLC (SqNSCLC) progressive to previous chemotherapy[33]. With a median OS of 9.2 mo *vs* 6 mo, nivolumab reduced the risk of death of 41%, with an HR of 0.59 (95%CI: 0.44-0.79), $P < 0.001$. The advantage was confirmed also for RR, PFS and safety profile, finally providing an unprecedented treatment option also in terms of tolerability.

Non-squamous cell lung cancer: With a slight delay and with not as brilliant but positive results, nivolumab was also approved for non-squamous NSCLC (non-SqNSCLC) treatment after failure of chemotherapy, on the basis of an analogous phase Ⅲ randomized trial demonstrating an improvement of median OS from 9.4 mo with docetaxel to 12.2 mo (HR = 0.73, 95%CI: 0.59-0.89, $P = 0.002$)[34]. In this study, nivolumab was associated with better OS and RR but not with longer PFS compared to chemotherapy. A crossing of the PFS curves suggested a delay of the benefit with nivolumab, consistent with the results of previous immune system modulating agents, probably reflecting a pattern of response typical of immunotherapy and the use of inadequate response assessment measurements for this type of drug[97].

Other thoracic malignancies: Among other thoracic tumors, small cell lung cancer (SCLC), malignant pleural mesothelioma (MPM) and thymic epithelial tumors (TETs), under the thrust of true unmet medical needs, came across immunotherapy with CKI.

Preliminary data for PD-1/PD-L1 blockade in SCLC were encouraging and currently ongoing phase Ⅲ studies are investigating CKI both in pretreated and untreated advanced SCLC patients[72,93] or as maintenance treatment after standard treatment either in extensive or in limited disease[91].

Great expectations have been made for MPM, because of the known relationship between neoplastic and inflammatory counterpart in this tumor, recognized to have a T-cell inflamed phenotype. At the moment, only preliminary data have been published and CKI are currently under proposal for further investigations in this disease. Finally, early phases studies are ongoing to test CKI immunotherapy also in TETs[98].

Renal cancer

After the pivotal trial Checkmate 025, nivolumab has vowed to became the cornerstone of previously treated metastatic renal cell carcinoma (mRCC) therapy, finally offering an OS improvement in a setting where targeted therapies have fallen short of expectation[99]. The median OS was 25 mo (95%CI: 21.8-not estimable) with nivolumab and 19.6 mo (95%CI: 17.6-23.1) with everolimus, with a HR of 0.73 and a RR of 25% *vs* 5% ($P < 0.001$). Also in terms of toxicity, nivolumab was superior to the standard treatment everolimus, with 19% *vs* 37% of AEs.

In the light of these results, nivolumab currently represents a new standard of treatment for mRCC after disease progression to first line antiangiogenic therapy. On this auriferous vein other phase Ⅲ randomized trials have been planned and their results are eagerly awaited. Worthy of note, a phase Ⅲ randomized trial with an innovative design is comparing the combination of lenvatinib and everolimus (which recently achieved great results in phase Ⅱ[100]) with the combination of lenvatinib and pembrolizumab *vs* the standard sunitinib. Such ambitious trials will probably provide the cornerstone of the future clinical practice in RCC[41,101].

After reaching the indication for second line treat-

Table 3 Phase III randomized clinical trials currently ongoing with PD-1/PD-L1 axis blockade in advanced setting for solid tumors

Trial name/NCT	Cancer type	Immune checkpoint inhibitor	Arms	Treatment line	Primary endpoint	Expected primary completion date	No. of patients
STOP-GAP[15]	Melanoma	PD-1 inhibitor (any)	Intermittent *vs* continuous therapy	Any	OS	2025	550
NCT02752074[16]	Melanoma	Pembrolizumab	Pembrolizumab + epacadostat *vs* pembrolizumab + placebo	I line	PFS	2018	600
MASTERKEY-265[17]	Melanoma	Pembrolizumab	Pembrolizumab + talimogene laherparepvec *vs* pembrolizumab + placebo	I line	PFS	2018	660
KEYNOTE-048[82]	HNSCC	Pembrolizumab	Pembrolizumab *vs* CT[1] + pembrolizumab *vs* CT[1]	I line	PFS	2018	780
KEYNOTE-040[38]	HNSCC	Pembrolizumab	Pembrolizumab *vs* methotrexate or docetaxel or cetuximab	From II line	OS	2017	466
KEYNOTE-204[39]	Hodgkin lymphoma	Pembrolizumab	Pembrolizumab *vs* brentuximab	From II line	PFS	2019	300
KEYNOTE-045[40]	Urothelial cancers	Pembrolizumab	Pembrolizumab *vs* paclitaxel, docetaxel or vinflunine	From II line	OS	2017[2]	470
NCT02811861[41]	Renal cell carcinoma	Pembrolizumab	Pembrolizumab + lenvatinib *vs* lenvatinib + everolimus *vs* sunitinib	I line	PFS	2020	735
KEYNOTE-426[102]	Renal cell carcinoma	Pembrolizumab	Pembrolizumab + axitinib *vs* sunitinib	I line	PFS, OS	2019	840
KEYNOTE-240[42]	HCC	Pembrolizumab	Pembrolizumab *vs* BSC	II line	PFS	2019	408
KEYNOTE-189[43]	NSqNSCLC	Pembrolizumab	Platinum and pemetrexed ± pembrolizumab	I line	PFS	2017	570
KEYNOTE-407[44]	SqNSCLC	Pembrolizumab	CT[1] ± pembrolizumab	I line	PFS	2018	560
KEYNOTE-042[45]	NSCLC PD-L1-positive	Pembrolizumab	Pembrolizumab *vs* platinum based CT[1]	I line	OS	2018	1240
KEYNOTE-010[46]	NSCLC	Pembrolizumab	Pembrolizumab *vs* docetaxel	From II line	OS	2019	1034
KEYNOTE-119[47]	Triple negative breast cancer	Pembrolizumab	Pembrolizumab *vs* monochemotherapy	II-III line	PFS	2017	600
KEYNOTE-355[48]	Triple negative breast cancer	Pembrolizumab	CT[1] + pembrolizumab *vs* CT[1] + placebo	I line	PFS	2019	858
KEYNOTE-177[49]	MSI-H or dMMR colorectal carcinoma	Pembrolizumab	Pembrolizumab *vs* CT[1]	I line	PFS	2019	270
KEYNOTE-181[50]	Esophageal/ esophago-gastric junction carcinoma	Pembrolizumab	Pembrolizumab *vs* monochemotherapy[1]	II line	PFS	2018	600
KEYNOTE-061[51]	Esophageal/ esophago-gastric junction adenocarcinoma	Pembrolizumab	Pembrolizumab *vs* paclitaxel	II line	PFS	2017	720
KEYNOTE-062[52]	Esophageal/ esophago-gastric junction carcinoma	Pembrolizumab	Pembrolizumab *vs* CT[1] + pembrolizumab *vs* CT[1]	I line	PFS	2019	750
JAVELIN Ovarian 200[53]	Ovarian cancer (platinum resistant)	Avelumab	Avelumab *vs* avelumab plus PLD *vs* PLD	From II line	OS	2018	550
JAVELIN Ovarian 100[54]	Ovarian cancer	Avelumab	CT[1] *vs* CT[1] followed by avelumab maintenance *vs* CT[1] + avelumab followed by avelumab maintenance	I line	PFS	2019	951
JAVELIN Renal 101[55]	Renal cell cancer	Avelumab	Avelumab with axitinib *vs* sunitinib	I line	PFS	2018	583
JAVELIN Bladder 100[56]	Urothelial cancer	Avelumab	Avelumab *vs* BSC (maintenance after CT[1])	I line maintenance	OS	2019	668

JAVELIN Gastric 100[57]	Adenocarcinoma of the stomach or of the gastro-esophageal junction	Avelumab	CT[1] continuation *vs* avelumab in maintenance after CT[1]	I line	OS	2018	666
JAVELIN Gastric 300[58]	Adenocarcinoma of the stomach or of the gastro-esophageal junction	Avelumab	Avelumab + BSC *vs* CT[1] + BSC *vs* BSC	III line	OS	2017	330
JAVELIN Lung 100[59]	NSCLC (PD-L1 positive)	Avelumab	Avelumab *vs* platinum based CT[1]	I line	PFS	2017	420
JAVELIN Lung 200[60]	NSCLC (PD-L1 positive)	Avelumab	Avelumab *vs* docetaxel	From II line	OS	2017	650
OAK[61]	NSqNSCLC	Atezolizumab	Atezolizumab *vs* docetaxel	From II line	OS	2017	1225
IMvigor211[62]	Bladder cancer	Atezolizumab	Atezolizumab *vs* monochemotherapy	II line	OS	2017	932
IMvigor130[63]	Urothelial carcinoma (ineligible for cisplatin)	Atezolizumab	Atezolizumab + CT[1] *vs* placebo + CT[1]	I line	PFS	2019	435
IMpower110[64]	NSqNSCLC	Atezolizumab	Atezolizumab *vs* platin + pemetrexed	I line	PFS	2019	570
IMpower111[65]	SqNSCLC	Atezolizumab	Atezolizumab *vs* gemcitabine + platin	I line	PFS	2017	ND
IMpower131[66]	SqNSCLC	Atezolizumab	Atezolizumab + nab-paclitaxel + carboplatin *vs* atezolizumab + paclitaxel + carboplatin *vs* nab-paclitaxel + carboplatin	I line	PFS	2023	1200
IMpower210[67]	NSCLC	Atezolizumab	Atezolizumab *vs* docetaxel	II line	OS	2019	563
IMpower130[68]	NSqNSCLC	Atezolizumab	Atezolizumab + nab-paclitaxel + carboplatin *vs* nab-paclitaxel + carboplatin	I line	PFS	2019	550
IMpower150[69]	NSqNSCLC	Atezolizumab	Atezolizumab + carboplatin + paclitaxel ± bevacizumab *vs* carboplatin + paclitaxel + bevacizumab	I line	PFS	2017	1200
IMpassion130[70]	Triple negative breast cancer	Atezolizumab	Atezolizumab + nab-paclitaxel *vs* placebo + nab paclitaxel	I line	PFS	2020	900
IMmotion151[71]	Renal cell carcinoma	Atezolizumab	Atezolizumab + bevacizumab *vs* sunitinib	I line	PFS	2020	900
IMpower133[72]	SCLC	Atezolizumab	Carboplatin and etoposide ± atezolizumab	I line	OS	2019	400
NCT02788279[73]	Colorectal carcinoma	Atezolizumab	Atezolizumab + cobimetinib *vs* atezolizumab *vs* regorafenib	From III line	OS	2019	360
KESTREL[74]	HNSCC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC	I line	PFS	2017	628
MYSTIC[75]	NSCLC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC	I line	PFS	2017	1092
Danube[76]	Bladder cancer	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC1	I line	PFS	2017	525
Lung-MAP[77]	SqNSCLC (biomarker-targeted)	Durvalumab, nivolumab	Docetaxel *vs* durvalumab vs erlotinib *vs* AZD4547 *vs* ipilimumab *vs* palbociclib *vs* rilotumumab *vs* taselisib	Any	PFS	2022	10000

CAURAL[78]	NSCLC T790M mutation positive	Durvalumab	AZD9291 + durvalumab *vs* AZD9291	II-III line	PFS	2018	350
NCT02369874[79]	HNSCC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC[1]	II line	OS	2018	720
NEPTUNE[80]	NSCLC	Durvalumab	Durvalumab + tremelimumab *vs* SOC[1]	I line	OS	2018	800
ARCTIC[81]	NSCLC	Durvalumab	Durvalumab *vs* durvalumab + tremelimumab *vs* SOC[1]	II-III line	OS	2016	730
NCT02224781[18]	Melanoma BRAFV600 mutated	Nivolumab	Dabrafenib + trametinib followed by ipilimumab + nivolumab *vs* ipilimumab + nivolumab followed by dabrafenib + trametinib	I line	OS	2019	300
NIBIT-M2[14]	Melanoma brain metastases	Nivolumab	Fotemustine *vs* ipilimumab + fotemustine *vs* ipilimumab + nivolumab	Any	OS	2018	168
CheckMate 026[37]	NSCLC PD-L1 positive (all)	Nivolumab	Nivolumab *vs* CT[1]	I line	PFS	2016[2]	535
CheckMate 651[83]	H&N SCC	Nivolumab	Nivolumab + ipilimumab *vs* platinum + fluorouracil + cetuximab	I line	OS	2020	490
CheckMate 459[84]	HCC	Nivolumab	Nivolumab *vs* sorafenib	I line	TTP	2017	726
NCT02267343[85]	Gastric cancer	Nivolumab	Nivolumab *vs* placebo	From II line	OS	2017	480
NCT02569242[86]	Esophageal cancer	Nivolumab	Nivolumab *vs* docetaxel/paclitaxel	From II line	OS	2019	390
CheckMate 214[87]	Renal cell carcinoma	Nivolumab	Nivolumab + ipilimumab *vs* sunitinib	I line	PFS	2019	1070
CheckMate 143[88]	Glioblastoma	Nivolumab	Nivolumab *vs* bevacizumab	II line	OS	2017	440
CheckMate 141[89]	H&N SCC	Nivolumab	Nivolumab *vs* cetuximab/ methotrexate/docetaxel monotherapy	Any	OS	2018	360
CheckMate 227[90]	NSCLC	Nivolumab	Nivolumab *vs* nivolumab + ipilimumab *vs* nivolumab + platinum doublet CT[1]	I line	OS	2018	1980
CheckMate 451[91]	SCLC	Nivolumab	Nivolumab *vs* nivolumab + ipilimumab *vs* placebo after platinum based CT[1]	Maintenance after I line	OS	2018	810
CheckMate 498[92]	Glioblastoma (unmethylated MGMT)	Nivolumab	Nivolumab + RT *vs* temozolomide + RT	I line	PFS	2019	550
CheckMate 331[93]	SCLC	Nivolumab	Nivolumab *vs* topotecan/amrubicin	II line	OS	2018	480
CheckMate 078[94]	NSCLC	Nivolumab	Nivolumab *vs* docetaxel	From II line	OS	2018	500
NCT02339571[95]	Melanoma	Nivolumab	Nivolumab + ipilimumab ± sargramostim	I line	OS	2021	400
CheckMate 401[96]	Melanoma	Nivolumab	Nivolumab + ipilimumab *vs* nivolumab	I line	OS	2021	615

[1]According to the standard of care and basing on the choice of the investigator; [2]The trial has results but it is still unpublished. OS: Overall survival; PFS: Progression free survival; HNSCC: Head and neck squamous cell carcinoma; HCC: Hepatocarcinoma; NSqNSCLC: Non-squamous non-small cell lung cancer; SqNSCLC: Squamous non-small cell lung cancer; CT: Chemotherapy; NSCLC: Non-small cell lung cancer; MSI-H: High microsatellite instability; dMMR: Deficient mismatch repair; PLD: Pegylated liposomal doxorubicin; SCLC: Small cell lung cancer; TTP: Time to progression; ORR: Objective response rate.

ment, also first line setting has been investigated, with the planning of interesting trials currently still ongoing. In previously untreated RCC patients, atezolizumab in combination with bevacizumab is being compared to sunitinib[71]; the same standard of treatment is in turn compared to pembrolizumab combined with axitinib[102] and then to nivolumab plus ipilimumab[87]. Eventually, also avelumab plus axitinib is being investigated *vs*

sunitinib[55]. In all cases, the control arm is represented by such a big standard of therapy (sunitinib) that, in case of positive results, the clinical practice for RCC will completely change, switching from angiogenesis inhibition to immune-checkpoint blockade.

Urothelial cancers

Since no significant improvements have been achieved in metastatic bladder cancer for long time, the impressive results of recent trials with CKI, in particular with the anti-PD-L1 atezolizumab, have given new hope to finally cure urothelial cancer[103,104].

Atezolizumab is currently been approved for treatment of urothelial cancer on the basis of a randomized phase Ⅱ trial comparing this anti-PD-L1 with standard treatment, demonstrating its advantage over chemotherapy in both platinum pretreated ineligible patients and in chemotherapy pretreated patients[105]. At the same time, phase Ⅲ studies in second line setting are ongoing and both atezolizumab and pembrolizumab have been compared to different second line chemotherapeutic regimens in all urothelial cancers: The trial with pembrolizumab has been recently early stopped due to the meeting of the primary endpoint (OS)[40,62]. Also avelumab and durvalumab reached phase Ⅲ investigation in bladder cancer, but in the first line setting; the latter combined with the anti-CTLA4 tremelimumab *vs* standard first line chemotherapy[56,76]. A further interesting study in metastatic urothelial cancer is recruiting naive patients ineligible to cisplatin to receive atezolizumab in combination with chemotherapy (gemcitabine and carboplatin) as first line treatment[63].

Not less significant the promising evidence about the role of CKI in the adjuvant setting of urothelial cancer: Atezolizumab is under investigation *vs* only observation after cystectomy in PD-L1 positive high risk muscle-invasive bladder cancer[23] and also nivolumab is being tested in this setting[28].

Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) undoubtedly a promising candidate for CKI because of the profound immune suppression from which is characterized. As the matter of fact, a phase Ⅲ randomized study comparing nivolumab to the standard of treatment in pretreated HNSCC patients was early stopped after the clear demonstration of an improvement in terms of OS for nivolumab[89]. This trial provided very promising results in platinum refractory disease, encouraging the planning of further phase Ⅲ studies, currently ongoing, also for pembrolizumab[38,82] and early phases trials with durvalumab and avelumab[106].

Despite an apparently not so favorable toxicity profile, also anti-CTLA4 antibodies are being tested in combination with anti-PD-1 or anti-PD-L1 agents in HNSCC. Phase Ⅲ studies with this therapeutic strategy are currently ongoing both in pretreated patients and in first line setting[74,79].

Other tumors

The PD-1/D-L1 axis has been targeted in other tumor types than those cited above, with an interesting rationale and supported by phase Ⅰ-Ⅱ experiences, despite still remaining in shadow waiting for phase Ⅲ results.

In ovarian cancer, despite several early phase studies currently ongoing with nivolumab, pembrolizumab, BMS936559 (an anti-PD-L1) and avelumab, the emerged response rates are relatively low, in front of a manageable safety profile[53,54,107].

Pembrolizumab, aside from early investigations in soft tissue and bone sarcomas[108], is currently under phase Ⅲ investigation in hepatocellular carcinoma[42], in esophageal and gastric carcinoma[50-52], in Hodgkin and non-Hodgkin lymphoma[39].

In these latter malignancies also nivolumab and pidilizumab, anti-PD-1 antibodies, besides from atezolizumab and durvalumab, anti-PD-L1 antibodies, are being evaluated in early phases[109]. Furthermore, different treatment lines of advanced gastric cancer are being tested with avelumab[57,58].

Some initial encouraging data are emerging from ongoing studies in favor of the employment of CKI also in central nervous system (CNS) malignancies, such as glioblastoma, where unmet clinical needs are leading to new investigations[88,92]. Disappointing results were instead obtained for pancreatic cancer, despite a certain evidence for durvalumab[110].

About colorectal cancer, despite the initial evidence to be not responsive to nivolumab, a subset of patients has been identified as potentially best responders to pembrolizumab, revealing that the mismatch repair (MMR) status can predict clinical benefit with enhanced responsiveness in tumors with microsatellite instability (MSI)[111]. With this rationale, phase Ⅲ randomized studies have been initiated in order to compare standard therapy with pembrolizumab in MSI colorectal cancer patients[49]. Furthermore, atezolizumab is currently under investigation alone or in combination with cobimetinib (mitogen activate protein kinase-inhibitor) *vs* regorafenib (antiangiogenic multi-kinase inhibitor) in all advanced colorectal tumors[73].

Eventually, a great interest for PD-1/PD-L1 blockade is represented by triple negative breast cancer: Phase Ⅲ trials are currently ongoing with pembrolizumab compared to chemotherapy and with atezolizumab combined with nab-paclitaxel both in neo-adjuvant and advanced setting[47,48,70,112].

Transversal challenges

Immune-related toxicity: The management of the "new toxicities" of CKI is transversal to all malignancies and to all cited antibodies, unavoidably involving other specialists beyond the oncologist, such as the endocrinologist and the immunologist in first line.

These immune-related adverse events (irAEs) are due to the infiltration of tissues by activated T-lymphocytes responsible of autoimmunity. As a consequence, the block of the immune-checkpoint can amplify any immune response in all organs: Skin, gastrointestinal tract, endocrine glands, lung, CNS, liver, kidney, hematological cells, muscular-articular system, heart and eyes can all be affected. Nevertheless, most of these irAEs are rare and only fatigue, rash, pruritus, diarrhea, nausea and arthralgia occurs in > 10% of cases. On the other hand, despite being rare, interstitial pneumonitis is the main life-threatening toxicity for anti PD-1/PD-L1 agents[113].

Potentially predisposing conditions for irAEs development could be represented by personal or family history of autoimmune disease (genetic determinants), by underlying silent autoimmunity, chronic viral infections or other personal ecological factors (such as the microbiome in the case of enterocolitis)[114].

The prevention, the anticipation, the detection and then the treatment (with multidisciplinary approach) and monitoring of irAEs are the principles of their correct clinical management. Depending on their severity, irAEs require temporary or permanent discontinuation of CKI therapy, use of high doses corticosteroids or, in severe cases, of anti-TNF treatment with infliximab. The current management guidelines are based on recent expert consensus recommendations published about the issue[115].

Response assessment: RECIST *vs* immune-related criteria: Based on survival analysis, traditional response evaluation criteria in solid tumors (RECIST) might underestimate the benefit of CKI[116].

The pattern of response of immunotherapy, radically different from those of standard chemotherapy and also of antiangiogenic agents, is frequently not captured by the conventional RECIST[117]. This led to the development of the immune-related response criteria (irRC)[118], assessing tumor burden as a continuous variable and evaluating percentage changes in several target lesions overtime. In this system, the appearance of new lesions does not mean progressive disease but it is considered and reassessed in the context of a dynamic evaluation. Moreover, the thresholds to determine progression or response (25% increase and 50% decrease) are higher than those of RECIST (20% increase and 30% decrease)[119]. Given the reported evidence, modified criteria are undoubtedly mandatory in the response assessment to the new immunotherapy, in order to prevent premature discontinuation of treatment.

PD-L1 expression as response predictor: In the context of solid tumors treated with PD-1/PD-L1 inhibitors, the predictive role of PD-L1 expression on tumor cells and, as more recently discovered, on immune infiltrating cells, represents an actual issue of great interest and constitutes a significant cue of discussion for clinical researchers[120].

Currently, on the basis of the state of art, the predictive value of PD-L1 on tumor cells is limited to NSCLC and melanoma, especially for anti-PD-1 antibodies, whilst a more predictive significance of PD-L1 expression on the immune cells infiltrating the tumor seems to emerge for urothelial cancers in the case of anti-PD-L1 antibodies[121,122]. Nevertheless, a great limit of such speculations is represented by the scarce reliance and reproducibility of the different methods used for the biomarker's detection, with controversial results depending on the staining technique, on the different anti-PD-L1 antibodies and finally on the sample used for immune-histochemical assay (primary tumor *vs* metastases samples, with the challenge of heterogeneity). Moreover, confusing data emerged from the use (and the lack of validation) of different cut-off for PD-L1 expression, from 1%, to 5%, to 50% threshold in different trials[120].

Aside from PD-L1 expression, further multiple factors have been explored and are currently undergoing investigations as predictive elements for response to CKI: Among these, an increasing interest is being acquired by the micro-environmental features of the tumor, such as the infiltrating immune cells sub-populations and their biomarkers expression[123].

Microsatellite instability and hyper-mutational status: The MSI phenotype, as a consequence of a defective DNA-MMR system, characterizes a subgroup of tumors harboring a large number of somatic mutations (high mutational load). Since these mutations have the potential to encode a great number of immunogenic neoantigens, a particular susceptibility of MSI-hypermutated cancers to PD-1/PD-L1 axis blockade have been hypothesized and more recently proven[124]. As the matter of fact, MSI tumors have a microenvironment characterized by abundant T-cell infiltrate, with activated $CD8^+$ cytotoxic T lymphocyte (CTL) and activated Th1 producing IFN-γ, high expression of PD-L1 (in particular by TILs and myeloid cells infiltrating the tumor) and great overexpression of immune-checkpoint related proteins[125]. All these elements configure the elective candidate cancer for immune-checkpoint inhibition and suggest to investigate CKI in all cancer types with MMR defects.

Additionally, tumors with polymerase E (POLE) mutations, despite stable microsatellites, have been demonstrated to contain a high mutational load. Also these POLE-ultra-mutated cancers are characterized by an active Th1/CTL microenvironment and upregulated immune checkpoints, constituting an ideal target for CKI therapy as well as MSI tumors[126].

In conclusion, among apparently resistant cancer types (such as colon cancer), CKI have been proven to exert an effect in case of MMR defects and trials on this selected population are currently ongoing to investigate

the efficacy of anti-PD-1 antibodies[49].

Immune system modulation with sequential or association strategies: Given the great benefit in terms of OS and the long lasting impact of CKI therapy on patients' survival in the responding cases, probably due to immunological memory, two major issues remain to be addressed: The sensitization of non-responders and the disease control in patients initially pseudo-progressive. With these aims, combination strategies have been planned and investigated in the last years, either combining immunotherapy with chemotherapy, radiotherapy and targeted agents or associating different CKI[127].

The strategy to increase the immunogenicity of tumors can be pursued through the enhancement of antigen presentation (boosting antigens release or stimulating APC function), the stimulation of major histocompatibility complex (MHC) class Ⅰ expression, the down-regulation of the T-reg cells and the stimulation of the T-cells infiltration. Some of these mechanisms can be achieved with promising combination strategies.

Chemotherapeutic agents are capable to induce immunogenic cancer death, generating a strong immune stimulation. Among these, cyclophosphamide have additionally been shown to reduce the number of circulating T-reg cells, removing a key element of immunosuppression, and moreover to sensitize tumor cells to T-cell mediated apoptosis, potentially boosting the effect of the immune checkpoint blockade[128-130]. Considering the criticism of a combination between CKI and chemotherapy, given expected short term immunosuppressive effect of the latter, in our opinion a sequential strategy could represent a good opportunity to take advantage of cell death and antigen release caused by an induction chemotherapy, in order to prepare a more immunogenic environment for the subsequent CKI[131].

A great interest for the potential stimulation of the immune-response through radiotherapy has been suggested by the evidence about the immune-mediated abscopal effect[132]. Aside from interesting case reports, clinical trials in this field are currently in early phases and eagerly awaited[133].

Targeted therapy combinations with immunotherapy are currently under investigation, in early phases, with interesting results[127]. The rationale of such strategies could be represented by the aim to obtain a more rapid RR and to boost PFS with the targeted agent, in expectation of the long-term effect on survival of the CKI.

Finally, the combination of anti-PD-1 and anti-CTLA4 antibodies, despite the increased immune-related toxicity, has been shown to improve the outcomes in a phase Ⅲ randomized trial in metastatic melanoma, early changing the standard of treatment a few years after the onset of the new immunotherapy with ipilimumab[134]. Several trials investigating such association of CKI are currently ongoing: The management of irAEs will probably represent the main criticism of such strategies[127].

Targeting PD-1/PD-L1 axis in adjuvant setting: The rationale for the PD-1/PD-L1 axis inhibition for adjuvant purposes is in the concept of "immunological memory", generated by the cancer-immunity cycle, starting from the release of cancer cell antigens also in the early phases of tumorigenesis. After the APC migration in the lymph nodes and the presentation of antigens in the context of MHC-I molecules to $CD8^+$ T cells, aside from effector T-lymphocytes capable of activation against cancer neo-antigens, memory T-cells are also generated. These quiescent lymphocytes are appointed to the subsequent immune-response and could contribute to avoid disease relapse[135].

Considering the widely acceptable toxicity profile of CKI, the proposal of using them as adjuvant therapy, to prevent relapses after surgery of early disease while maintaining a good quality of life, appears very favorable. In support of this, we have the approval of the CTLA4 inhibitor ipilimumab for adjuvant treatment in melanoma, on the basis of a recent pivotal trial[136]. For PD-1/PD-L1 axis inhibitors, nevertheless, the investigation in adjuvant setting is quite early, in spite of a more favorable safety management. A noteworthy issue about immune-adjuvant treatment with these compounds (unlike the case of ipilimumab) is the correct duration of therapy, ranging from one to more years in different planned trials. The currently ongoing studies are reported in Table 2.

PERSPECTIVES

Considering the wide range of settings and combinations covered by the ongoing clinical trials with CKI treatment, we think that the future directions for immunotherapy are still to be written and they are probably different basing on cancer types. The reason of this latter statement, not so obvious as it may seem, is likely due to the other different therapies to whom immune-checkpoint blockade needs to be sequenced and alternated in each tumor, more than to a real difference in the target, which is always represented by the immune system and by its relationship with the tumor rather than by the tumor itself.

From this point of view, a key issue could be represented by the immunomodulating potential of the current standard of treatment in each case, sometimes widely unknown and rarely explored before the "immunotherapy era"[137].

The great advantage of anti-PD-1/PD-L1 agents is undoubtedly represented by their very favorable safety profile, with large tolerability in almost all patients. Combinations of CKI with standard chemotherapy or targeted therapies, despite possibly more effective, have the risk of became unsustainable both in terms of costs and of toxicity, significantly impacting on the final outcome. Nevertheless, alternating targeted and

immunotherapy might permit to modulate tumor metabolism, inflammation and immune infiltration, allowing to modify the relationship between cancer and immune system.

Thus, in order to fully take advantage of its potential, the winning strategy with immune-checkpoint blockade could be represented by an ingenious sequence, exploiting the immunomodulating properties of previous and subsequent drugs with the aim of boosting immune system activation against the tumor.

CONCLUSION

The onset of PD-1/PD-L1 targeted therapy has demonstrated the importance of this axis in the immune escape across almost all human cancers. Despite being burdened by some issues not still addressed, such as the correct duration of therapy in the responsive patients, the new CKI allowed to significantly prolong survival and to generate durable response, demonstrating remarkable efficacy in a wide range of cancer types. However, such benefit is not extended to all patients, and some of them experienced immune escape despite therapy. The investigation about mechanisms leading to the development of primary or secondary immune escape must represent the key element of future studies in the whole immuno-oncology, with the aim of resensitize these patients to the immune checkpoint blockade. The future approach to the problem may be represented by a personalized cancer immunotherapy, allowed only by multiparameter biomarkers approaches, as interestingly suggested by Kim *et al*[138] in a recent review about the "step to success (or failure)" to PD-1/PD-L1 blockade. In their proposal, a hypothetical algorithm could provide the assessment of specific immune-related biomarkers in each patient's tumor, allowing to create a personal mapping according to which characteristics the oncologist could chose (or exclude) the optimal immunotherapy or immunotherapeutic combination for each single case.

Waiting for the possible realization of such sophistication of therapy, the immune checkpoint blockade in oncology is currently experiencing promising huge advances, shifting the classical paradigm of anticancer treatment from targeting the tumor to targeting the immune system and increasing our hopes to gain the immune control of oncological disease.

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ORIGINAL ARTICLE

Basic Study

Nanoparticle-linked antagonist for leptin signaling inhibition in breast cancer

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Abstract

AIM

To develop a leptin peptide receptor antagonist linked to nanoparticles and determine its effect on viability of breast cancer cells.

METHODS

The leptin antagonist, LPrA2, was coupled *via* EDAC [1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide] to iron oxide nanoparticles (IONP-LPrA2) to increase its efficacy. IONP-LPrA2 conjugation was confirmed by Western blot and nanoparticle tracking analysis. Human triple negative breast cancer (TNBC) MDA-MB-231, HCC1806 and estrogen receptor positive (ER^+) MCF-7 cells were analyzed for the expression of the leptin receptor, Ob-R. The effects of leptin and antagonist on levels of leptin-induced STAT3 phosphorylation and cyclin D1, cell cycle progression, cell proliferation, and tumorsphere formation in breast cancer cells were determined. Doses of the chemotherapeutics [cisplatin

(Cis), cyclophosphamide (CTX), doxorubicin (Dox) and paclitaxel (PTX)] to effectively reduce cell viability were calculated. The effects of combination treatments of IONP-LPrA2 and chemotherapeutics on cell viability were determined.

RESULTS

Western blot analysis of coupling reaction products identified IONP-LPrA2 at approximately 100 kD. IONP-LPrA2 significantly decreased leptin-induced pSTAT3 levels in HCC1806 cells and drastically decreased cyclin D1 levels in all cell lines. IONP-LPrA2 significantly reduced leptin-induced S phase progression and cell proliferation in all breast cancer cell lines and the formation of tumorspheres in MDA-MB-231 cells. Also, IONP-LPrA2 showed an additive effect on the reduction of breast cancer cell survival with chemotherapeutics. Cis plus IONP-LPrA2 produced a significant reduction in the survival of MDA-MB-231 and HCC1806 cells. CTX plus IONP-LPrA2 caused a significant decrease in the survival of MDA-MB-231 cells. Dox plus IONP-LPrA2 caused a marked reduction in the survival of HCC1806 cells. Although, PTX plus IONP-LPrA2 did not have a major effect on the viability of the breast cancer cells when compared to PTX alone.

CONCLUSION

Present data indicate that IONP-LPrA2 may be a useful adjuvant for chemotherapeutic treatment of breast cancer, particularly for TNBC which lacks targeted therapeutic options.

Key words: Triple negative breast cancer; Obesity; Leptin; Leptin peptide receptor antagonist 2; Iron oxide nanoparticles; Chemotherapy adjuvant

Core tip: Breast cancer is the second leading cause of cancer deaths in women. Triple negative breast cancer is an aggressive subtype that lacks targeted therapy. Obesity is a risk factor for breast cancer and is associated with high leptin levels. Leptin induces the expression of cell cycle associated proteins advancing cell cycle progression. Leptin also increases breast cancer stem cell growth, which promotes chemotherapeutic resistance. We have developed a leptin antagonist linked to iron oxide nanoparticles (IONP-LPrA2) which significantly inhibits leptin-induced cell proliferation and survival of breast cancer cells treated with chemotherapeutics. IONP-LPrA2 can increase chemotherapeutic efficacy in breast cancer.

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INTRODUCTION

The American Cancer Society estimates that there will be nearly 300000 new breast cancer cases diagnosed worldwide and approximately 50000 women will die from breast cancer in 2016[1]. Triple negative breast cancer (TNBC) accounts for 15% of all breast cancer diagnoses. TNBC is a subtype of breast cancer characterized by the lack of expression of the estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor-2 (HER2)[1,2]. Due to the absence of receptor expression; this form of breast cancer, which predominantly affects younger, African American and Hispanic patients lacks targeted therapeutic options[3]. TNBC patients are commonly treated with chemotherapy; however these patients make up approximately 30% of breast cancer-related deaths annually[4]. This necessitates the development of targeted therapies for this more aggressive form of the disease.

There are many factors that increase the risk of developing TNBC including environment, genetic susceptibility, and obesity[5]. Obesity has a negative impact on breast cancer patient survival and, like TNBC, is associated with an increased risk of recurrence[6]. Obesity is correlated to high levels of leptin, a cytokine produced by adipose tissue which regulates satiety. The leptin signaling pathway occurs in approximately 80% of breast cancers[7]. The binding of leptin to its receptor, Ob-R, leads to activation of pathways involved in cell proliferation, migration, and survival[8]. Leptin is a survival factor in breast cancer and may have the ability to limit the effectiveness of chemotherapy drugs by activating the JAK2/STAT3, MAPK/ERK, and PI3/Akt signaling pathways[9,10]. Therefore leptin signaling inhibition has become a promising therapeutic area for breast cancer, particularly in the case of TNBC for which there is no targeted therapy[11].

The binding of leptin to Ob-R upregulates Notch, interleukin 1 (IL-1), vascular endothelial growth factor (VEGF), and its receptor VEGFR2; which promote breast cancer cell survival and angiogenesis[12]. The harmful effects of leptin signaling on breast cancer onset and progression have been shown to be diminished by the leptin peptide receptor antagonist 2 (LPrA2)[13]. LPrA2 and the pegylated form (PEG-LPrA2) have been shown to cause a delay in cancer onset and progression as well as a reduction in 4T1-tumor growth in BALB/C mice[14]. Additionally, PEG-LPrA2 has been shown to decrease MDA-MB-231 and MCF-7-tumor growth in SCID mice[15]. In another study, diet-induced obese (DIO) C57BL/6J mice treated with the carcinogen 7, 12-Dimethylbenz (a) anthracene (DMBA) along with PEG-LPrA2 did not develop tumors[16]. The anti-tumor activity of LPrA2 provides mounting evidence for its usefulness in cancer therapy.

The leptin signaling pathway plays a major role in breast cancer cell growth, angiogenesis, as well as metastasis and invasion[8]. Although the leptin

antagonist LPrA2 attenuates leptin signaling, it is limited by its insolubility in water and short half-life of 1-2 h[14,17,18]. Here we describe the coupling of LPrA2 to a nanoparticle delivery system which uses iron oxide nanoparticles (IONPs) to capture multiple LPrA2 peptides. We assessed the conjugation of LPrA2 to IONPs (IONP-LPrA2) to determine the inhibitory effect on breast cancer cell growth and survival. Because LPrA2 decreases breast cancer tumor growth and chemotherapy is widely used in the treatment of breast cancer, we sought to assess if combining IONP-LPrA2 and chemotherapeutic drugs would allow for reduction of the effective dose. Thus, we evaluated the survival of human breast cancer cell lines with IONP-LPrA2 and a panel of anti-cancer drugs.

MATERIALS AND METHODS

Reagents and antibodies

IONPs were obtained from Ocean Nanotech San Diego, CA. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), Sulfo-NHS, and other chemicals were purchased from Sigma Aldrich St. Louis, MO. Ob-R (sc-8325), Cyclin D1 (sc-246), pSTAT3 (sc-8059), STAT3 (sc-8019) antibodies were purchased from Santa Cruz Biotechnology Santa Cruz, CA. Anti-rabbit and anti-mouse conjugated to horseradish peroxidase were obtained from Bio-Rad Laboratories Hercules, CA. Dulbecco's Modified Eagles Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMEM), Protease and Phosphatase Inhibitor cocktails, Penicillin/Streptomycin, Slide-a-lyzer dialysis cassette, and Western blotting chemiluminescence substrate were purchased from Thermo Fisher Scientific Rockford, IL. Mammocult complete medium was obtained from Stem Cell Technologies Vancouver, BC. Fetal bovine serum was obtained from Med Supply Partners Atlanta, GA. Leptin was purchased from R and D Systems Minneapolis, MN. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from Molecular Probes Eugene, OR. Annexin V/fluorescein Isothiocyanate (FITC) and propidium iodide (PI) were obtained from Nexcelom Bioscience Boston, MA. Cisplatin (Cis) was purchased from Millipore Billerica, MA. Cyclophosphamide (CTX), paclitaxel (PTX), and doxorubicin (Dox) were obtained from SelleckChem Houston, TX.

Nanoparticle conjugation

LPrA2 was synthesized as described[8,19]. LPrA2 was desalted using the slide-a-lyzer dialysis cassette (Thermo Fisher). LPrA2 was conjugated to IONPs (Ocean Nanotech) by the outlined method[20].

Western blot analysis

IONP-LPrA2 was separated by SDS-PAGE. LPrA2 and LPrA2-Scramble (Sc) were used as positive and negative controls, respectively. The peptides were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were probed with an LPrA2 antibody, purified from antigen injected rabbit bleeds. Anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) was used for further detection of the peptides. Chemiluminescent detection of the bands was displayed by Western blotting substrate (Thermo Fisher).

Nanoparticle tracking analysis

Dilutions of 1:10000 of the bound and unbound particles were sonicated for 30 min. The size and distribution of the conjugated and unconjugated IONPs were determined by the NanoSight (Malvern Instruments Ltd., Worcestershire, United Kingdom).

Cell culture

Human ER$^+$ MCF-7 cells in addition to TNBC MDA-MB-231 and HCC1806 cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured in DMEM (Thermo Fisher) with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher) and maintained in an incubator at 37 ℃ with 5% CO_2.

Cell lysis and immunoblotting analysis

Cells were seeded at 2 × 10^5 in 6 well cell culture plates and grown to 70%-80% confluence. The cells were treated with leptin (1.2 nmol/L) (R and D Systems), or IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. Basal cells served as untreated controls. The cells were lysed with RIPA buffer (Sigma) containing protease/phosphatase inhibitors (Thermo Fisher). Proteins were pulled down by Immunoprecipitation. Immunoblotting analysis was performed as described[21]. The membranes were incubated with Ob-R, cyclin D1, pSTAT3, and STAT3 (Santa Cruz Biotechnology) antibodies overnight at 4 ℃. GAPDH (Sigma) was used as a protein loading control. Relative protein levels were determined by Image J software (National Institute of Health, NIH).

Cell cycle analysis

Cells were seeded at 2 × 10^5 in 6 well cell culture plates and grown to 70%-80% confluence. They were treated with IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) at indicated concentrations for 24-48 h. Leptin and unconjugated LPrA2 served as controls. The cells were trypsinized, washed with 1 × PBS, and resuspended in cold 100% methanol (Sigma). The were stored at -20 ℃ prior to analysis (< 1 wk). Afterward, the cells were centrifuged to remove the methanol. They were resuspended in 50 μL PI (Nexcelom) and incubated at 37 ℃ for 40 min. The cells were centrifuged to remove the PI, resuspended in 1 × PBS, and analyzed by the Nexcelom Cellometer Vision® image based cytometer to determine the percentage of cells in the S phase of the cell cycle.

MTT assay

Cells were seeded at 5 × 10^3 in 96 well cell culture plates and grown to 70%-80% confluence. The cells were treated with leptin (1.2 nmol/L), or IONP-LPrA2

(0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. Basal cells served as untreated controls. The media was removed from the cells, the wells were washed with 1 × PBS, and 200 μL of IMEM (Thermo Fisher) together with 10 μL of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5 mg/mL in PBS, (Molecular Probes) were added. The plates were incubated for 4 h at 37 ℃. Following incubation the media was removed, 50 μL of Dimethyl sulfoxide was added to the wells, and the plates were incubated at 37 ℃ for 30 min. The absorbance was read at 540 nm using a microplate reader (Molecular Devices) to measure cell proliferation.

Tumorsphere formation

MDA-MB-231 cells were seeded at 5 × 10^3-2 × 10^4 cells/mL in low attachment plates and grown for 1-2 wk in Mammocult complete medium (Stem Cell Technologies) supplemented with heparin and hydrocortisone and treated with leptin (1.2 nmol/L), or IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L). Basal tumorspheres served as untreated controls. The tumorspheres were visually assessed by light microscopy. The size of the tumorspheres were determined and the number of tumorspheres were counted manually in triplicate.

Apoptosis assay

Cells were seeded at 2 × 10^5 in 6 well cell culture plates and grown to 70%-80% confluence. They were treated with the chemotherapeutic drugs: Cis (Millipore), CTX, PTX, and Dox (SelleckChem) in 5% FBS with or without IONP-LPrA2 for time periods ranging from 1-6 d. Before trypsinizing, the supernatants were transferred into microfuge tubes for subsequent analysis. The trypsinized cells were added to the supernatants and centrifuged. The pellets were washed with 1 × PBS and resuspended in Annexin V binding buffer (Nexcelom). Annexin V/FITC, and PI, 5 μL each (Nexcelom) were added with mixing. The samples were incubated in the dark at room temperature for 15 min. The cells were washed with 1 × PBS, centrifuged, and resuspended in Annexin V binding buffer to a concentration of 3 × 10^4 cells per 20 μL. The samples were analyzed by the Cellometer Vision. The viability was determined by multiplying the percentage of live cells by the total cell count.

Statistical analysis

All experiments were performed in triplicate. One-way ANOVA (SigmaPlot) was used to determine statistical significance among treatment groups and controls. Data presented as the average ± standard deviation (SD). *P* values of $P < 0.05$ were considered statistically significant.

Biostatistics statement

The statistical review was performed by Ward Kirlin, PhD. The appropriate ANOVA of variance was performed on the data presented in this paper, and levels of statistical significance are based on the F-values and Tukey's multiple comparisons between group means as determined using SigmaPlot (Systat Software, Inc.). Mean + SDs are indicated in the graphical analysis, based on replicates of densitometry analysis of Western blots, the percentage of cells in S-phase of the cell cycle, or percentage of proliferating cells as indicated in the figures.

RESULTS

Generation and characterization of IONP-LPrA2

The leptin antagonist, LPrA2, has been shown to inhibit breast cancer growth and progression *in vitro* as well as *in vivo*[2,22,23]. To increase its efficacy, LPrA2 was conjugated to IONPs. IONPs are amphiphilic and have a 10 nm core[20]. The binding of LPrA2 to IONPs was facilitated by EDAC, which activates the carboxyl group on the IONP surface and allows the formation of an amide bond with the amino group of LPrA2 (Figure 1A). To confirm the binding of the LPrA2 peptides to the nanoparticles, the conjugates were analyzed by SDS-PAGE and Western blot. With LPrA2 antibody incubation, bands were detected at approximately 100 kD, indicating conjugated LPrA2, and approximately 3 kD indicating unbound LPrA2. Unconjugated LPrA2 and LPrA2-Sc were used as positive and negative controls, respectively (Figure 1B). To further characterize IONP-LPrA2, 1:10000 dilutions of the conjugated and unconjugated IONPs were measured by NanoSight nanoparticle tracking analysis (Malvern); in which the left and right Y-axes show particle number and percent distribution, and the X-axis displays particle size. The size of the unconjugated IONP was found to be 14 nm while conjugated IONP-LPrA2 measured 20 nm. This data suggests that the conjugation of LPrA2 to IONPs was successful.

Ob-R expression and effect of IONP-LPrA2 on leptin-induced pSTAT3 and cyclin D1 levels in human breast cancer cells

In order to determine the effects of IONP-LPrA2 on leptin signaling inhibition, we first had to confirm expression of the leptin receptor, Ob-R, in the human breast cancer cell lines. Immunoprecipitation and subsequent Western blot analysis showed Ob-R expression in MDA-MB-231, HCC1806, and MCF-7 cells (Figure 2A). Leptin signaling activates the JAK2/STAT3, MAPK/ERK, and PI3/Akt signaling pathways, which are implicated in its anti-apoptotic activity[9]. For this reason, we aimed to determine the effect of IONP-LPrA2 treatment on active/phosphorylated, pSTAT3. Leptin significantly increased the level of pSTAT3 in MDA-MB-231 and HCC1806 cells. IONP-LPrA2 significantly inhibited the effect of leptin on pSTAT3 levels in HCC1806 cells. No significant changes occurred in pSTAT3 levels in MCF-7 cells treated with

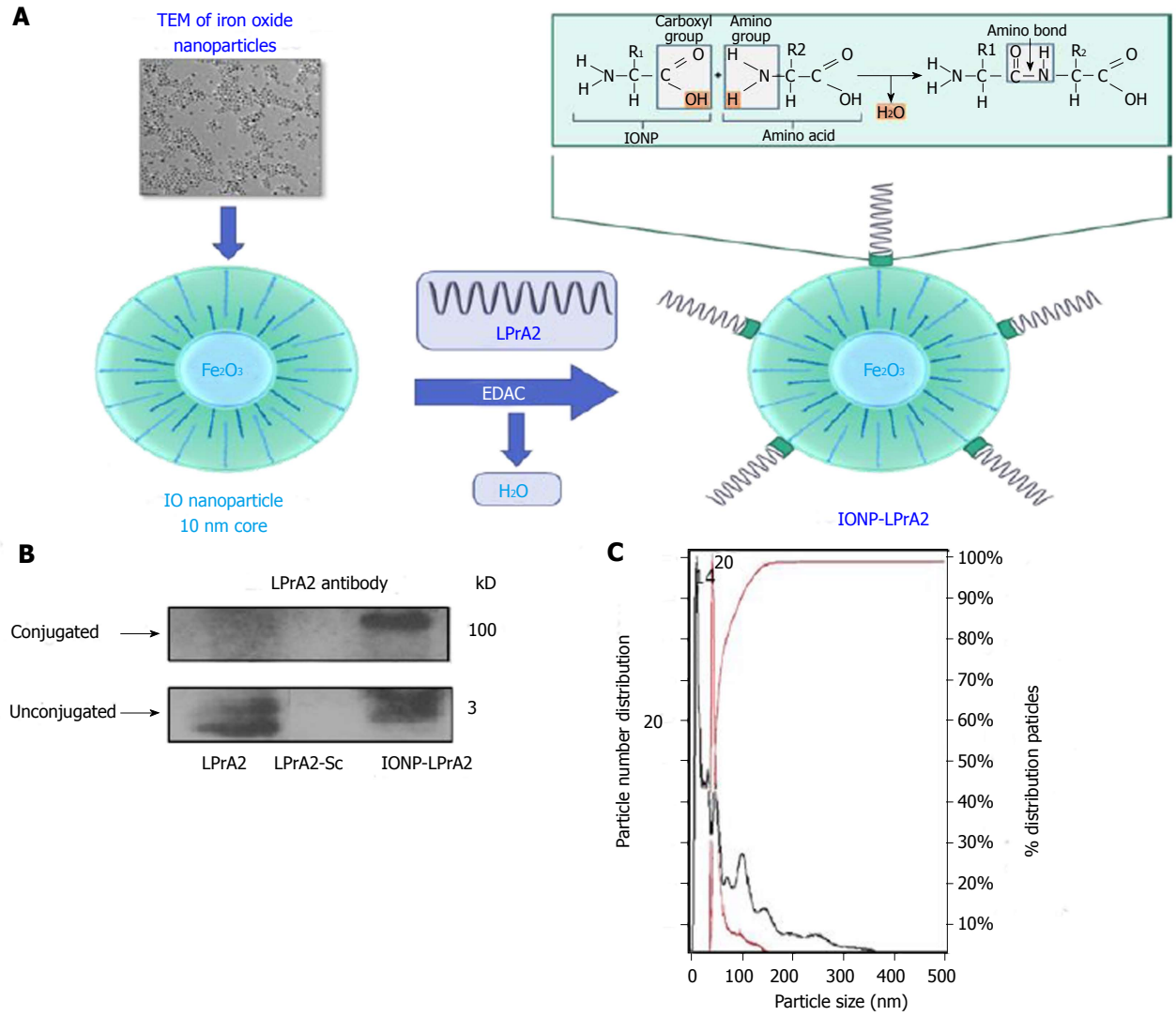

Figure 1 Generation and characterization of iron oxide nanoparticles-LPrA2. A: Conjugation of iron oxide nanoparticles (IONP)-LPrA2. LPrA2 was conjugated to IONPs *via* 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), which activates the carboxyl group on the IONP surface allowing it to form a covalent bond with the amino group of LPrA2 (displayed by TEM, transmission electron microscopy, Ocean Nanotech); B: Western blot confirmation of IONP-LPrA2 conjugation. Conjugated IONP-LPrA2 (100 kD) was detected by Western blot with an LPrA2 antibody, purified from antigen injected rabbit bleeds. Unconjugated LPrA2 (3 kD) and the scrambled peptide LPrA2-Sc (3 kD) served as positive and negative controls, respectively; C: NanoSight analysis of unconjugated and conjugated IONPs. The particle size of unconjugated IONP (14 nm) shown in black and the conjugated IONP-LPrA2 (20 nm) shown in red were determined by nanoparticle tracking analysis. The hyperbolic curve shows that the particles are 100% homogeneous.

leptin and IONP-LPrA2 (Figure 2B and C). Because leptin has been shown to increase cyclin D1 levels in breast cancer cells[14,15], we sought to determine the effect of IONP-LPrA2 treatment on cyclin D1 expression in MDA-MB-231, HCC1806, and MCF-7 breast cancer cells. Leptin significantly induced cyclin D1 expression in all cell lines (Figure 2B and C). The addition of IONP-LPrA2 significantly inhibited the effect of leptin on cyclin D1 expression in all cell lines (Figure 2B and C). These results suggest that IONP-LPrA2 abrogates the effect of leptin on leptin-induced signaling pathways.

IONP-LPrA2 inhibits leptin-induced cell cycle progression of human breast cancer cell lines

Leptin has been shown to increase expression of the cell cycle associated protein, cyclin D1[14,15]. To illustrate the effect of leptin on cell cycle progression, the number of cells in the S phase was determined by cell cycle analysis with the Cellometer Vision (Nexcelom). MDA-MB-231, HCC1806, and MCF-7 human breast cancer cells lines were treated with leptin (1.2 nmol/L) and IONP-LPrA2 plus leptin in order to determine its antagonistic effect. The cells were treated with IONP-LPrA2 concentrations ranging from 0.0018-0.036 pmol/L. MDA-MB-231 and HCC1806 TNBC cell lines were treated for 24 h while the ER^+ MCF-7 cells were treated for 48 h to produce an effect. Treatment with leptin caused a significant increase in cell cycle progression in HCC1806 (Figure 3B) and MCF-7 (Figure 3C), but had no significant effect on MDA-MB-231 cells (Figure 3A). Treatment with IONP-LPrA2 plus leptin abrogated leptin-induced cell cycle progression at 0.0018-0.0036 pmol/L

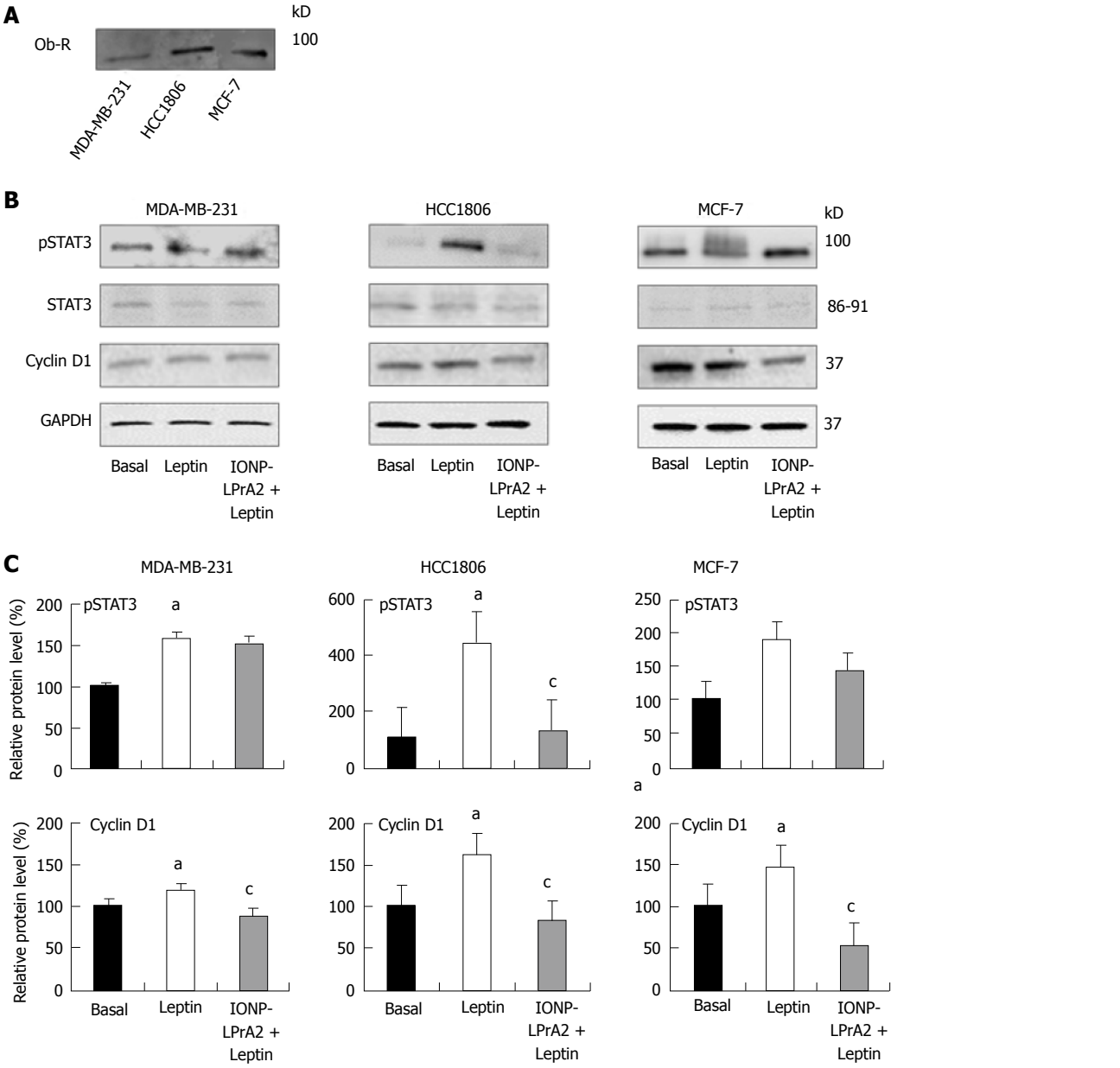

Figure 2 Ob-R expression and effect of iron oxide nanoparticles-LPrA2 on leptin-induced pSTAT3 and cyclin D1 levels in human breast cancer cells. A: Detection of Ob-R expression. The expression of Ob-R was detected by Western blot in MDA-MB-231, HCC1806, and MCF-7 cells; B: Iron oxide nanoparticles (IONP)-LPrA2 inhibition of leptin-induced pSTAT3 and cyclin D1 levels. Lysates were obtained from MDA-MB-231, HCC1806, and MCF-7 cells treated with leptin (1.2 nmol/L) or IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. pSTAT3 and cyclin D1 levels were detected by Western blot. STAT3 served as a loading control for pSTAT3. GAPDH served as a loading control for cyclin D1; C: Densitometric analysis of pSTAT3 and cyclin D1 levels. Graphs represent quantitative analysis of pSTAT3 and cyclin D1 levels in MDA-MB-231, HCC1806, and MCF-7 cells with Image J software. Relative protein level was significantly increased in leptin treated cell lines compared to basal (untreated) cells, $^{a}P < 0.05$. Relative protein level in cells pretreated with IONP-LPrA2 and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

in MDA-MB-231, at 0.0018-0.036 pmol/L in HCC1806, and at 0.0018-0.0072 in MCF-7 cells (Figure 3). This data elucidated the effective dilution of IONP-LPrA2 for abrogation of leptin-induced cell cycle progression in each of the cell lines.

IONP-LPrA2 inhibits leptin-induced cell proliferation in human breast cancer cells

Leptin signaling stimulates breast cancer cell survival and proliferation[8]. To ascertain the manner in which IONP-LPrA2 affects cell proliferation, an MTT assay was performed. MDA-MB-231, HCC1806, and MCF-7 cell were treated with leptin (1.2 nmol/L) and IONP-LPrA2 (0.0036) plus leptin (1.2 nmol/L). Leptin treatment significantly increased cell proliferation and IONP-LPrA2 significantly diminished the effect of leptin in all of the cell lines (Figure 4). This data indicates that IONP-LPrA2 prevents leptin induction of cell proliferation.

IONP-LPrA2 decreases MDA-MB-231 tumorsphere formation

Self-renewal is a hallmark of cancer. Leptin has been

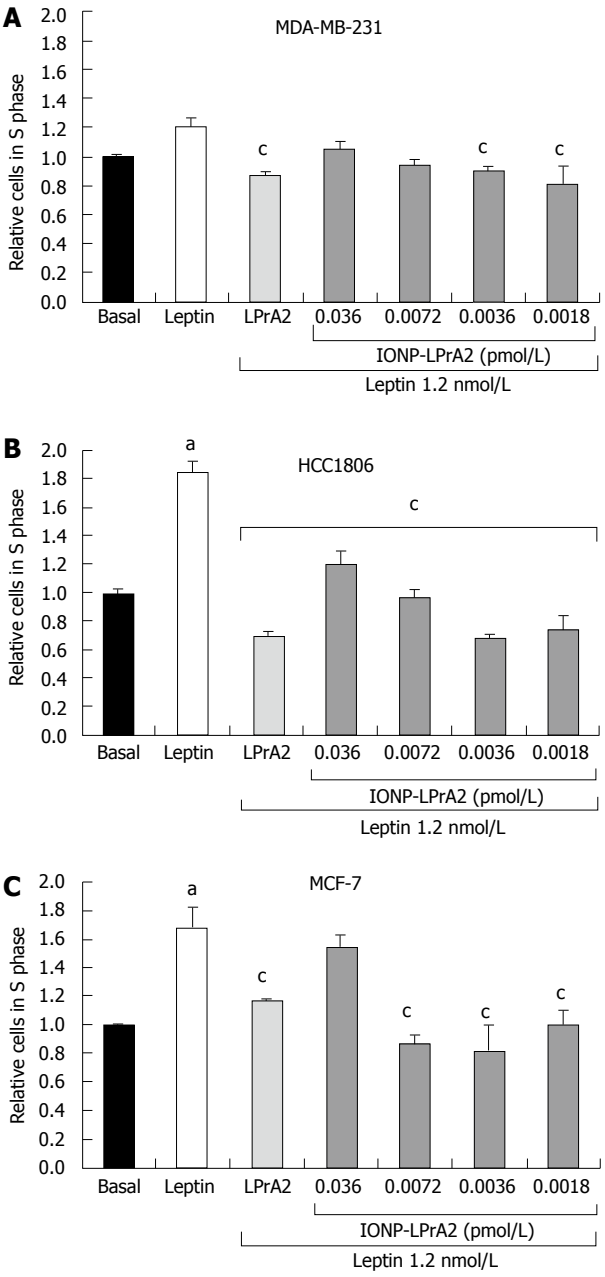

Figure 3 Iron oxide nanoparticles-LPrA2 inhibits leptin-induced cell cycle progression of human breast cancer cell lines. Iron oxide nanoparticles-LPrA2 inhibits S phase progression in breast cancer cells. A: MDA-MB-231; B: HCC1806; C: MCF-7. The cells were seeded in 6 well plates and treated with leptin (1.2 nmol/L), LPrA2 (1.2 nmol/L) plus leptin (1.2 nmol/L), or IONP-LPrA2 at indicated concentrations plus leptin (1.2 nmol/L) for 24-48 h. The percentage of cells in S phase was determined by cell cycle analysis, a measure of propidium iodide (PI) fluorescence. Relative percentage of cells in S phase was significantly increased in leptin treated cell lines compared to basal (untreated) cells, $^{a}P < 0.05$. Relative percentage of cells in S phase pretreated with leptin antagonists and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

shown to increase self-renewal and breast cancer stem cell (BCSC) growth[24]. To learn how IONP-LPrA2 affects BCSC growth, tumorsphere formation was assessed. MDA-MB-231 TNBC cells were treated with leptin (1.2 nmol/L) and IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L). Untreated, basal, MDA-MB-231 cells developed few small and medium tumorspheres

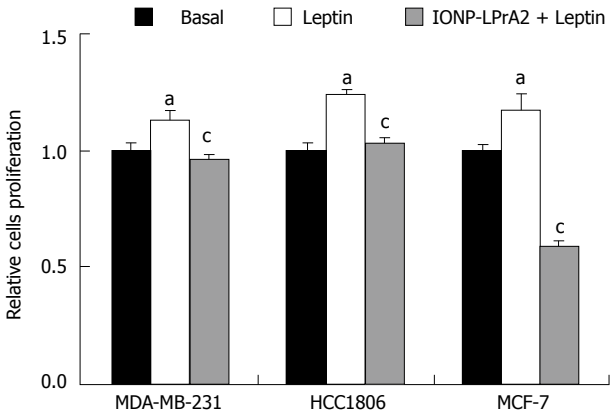

Figure 4 Iron oxide nanoparticles-LPrA2 inhibits leptin-induced cell proliferation in human breast cancer cells. MDA-MB-231, HCC1806, and MCF-7 cells were seeded in 96 well plates and treated with leptin (1.2 nmol/L) and iron oxide nanoparticles (IONP)-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L) for 24-48 h. Cell proliferation was determined by MTT assay. Relative percentage of proliferating cells was significantly increased in leptin treated cell lines compared to basal (untreated) cells, $^{a}P < 0.05$. Relative percentage of proliferating cells pretreated with IONP-LPrA2 and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

(100-200 μm), cells treated with leptin showed a significant increase in the development of medium (200 μm) and large tumorspheres (> 200 μm) in comparison to basal. Cells treated with IONP-LPrA2 plus leptin displayed a significant decrease in medium tumorsphere growth relative to the leptin treated (Figure 5). This data shows that IONP-LPrA2 treatment may decrease BCSC growth.

The effect of chemotherapeutics on survival of breast cancer cell lines

Chemotherapy is among the most common treatments for breast cancer in addition to radiation and surgery[25]. To determine the effective dose of chemotherapeutics, cells were treated with a panel of anti-cancer drugs and viability was tested by the Annexin V FITC/PI Assay (Nexcelom). MDA-MB-231, HCC1806, and MCF-7 cells were treated with Cis (0.001-1.1 μmol/L), CTX (0.01-100 μmol/L), Dox (0.01-50 μmol/L), and PTX (0.05-1.0 μmol/L) for time periods ranging from 1-6 d to determine an effective dose to reduce cell viability (Figure 6). Cis and Dox reduced cell viability in 24 h while CTX and PTX treated cells required up to 6 d to produce an effect. All cell lines displayed a similar response to Cis and PTX (Figure 6A and D). MDA-MB-231 cells appeared to be more sensitive to CTX and Dox (Figure 6B and C).

Determination of the effect of IONP-LPrA2 on survival of breast cancer cells treated with chemotherapeutics

Chemotherapy has many detrimental side effects; because of this it is advantageous to utilize adjuvant therapies in order to reduce the effective dose. To determine the adjuvant potential of IONP-LPrA2, cells were treated with chemotherapeutics combined with IONP-LPrA2 and analyzed for viability by the Annexin

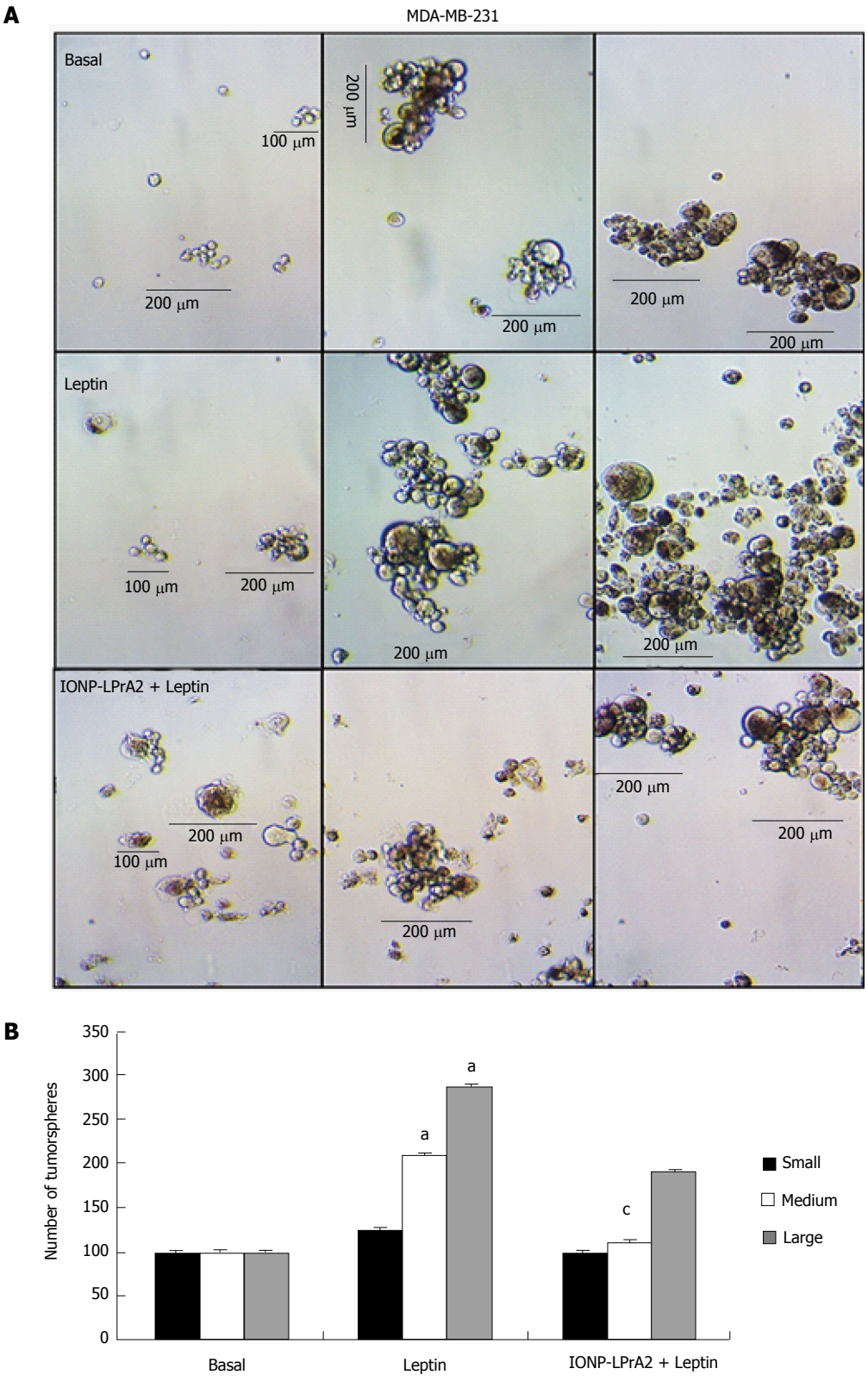

Figure 5 Iron oxide nanoparticles-LPrA2 decreases MDA-MB-231 tumorsphere formation. A: Iron oxide nanoparticles (IONP)-LPrA2 attenuation of leptin-induced tumorsphere formation. MDA-MB-231 cells were grown in low attachment plates with mammocult medium for 1-2 wk, under treatment with leptin (1.2 nmol/L) and IONP-LPrA2 (0.0036 pmol/L) plus leptin (1.2 nmol/L). Tumorspheres were counted. Tumorspheres were grouped according to size: Small (< 100 μm), medium (100-200 μm) and large (> 200 μm); B: Effect of leptin and IONP-LPrA2 on number and size of tumorspheres. Graph represents quantitative analysis of small, medium, and large tumorspheres in response to leptin and IONP-LPrA2 treatment. The number of colonies was significantly increased in leptin treated cells compared to basal (untreated) cells, $^{a}P < 0.05$. The number of colonies pretreated with IONP-LPrA2 and then leptin differed significantly from those treated with leptin alone, $^{c}P < 0.05$.

V FITC/PI Assay (Nexcelom). MDA-MB-231, HCC1806, and MCF-7 cells were treated with chemotherapeutics

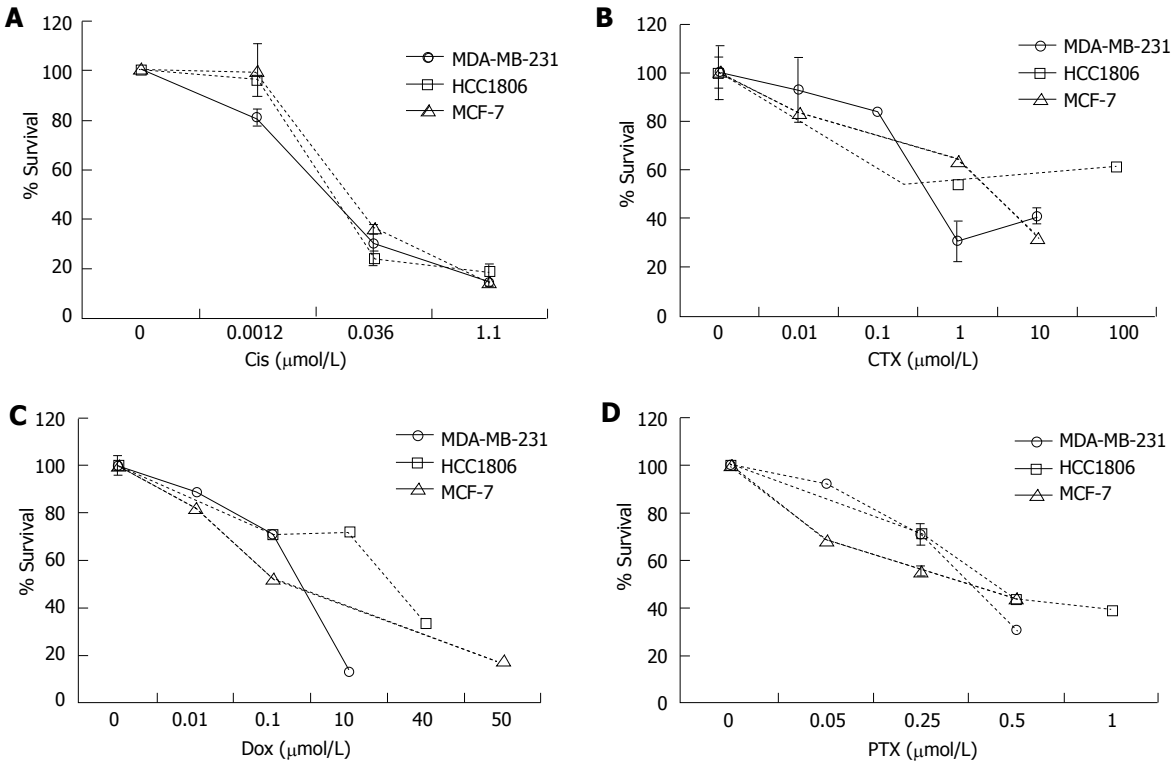

Figure 6 The effect of chemotherapeutics on survival of breast cancer cell lines. The effective dose of the chemotherapeutics. A: Cisplatin (Cis); B: Cyclophosphamide (CTX); C: Doxorubicin (Dox); D: Paclitaxel (PTX) were determined in MDA-MB-231, HCC1806, and MCF-7 cells. The cells were seeded in 6 well plates and treated with Cis (0.001-1.1 μmol/L), CTX (0.01-100 μmol/L), Dox (0.01-50 μmol/L), and PTX (0.05-1.0 μmol/L) for 1-6 d. Percent survival was determined by the Annexin V/FITC and PI assay. The relative survival was determined by multiplying the percentage of live cells by the total cell count.

at concentrations determined in Figure 6 in media containing 5% FBS to mimic physiological leptin levels, in addition to IONP-LPrA2 (0.0036 pmol/L) for time periods ranging from 1-6 d. The treatment concentrations were MDA-MB-231 (Cis 0.001 μmol/L, CTX 0.5 μmol/L, Dox 0.4 μmol/L, PTX 0.5 μmol/L); HCC1806 (Cis 0.036 μmol/L, CTX 1 μmol/L, Dox 10 μmol/L, PTX 0.5 μmol/L); and MCF-7 (Cis 0.036 μmol/L, CTX 5 μmol/L, Dox 0.01 μmol/L, PTX 1 μmol/L). MDA-MB-231 TNBC cells treated with IONP-LPrA2 displayed a significant decrease in viable cells when dosed with Cis and CTX (Figure 7A and B). HCC1806 TNBC cells treated with IONP-LPrA2 showed a significant reduction in viable cells when dosed with Cis and Dox (Figure 7A and C). ER+ MCF-7 cells treated with IONP-LPrA2 did not show a significant decrease in viable cells when treated with chemotherapeutics (Figure 7). Although cells were treated with PTX for up to 6 d to reduce cell viability, IONP-LPrA2 showed no additional decrease in viability when combined with PTX (Figure 7D). PTX is an anti-microtubule agent which acts on the M phase of the cell cycle while the other chemotherapeutics act on DNA which affects the S phase[2]. This data suggests that IONP-LPrA2 increases the potency of chemotherapeutics on TNBC cells, particularly anti-cancer drugs which target DNA.

DISCUSSION

In spite of methods for early detection of breast cancer, it remains the second leading cause of cancer deaths in women in the United States[1]. TNBC is a subtype of breast cancer characterized by the lack of hormone receptor expression. The absence of hormone receptors makes this more aggressive form of breast cancer even more difficult to treat. Obesity is often associated with poorer outcomes in individuals with breast cancer, particularly those with TNBC[25]. Obesity is characterized by an excess of the inflammatory cytokine, leptin. Elevated leptin levels display a significant correlation with metastasis and lower breast cancer patient survival[26]. The leptin antagonist, LPrA2 has been shown to inhibit leptin signaling in breast and other cancer types, but the actions of LPrA2 are restricted by its low MW of < 3 kD, short half-life, and insolubility in water[8,27]. IONP-LPrA2 was developed to circumvent these limitations. IONPs conjugated to other peptides, such as the amino terminal fragment of urokinase type plasminogen activator (ATF-uPA) are stable for more than 48 h in *in vivo* imaging experiments[20]. IONPs are amphiphilic, small (10 nm core size), and uniformly sized to facilitate delivery which prevents phagocytosis[28]. The characteristics of IONPs make them an ideal delivery system for LPrA2 to target and treat breast cancer. In the present study, IONP-LPrA2 was used to evaluate its ability to inhibit leptin signaling in human breast cancer cells. The data indicates that IONP-LPrA2 abrogates cell cycle progression and acts as an adjuvant when administered with chemotherapeutics.

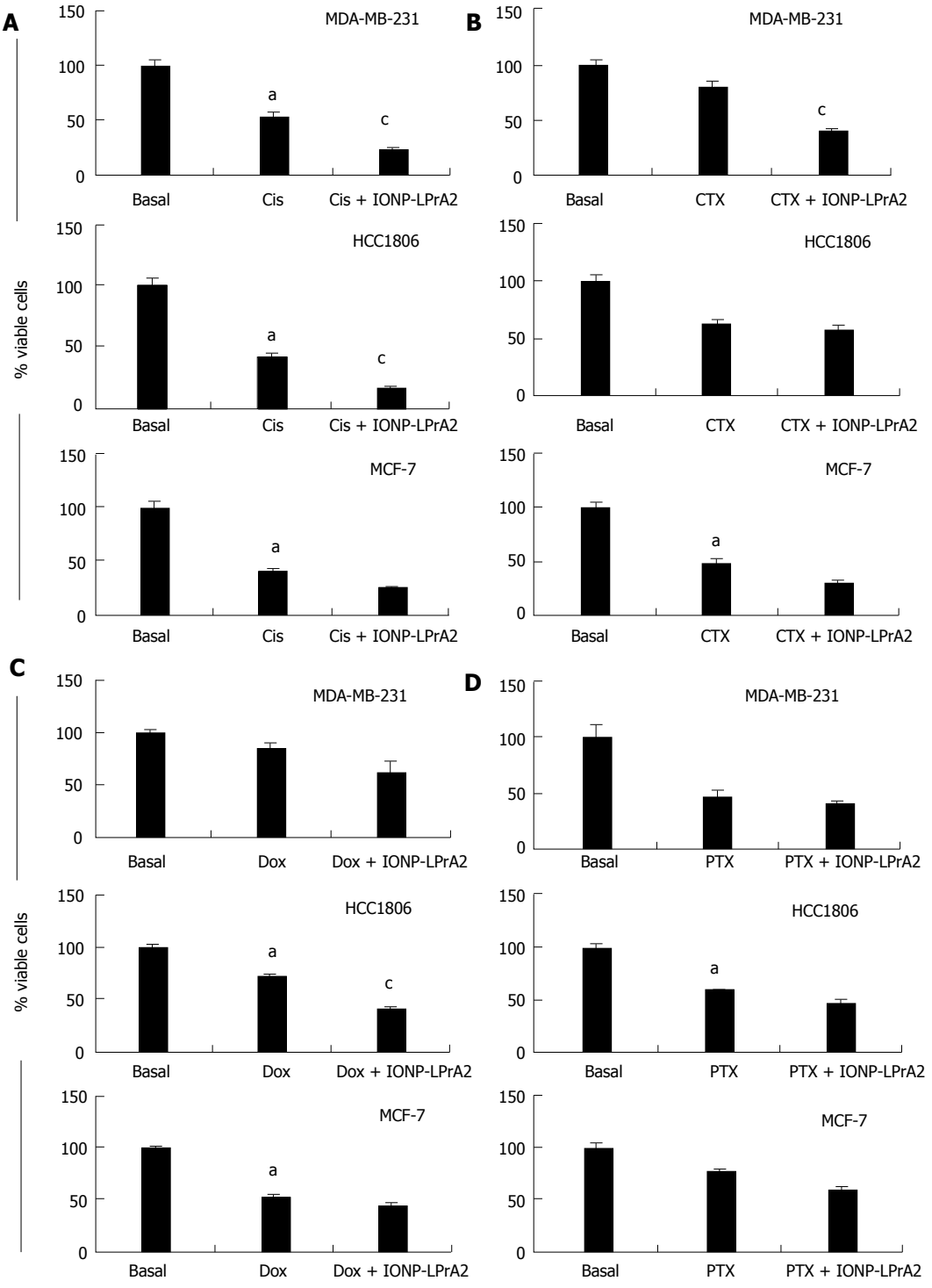

Figure 7 Determination of the effect of iron oxide nanoparticles-LPrA2 on survival of breast cancer cells treated with chemotherapeutics. MDA-MB-231, HCC1806, and MCF-7 cells were treated with an effective dose of the chemotherapeutics. A: Cisplatin (Cis); B: Cyclophosphamide (CTX); C: Doxorubicin (Dox); D: Paclitaxel (PTX) plus IONP-LPrA2 (0.0036 pmol/L). The cells were seeded in 6 well plates and treated with chemotherapeutics at effective concentrations determined in Figure 6 for 1-6 d. The treatment concentrations were MDA-MB-231 (Cis 0.001 μmol/L, CTX 0.5 μmol/L, Dox 0.4 μmol/L, PTX 0.5 μmol/L); HCC1806 (Cis 0.036 μmol/L, CTX 1 μmol/L, Dox 10 μmol/L, PTX 0.5 μmol/L); and MCF-7 (Cis 0.036 μmol/L, CTX 5 μmol/L, Dox 0.01 μmol/L, PTX 1 μmol/L). Percent of survival was determined by the Annexin V/FITC and PI assay. The relative survival was determined by multiplying the percentage of live cells by the total cell count. Perecent viability was significantly decreased in cells treated with chemotherapeutic compared to basal (untreated) cells, $^{a}P < 0.05$. Cells treated with chemotherapeutic and IONP-LPrA2 differed significantly from those treated with chemotherapeutic alone, $^{c}P < 0.05$.

Decreased levels of pSTAT3 and cyclin D1 with IONP-LPrA2 treatment were shown by Western blot. Cyclin D1 is a cell cycle regulatory gene. STAT3 is a transcription factor responsible for the regulation of cyclin D1[10]. Decreased levels of pSTAT3 with IONP-LPrA2 treatment were seen at time points as early as 5-15 min post

treatment. Previous studies have shown that leptin is mitogenic and increases cyclin D1 in ER^+ MCF-7 breast cancer cells[14,15]. Because leptin increases cyclin D1 and IONP-LPrA2 inhibits the effect of leptin, utilizing agents that target cyclin D1 may be a plausible method to treat breast cancer. In this study, we have shown that IONP-LPrA2 decreases pSTAT3 and cyclin D1. The decreased levels of these leptin-induced targets may inhibit cell cycle progression in ER^+ MCF-7 cells as well as MDA-MB-231 and HCC1806 TNBC cells.

Inhibition of cell cycle progression by IONP-LPrA2 was displayed by image based cytometry. Leptin has been shown to increase levels of cyclin D1[14,15]. In this study, we show that IONP-LPrA2 decreases cyclin D1 expression, but the effect on cell cycle progression was yet to be determined. Here we show that IONP-LPrA2 treatment decreases the percentage of cells in the S phase of the cell cycle, where DNA is synthesized, as or more effectively than LPrA2 alone. Interestingly, the greatest decrease in the percentage of cells in S phase with IONP-LPrA2 treatment was seen in HCC1806 TNBC cells derived from a non-metastatic squamous cell carcinoma in contrast to MCF-7 and MDA-MB-231 cells derived from metastatic adenocarcinomas. This data suggests that IONP-LPrA2 inhibition of cell cycle progression may reduce the advancement of breast cancer, and may be particularly beneficial in the treatment of non-metastatic and squamous cell carcinomas.

Chemotherapy is the first line of treatment for TNBC. Although TNBC is generally more responsive to chemotherapy than other forms of breast cancer, there is an increased risk of developing drug resistance[29]. BCSC growth and self-renewal play an important role in breast cancer drug resistance and leptin increases the risk[24]. These cells express molecular markers for breast cancer, $CD44^+CD24^-/ALDH^+$[10]. We have demonstrated that leptin induces *in vitro* BCSC, tumorsphere, formation and treatment with IONP-LPrA2 attenuates the effect of leptin in MDA-MB-231 TNBC cells. These results indicate that IONP-LPrA2 prevents BCSC formation and may decrease chemoresistance in TNBC.

Chemotherapeutic treatment of breast cancer is plagued with high toxicity. Toxic side effects and the development of drug resistance are cause for the development of adjuvant therapies. The need for adjuvant therapies is exacerbated in TNBC patients who often experience relapse and develop resistance to chemotherapy[29]. TNBC is commonly treated with combination chemotherapy[25]. Here, we treated breast cancer cells with a panel of commonly used chemotherapeutics (Cis, CTX, Dox and PTX) in addition to IONP-LPrA2 to test its ability to decrease cell viability more than the drugs alone. We demonstrated that TNBC cells, MDA-MB-231 displayed a significant decrease in viability with Cis and CTX plus IONP-LPrA2; and HCC1806 showed a significant reduction in live cells when treated with Cis and Dox plus IONP-LPrA2. ER^+ MCF-7 cells treated with chemotherapeutics plus IONP-LPrA2 did not show a significant decrease in viable cells. Also, there was no significant decrease in viability in the cells treated with PTX plus IONP-LPrA2. This may be due, in part, to PTX's anti-microtubule action, which affects the M phase of the cell cycle[25]. Cis, CTX, and Dox act on DNA which affects the S phase[25]. These drugs may work synergistically with IONP-LPrA2, which also appears to act on the S phase. These data indicate that IONP-LPrA2 may act as a chemotherapeutic adjuvant by decreasing viability, thereby decreasing the effective dose in TNBC.

In conclusion, IONP-LPrA2 was found to decrease the level of leptin-induced targets pSTAT3 and cyclin D1. IONP-LPrA2 decreased DNA synthesis during the S phase of the cell cycle and reduced proliferation in both ER^+ and TNBC cells. When combined with chemotherapeutics, particularly drugs targeting the S phase, IONP-LPrA2 showed an additive effect on the reduction of live breast cancer cells. These findings indicate that IONP-LPrA2 may be useful in the prevention of tumor cell growth and proliferation in breast cancer. Further, treatment with IONP-LPrA2 may allow for lower chemotherapeutic dosing. These results are potentially beneficial for obese patients with elevated leptin levels, whom have a higher incidence and thus poorer outcome of TNBC. Taken together, the present data provides confirmation of our hypothesis that IONP-LPrA2 treatment may be useful in impairing tumor growth and when given in combination with the indicated chemotherapeutics has the potential to increase drug effectiveness. These data indicate that there is a synergistic effect with IONP-LPrA2 and chemotherapeutics which affect the S phase of the cell cycle *in vitro*.

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COMMENTS

Background

Obesity and high leptin levels are strongly associated with breast cancer relapse, drug resistance, and poorer patient outcomes. Overexpression of leptin and its receptor, Ob-R, induce breast cancer cell growth and proliferation. Triple negative breast cancer (TNBC) is a subtype of breast cancer which comprises approximately 15% of cases and is an aggressive form of the disease with no targeted therapy. TNBC chemotherapeutic treatment often leads to chemoresistance and shows several undesirable side effects. Leptin is proliferative and is a survival factor for breast cancer treated with chemotherapeutics. Therefore, the authors have developed a leptin peptide receptor antagonist coupled to iron oxide nanoparticles (IONP-LPrA2), which successfully inhibits leptin signaling as well as increases chemotherapeutic effectiveness in breast cancer and is particularly promising for TNBC treatment.

Research frontiers

IONP-LPrA2 could be a new and effective biological for blocking pro-oncogenic and drug resistance effects of leptin in breast cancer, especially in obese patients suffering from TNBC that are treated with chemotherapeutics.

Innovations and breakthroughs

This study describes for the first time the production and characterization of a new biological bound to nanoparticles that can effectively block leptin signaling inducing proliferation and survival in breast cancer cells treated with chemotherapeutics.

Applications

In recent years, IONPs have become an important tool for biomedical applications. The use IONPs has been employed in vaccinations, drug delivery, MRI, and molecular imaging. The authors' data suggests combining IONPs with the leptin antagonist, LPrA2, prevents the growth of breast cancer cells and acts as a chemotherapeutic adjuvant by reducing the effective dose.

Terminology

Leptin signaling occurs when the hormone is secreted by the adipose tissue and binds to its receptor, Ob-R. Breast cancer, particularly in obese individuals, is associated with high levels of leptin. Leptin signaling leads to increased breast cancer cell growth, proliferation and drug resistance. The inhibition of leptin signaling with the nanoparticle-linked leptin antagonist, IONP-LPrA2, provides a promising new way to improve breast cancer chemotherapy.

Peer-review

This manuscript provides useful information to the medical students, clinicians, and researchers in this field, therefore, is acceptable for publication.

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ORIGINAL ARTICLE

Basic Study

NDRG2 gene copy number is not altered in colorectal carcinoma

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Abstract

AIM

To investigate if the down-regulation of *N-myc Downstream Regulated Gene 2* (*NDRG2*) expression in colorectal carcinoma (CRC) is due to loss of the *NDRG2* allele(s).

METHODS

The following were investigated in the human colorectal cancer cell lines DLD-1, LoVo and SW-480: NDRG2 mRNA expression levels using quantitative reverse transcription-polymerase chain reaction (qRT-PCR); interaction of the *MYC* gene-regulatory protein with the *NDRG2* promoter using chromatin immunoprecipitation; and *NDRG2* promoter methylation using bisulfite sequencing. Furthermore, we performed qPCR to analyse the copy numbers of *NDRG2* and *MYC* genes in the above three cell lines, 8 normal colorectal tissue samples and 40 CRC tissue samples.

RESULTS

As expected, NDRG2 mRNA levels were low in the three colorectal cancer cell lines, compared to normal colon. Endogenous MYC protein interacted with the *NDRG2* core promoter in all three cell lines. In addition, the *NDRG2* promoter was heavily methylated in these cell lines, suggesting an epigenetic regulatory mechanism. Unaltered gene copy numbers of *NDRG2* were observed in the three cell lines. In the colorectal tissues, one normal and three CRC samples showed partial or complete loss of one *NDRG2* allele. In contrast, the *MYC* gene was amplified in one cell line and in more than 40% of the CRC cases.

CONCLUSION
Our study suggests that the reduction in *NDRG2* expression observed in CRC is due to transcriptional repression by MYC and promoter methylation, and is not due to allelic loss.

Key words: N-myc downstream-regulated gene 2; Colorectal carcinoma; MYC; Tumor suppressor; Allelic loss; Gene amplification; Copy number

Core tip: *NDRG2* is a putative tumor suppressor gene whose expression is reduced in many cancer forms, including colorectal carcinoma (CRC). We set out therefore to investigate if down-regulation of *NDRG2* expression was due to loss of one or both alleles and/or to other mechanisms. In our paper, we show that allelic loss of *NDRG2* is a rare event in CRC. To our knowledge, this is the first study that has specifically investigated gene copy number of *NDRG2* in CRC. Furthermore, our results suggest that *MYC* is amplified in more than 40% of CRC cases. MYC is known to repress transcription of *NDRG2*. Our results lead us to suggest that it is the transcriptional control of *NDRG2* expression, including repression by MYC and epigenetic regulation, that results in decreased *NDRG2* mRNA levels in CRC, rather than allelic loss of *NDRG2*.

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INTRODUCTION

N-myc downstream regulated gene 2 (*NDRG2*) is one of four genes belonging to the *NDRG* gene family. Common for these genes is an NDR domain, a protein motif covering almost the entire protein, but the cellular functions of these genes are currently unclear[1,2]. *NDRG2* expression has been found to be down-regulated in several human cancers including colorectal carcinoma (CRC), hepatocellular carcinoma, glioblastoma and thyroid cancer[3-7]. *NDRG2* is a candidate tumor suppressor gene, with a better overall survival for CRC, hepatocellular carcinoma and glioma patients displaying expression of the gene compared to low or no expression[8-12]. Further evidence of the tumor suppressor function of NDRG2 comes from the observation that NDRG2-lacking mice develop various types of tumors, and from xenograft studies showing that NDRG2-expressing tumor cells implanted in nude mice form smaller tumors and fewer metastases than control cells[13-15]. NDRG2 has a number of downstream targets, including activation of phosphatase and tensin homolog, a known tumor suppressor in the PI3K-AKT pathway[13,16].

Several mechanisms have been suggested as possible regulators of *NDRG2* expression, of which epigenetic silencing, due to promoter hypermethylation, is the most widely observed[4,8,9,13,14,17]. However, other regulatory mechanisms may also play a role. One example could be the transcription factor *MYC*, which is characterised as a proto-oncogene often altered in human cancers[18]. The biological function of MYC seems to be to either activate or repress the transcription of target genes[19,20]. Zhang *et al*[21] have previously shown that ectopically expressed MYC is able, *via* Miz-1, to interact with and to repress transcription from the *NDRG2* promoter. Moreover, correlation of high *MYC* with reduced *NDRG2* expression has been observed in different cancers and cancer cell lines[15,22-24]. However, an inverse relation between *MYC* levels and *NDRG2* expression seems not to apply to all cancer types[25].

CRC is, like most other cancers, a malignant disease with a combination of both genetic and epigenetic changes. One of these changes is chromosome instability, which affects one or several chromosomal regions. Many groups have analysed changes in gene copy numbers in CRC by different approaches and found numerous chromosomal gains and losses[26-29]. In the study by Lagerstedt *et al*[29], the status of CRC samples classified as Dukes stages A-D was analysed, showing an increasing frequency of allelic losses at more severe stages (Dukes C and D). According to their data, allelic deletions in chromosome 14, containing the *NDRG2* gene, is already found at earlier stages (Dukes A and B) and becomes more frequent at the later stages. Although chromosome 14 is not considered one of the deletion hot spot regions, such as chromosome 8p or 18q[27,28,30,31], we hypothesised that deletions in chromosome 14 could lead to loss of one or both of the *NDRG2* alleles. On the other hand, the *MYC* gene is found on chromosome 8q, and gains of this large chromosome arm are frequently found in CRC[26,28,32]. Analysing the gene copy number of *MYC* is therefore of interest with regards to its possible regulatory effect on *NDRG2*.

In this study, we demonstrate a frequent increase in the gene copy number of *MYC* in CRC. In contrast, we find that changes in the copy number of the *NDRG2* locus are rare in CRC, and we suggest that reduced expression of NDRG2 in CRC is due to epigenetic and MYC-related transcriptional repression.

MATERIALS AND METHODS

Cell lines and genomic DNA

The DLD-1, LoVo and SW-480 colorectal cancer cell lines were a gift from Associate Professor Ole Vang, Roskilde University. Cells lines were incubated and maintained at 37 ℃ in an environment of humidified air with 5% CO_2 in McCoy's 5A + GlutaMaxTM-1 media with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Invitrogen). RNA from cell lines was purified with

the SV total RNA isolation kit (Promega) and genomic DNA was purified by ethanol precipitation after an overnight Proteinase K treatment. Reference human genomic DNA, purified from blood lymphocytes, was obtained from Roche Diagnostics, United States (Cat. No.11691112001). As a normal colonic control we used commercially available DNA (BioChain Institute Inc., D4234090). Human colon genomic DNA from tissue classified as either normal or tumorigenic was obtained from BioChain Inc, United States (Cat. no. D8235090-1; Supplementary Table S1). The commercial supplier confirms that tissue and data collection were ethically approved by their Institutional Regulatory Board and that informed consent was obtained from all human subjects.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) kit from Abcam (Ab500) was used according to the instructions, with inclusion of a final ethanol precipitation to increase the DNA concentration. Antibody against MYC (Abcam, ab56-100) was used at a concentration of 5 μg per reaction. The primers used in the PCR step were designed to cover the core promoter region in *NDRG2* (-80 to +93, Figure 1A) and their sequences were (5'-3'): CTTGAGGCATTGACCCCAGAG and CTCTTTGCTGCGTCCCGAC.

Bisulfite treatment and sequencing

Bisulfite treatment of genomic DNA was performed as previously described[33], using glycogen as carrier, and the precipitated DNA was redissolved in TE buffer, amplified by PCR and sequenced directly. The primers were designed to cover 16 CpG sites in the promoter region in *NDRG2* (Figure 1A) and their sequences were (5'-3'): TTTTCGAGGGGTATAAGGAGAGTTTATTTT and CCAAAAACTCTAACTCCTAAATAAACA[34]. A positive control with *in vitro* methylated (IVM) DNA was prepared by mixing 2 μL NEB2 buffer, 1 μL 20 x S-adenosylmethionine (New England Biolabs, B9003S), 200 ng reference human genomic DNA and 1 μL SssI methyltransferase (New England BioLabs, M0226S) in a total of 20 μL. Samples were incubated at 37 ℃ overnight with occasional addition of 2 μL 20 x S-adenosylmethionine to ensure sufficient methyl-donor substrate. The following description was used for each CpG site: Unmethylated (no methylation signal); weakly methylated (methylation signal was less than or approximately equal to unmethylated signal); and strongly methylated (methylation signal was greater than unmethylated signal).

Quantitative real-time PCR

Determination of gene copy number was based on the LightCycler technology using SYBR Green. The sequences of the primers were (5'-3'): *NDRG2* (5' end): CCCCTTGCCTTCTAACTTCCCA and ACAGCCCCTCCTCCCACCTT; *NDRG2* (3' end): GGGGTGAACGAAGAACAAAACAAAG and CGAGGGAGACGGTGAGATGAGG; *MYC*: CCAGAGGAGGAACGAGCTAA and TTGGACGGACAGGATGTATG; *GFAP*: TGACCCTCTCCACCCCATAGTGAC and CAGCAGCAGTGCCCTGAAGATTAG; and *MECP2*: TCAGAGGGTGTGCAGGTGAA and TTGAAAAGGCATCTTGACAAGGA. In a validation experiment using a control sample, a dilution series was produced and assayed for *NDRG2, MYC*, *GFAP* and *MECP2*. When C_t values were plotted against log dilution it was shown that the assays are quantitative over a range of 625-fold dilution for *NDRG2* (5' end), *NDRG2* (3' end), *MYC*, *MECP2* and 125 for *GFAP*. All samples were quantified in triplicates and mean C_t values were normalised to *GFAP* and used to calculate delta delta C_t (ddCt) relative to the reference human genomic DNA[35]. Copy number was defined as a loss for ddCt < 0.75 and as a gain for ddCt > 1.25. Quantification of *NDRG2* mRNA expression levels in colorectal cancer cell lines, using qRT-PCR and normalisation to β-actin, was carried out as previously described[25].

Statistical analysis

All statistical tests were carried out using GraphPad Prism 4 software and *P* values of < 0.05 were considered significant. An unpaired two-tailed *t*-test was used to compare the means of normal-distributed data for the two groups (normal *vs* tumor). The null hypothesis is that there is no difference between the two groups. When data of the two groups did not have equal variance, by *F* test analysis, we used a Mann-Whitney test.

RESULTS

NDRG2 expression is down-regulated in colorectal cancer cell lines

In order to examine how *NDRG2* expression is regulated in colorectal cancer, we chose to work with three cell lines. First of all, we quantified *NDRG2* mRNA levels in the three colorectal cancer cell lines DLD-1, LoVo and SW-480 and observed no or very low expression of *NDRG2*, when normalised to β-actin and compared to human colon mRNA from healthy controls (Table 1).

MYC binds to the NDRG2 gene promoter in colorectal cancer cell lines

We were interested in seeing whether endogenous MYC was bound to the *NDRG2* promoter in these cell lines, since ectopically expressed MYC is a transcriptional repressor of *NDRG2*[21]. A ChIP experiment did indeed show binding of endogenous MYC protein to the core promoter region of *NDRG2* in all three colorectal cancer cell lines (Figure 1B).

The NDRG2 promoter is heavily methylated in colorectal cancer cell lines

In silico analysis of the NDRG2 promoter predicted a CpG island between -380 and +1471 relative to the transcriptional start site (%GC = 66.3, observed/expected CpG = 0.673, cpgislands.usc.edu/cpg.aspx).

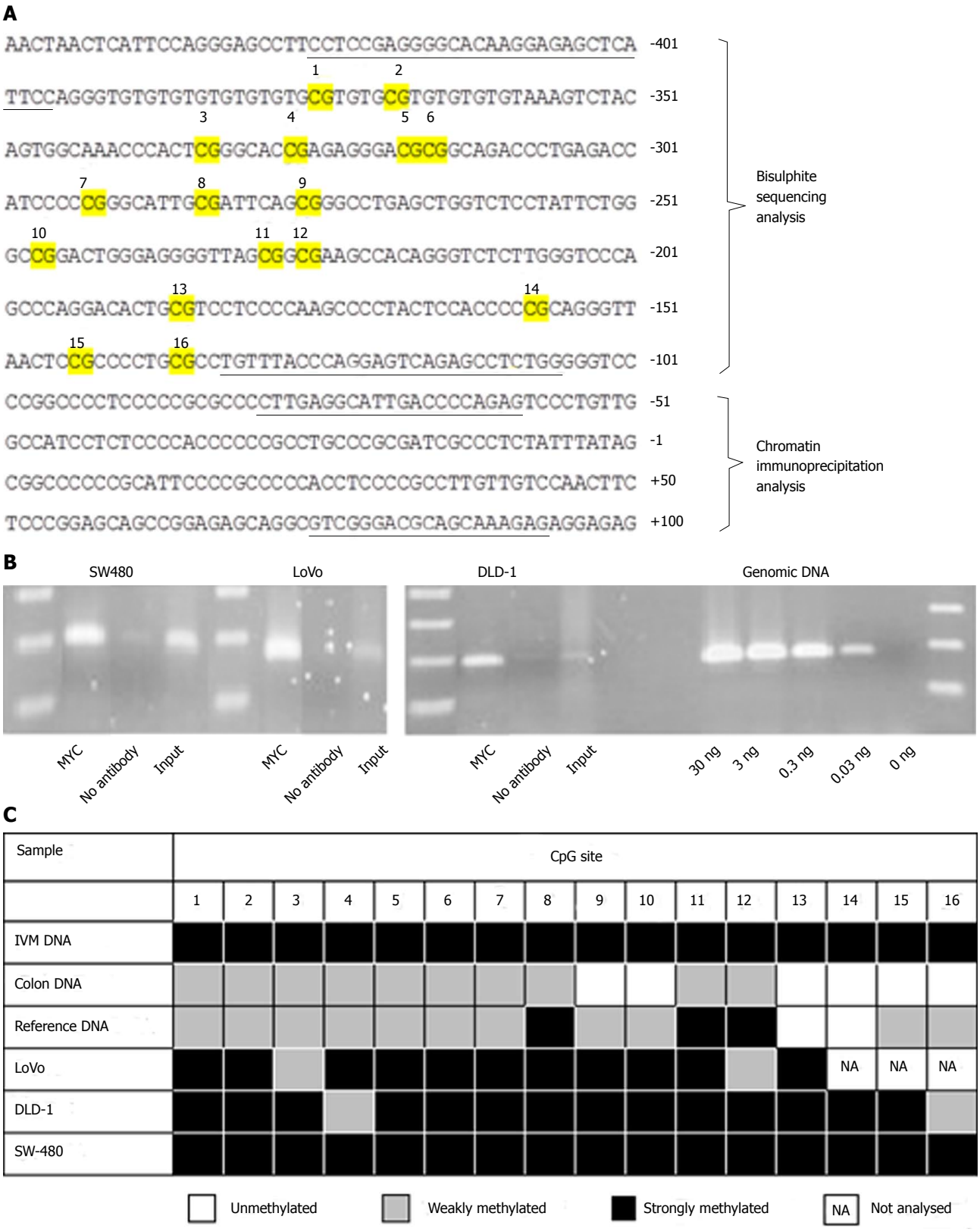

Figure 1 Epigenetic and chromatin immunoprecipitation analysis of the *NDRG2* promoter in three colorectal cancer cell lines. A: The *NDRG2* gene sequence around the transcriptional start site at +1. Primer-binding regions for PCR are underlined and CpG sites subjected to methylation analysis are numbered 1 to 16; B: Endogenous MYC interacts with the *NDRG2* core promoter. ChIP analysis was carried out on SW-480, LoVo and DLD-1 cell extracts using antibody against the transcription factor MYC. "No antibody" was without antibody and "input" served as a positive control. Genomic DNA was used as positive control for the PCR reaction; C: The NDRG2 promoter is hypermethylated in three colorectal cancer cell lines. Bisulfite sequencing was carried out on human genomic DNA from LoVo, DLD-1 and SW-480 cell lines, normal colonic DNA, reference DNA and *in vitro* SssI-methylated (IVM) DNA. Each CpG site was rated as unmethylated, weakly methylated (≤ 50% methylated), or strongly methylated (> 50% methylated).

Table 1 Mean values of normalised levels of *NDRG2* mRNA in colorectal cancer cell lines and healthy colonic tissue

Sample	mRNA level
DLD-1 cell line	0
LoVo cell line	0.005
SW-480 cell line	0.001
Control human colon[a]	0.034 ± 0.009

All samples were analysed in technical triplicates and normalised to β-actin mRNA levels. [a]Previously published data for the mean ± standard deviation for 15 individuals[3].

To establish the methylation status of the *NDRG2* proximal promoter in all three cell lines, we carried out bisulfite treatment and sequencing of the region from -426 to -107, which contains 16 CpG sequences. Bisulfite treatment converts all unmethylated cytosines into uracils, while cytosines with a methyl group attached remain unaltered. As controls, we compared our results with healthy colon genomic DNA, reference genomic DNA from normal blood lymphocytes, and IVM genomic DNA. As presented in Figure 1C, the normal colon genomic DNA and reference genomic DNA sample were predominantly weakly methylated, whereas the *in vitro* methylated control was completely methylated at all cytosines. The three colorectal cancer cell lines, LoVo, DLD-1 and SW-480, displayed strong methylation at the majority of CpG sites (Figure 1C).

NDRG2 gene copy number is not altered in colorectal cancer

We wished to determine the allelic copy numbers of both *NDRG2* and *MYC* in human colorectal carcinoma. By combining qPCR with the mathematical delta delta C_t equation (ddCt), we were able to quantify both losses and gains of these genes. Our experimental setup was validated by analysing the copy numbers of the X-chromosome linked *MECP2* gene in males and females - with the expected one and two X-chromosomes, respectively. As visualised in Figure 2, DNA from 3 females were scored with a ddCt value close to 1.00, which means that the same gene copy ratio between *MeCP2* and *GFAP* was present in both the analysed samples and the reference female genomic sample. A ddCt value of 1.00 therefore represents the normal two alleles. On the contrary, males displayed a ddCt value of approximately 0.50, which represents one allele. Finally, we tested our setup on an unknown sample clearly showing the pattern for male DNA. The conclusion was, therefore, that our setup clearly could differentiate between females and male, *i.e.*, one and two alleles, and has the potential to analyse the copy numbers of *NDRG2* and *MYC*.

We have previously published data showing a statistically significant down-regulation of *NDRG2* mRNA in CRC[3], and the main aim in the present study has therefore been to analyse if allelic loss of *NDRG2* could explain cases of decreased *NDRG2* mRNA levels. For a thorough investigation of *NDRG2,* we selected two regions of the genomic sequence of *NDRG2*, one lying in the 5′ part of the sequence and the other lying in the 3′ end. We first analysed the three colorectal cancer cell lines for both *NDRG2* and *MYC* and found no changes in the copy number of *NDRG2*, in contrast to *MYC*, for which we observed copy number loss in the LoVo cell line, the normal two alleles in DLD-1 cells and a clear copy number gain in SW-480 (Table 2). This latter result is in agreement with a previous study showing a 5 to 10-fold genomic amplification of *MYC* in SW-480 cells[36].

We next analysed 8 normal and 40 CRC tissue samples. In one case out of the eight normal samples, our data indicated copy number loss at the 5′ end of the *NDRG2* gene; otherwise, none of the samples showed any copy number alterations for *NDRG2* (Table 3). As summarised in Table 3, 29 out of the 40 CRC samples (72%) had an unaltered copy number, 2 samples showed loss at either the 5′ or the 3′ end of *NDRG2*, and only in one case did we observe loss at both ends of the gene. In contrast, we found complete copy number gain of *NDRG2* in 3 cases and partial gain in 9 cases (Supplementary Table S2).

Finally, we determined the copy numbers of *MYC* in the same 8 normal and 40 CRC samples, and observed one case of genomic amplification in the normal samples. Otherwise, we did not find any allelic changes in the normal samples (Table 3). For the 40 CRC samples, we observed copy number loss in 4 cases, the normal two copies in nearly half the cases (19 out of 40), and copy number gains of the *MYC* gene in the remaining 17 samples (42.5%) (Supplementary Table S1). However, the observed differences in copy number between normal and CRC tissue did not reach statistical significance (Mann-Whitney test, Table 3).

DISCUSSION

We and others have previously published data showing a statistically significant reduction in *NDRG2* mRNA levels in CRC compared to normal colorectal tissue samples[3,12,23]. Similar findings have been observed in other cancers including gliomas, hepatocellular carcinoma, breast cancer, thyroid cancer and meningioma[5-7,25,37]. Exactly how and why *NDRG2* expression is reduced is not fully understood, but repression by the MYC transcription factor is likely to be involved in some cases, just as promoter hypermethylation seems to play an important role[4,14,21,34]. Here, we show that 16 potential methylation sites in the proximal promoter of *NDRG2* are heavily methylated in all three colorectal cancer cell lines tested. Methylation of the analysed region from -426 to -107 could reduce accessibility to the transcription factors WT1 and HIF1α, which have binding sites in this region[38,39] and/or result in transcriptional silencing. In support of this, previous studies have shown that reversal of methylation by 5-aza-2′-deoxycytidine treatment leads to increased NDRG2 mRNA levels in the colorectal cancer cell lines CaCo2, HCT116 and SW480[34]. Furthermore,

Table 2 ddCt values and corresponding copy numbers for the *NDRG2* and *MYC* genes in colorectal cancer cell lines

Cell line	*NDRG2* - 5′ end		*NDRG2* - 3′ end		MYC	
	ddCt ± SD	Copy number	ddCt ± SD	Copy number	ddCt ± SD	Copy number
LoVo	1.23 ± 0.47	2	1.12 ± 0.51	2	0.91 ± 0.31	2
DLD-1	1.04 ± 0.23	2	1.08 ± 0.50	2	0.74 ± 0.22	Loss
SW-480	1.04 ± 0.26	2	0.94 ± 0.44	2	4.88 ± 0.30	Gain

Copy number loss is defined as ddCt < 0.75 and a gain is defined as ddCt > 1.25. ddCt: Delta delta Ct; SD: Standard deviation.

Table 3 Alteration in copy numbers for the *NDRG2* and *MYC* genes in colorectal tissue

Colorectal tissue	Number of samples	Loss ddCt < 0.75	Unaltered ddCt 0.75-1.25	Gain ddCt > 1.25	Normal *vs* CRC
NDRG2 - 5′ end					
Normal	8	1	7	0	
CRC	40	2	29	9	$P = 0.194^a$
NDRG2 - 3′ end					
Normal	8	0	8	0	
CRC	40	2	32	6	$P = 0.470^a$
MYC					
Normal	8	0	7	1	
CRC	40	4	19	17	$P = 0.135^b$

[a]*P* value for comparison of ddCt values (supplementary table S2) in normal and CRC samples using an unpaired two-tailed *t* test; [b]*P* value for comparison of ddCt values (supplementary table S2) in normal and CRC samples using a Mann-Whitney test. ddCt: Delta delta Ct.

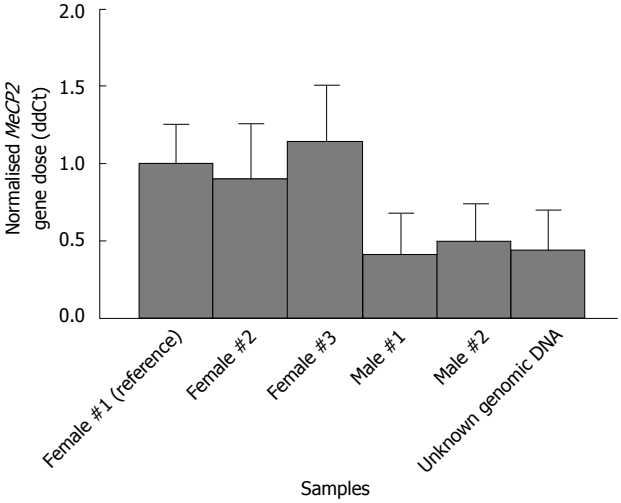

Figure 2 Validation of the gene copy number experimental setup. Bar diagram showing the calculated delta delta Ct values (ddCt) of the X-linked *MeCP2* gene normalised to *GFAP*, giving the expected result (one copy in males and two copies in females). A ddCt value of 1.00 in the reference female genomic sample represents the normal two alleles. Data are presented as mean (filled bars) and SD (whiskers).

DNA methylation at the *NDRG2* promoter was shown to be significantly higher in CRC tissue compared to normal colonic tissue from the same patients[14,34].

Our ChIP experiments on three colorectal cancer cell lines showed that endogenous MYC interacts with the *NDRG2* core promoter. Although MYC is considered a classical transcription factor, it is also involved in the maintenance of chromatin structure[40,41]. For example, MYC has been shown to recruit DNA methyltransferase 3a to the promoter region of a gene to exert its repressive activity[42]. Thus, we suggest that MYC could be involved in the regulation of *NDRG2* by recruitment of other proteins to produce an epigenetic silencing of *NDRG2*.

However, the suggested regulatory mechanisms cannot explain all cases of down-regulation of *NDRG2* expression, and we were therefore interested in looking at allelic loss to see if this genetic event could contribute to the decreased *NDRG2* mRNA levels observed in CRC. To investigate this question, we designed an experimental setup making it possible to quantify the copy numbers of any gene. In a validation experiment, we could easily differentiate between one or two copies of the X-chromosome linked gene *MECP2*. Our data indicate that allelic loss at the *NDRG2* locus is not very frequent in CRC. On the contrary, a subset of CRC cases showed gains of one or both ends of the *NDRG2* gene, which might lead to elevated levels of *NDRG2* mRNA. These findings were unexpected, since allelic losses in chromosome 14 are more frequently observed than gains[27,28]. Although we have only looked at copy number changes in CRC, our results might be applied to other cancers and could explain why we observed an increase in *NDRG2* levels in approximately 8% of 154 paired normal and tumor samples analysed from 19 different tumor types[25].

The proto-oncogene *MYC* is located on chromosome 8 at the q24.12 region, and several groups have shown amplification of chromosome 8q[27,28,43]. Indeed, we observed an increase in *MYC* gene copy numbers in nearly every second CRC sample, confirming a frequent gain at this particular gene locus. However, we did not detect the same high percentage of *MYC* amplification as a previous study focusing on the 8q24 region, which revealed that nearly 80% of the cases analysed had some kind of gene amplification[32]. Since MYC has the potential to repress *NDRG2* transcription[21], increased copy numbers of the *MYC* gene could lead to higher levels of MYC protein and thereby a reduced level of *NDRG2* mRNA.

Finally, copy number loss of the 5′ end of *NDRG2* and a gain of *MYC* were observed in separate normal samples and might indicate a rare, but real, genomic alteration in healthy tissue. An alternative explanation is that since all normal samples were obtained from patients diagnosed with CRC and classified as normal, the tissue might be at an early pre-malignant stage with

no visual changes, but where genetic abnormalities had already occurred.

In conclusion, we observed *NDRG2* promoter hypermethylation and interaction of endogenous MYC with the core promoter in three colorectal cancer cell lines, together with absent or low *NDRG2* mRNA expression. Frequent allelic loss was not found at the *NDRG2* locus in the colorectal cancer cell lines and tissue samples from either normal or tumor tissues. In contrast, we observed partial or complete NDRG2 copy number gains in more than 25% of the CRC cases, compared to none in the normal samples. We also found that more than 40% of CRC cases displayed *MYC* amplification, which indicates that the level of *MYC* mRNA is elevated in CRC. We conclude that epigenetic silencing and transcriptional repression by MYC are likely to be more important than copy number loss for the reduced levels of *NDRG2* mRNA observed in CRC.

COMMENTS

Background

A frequent change observed in colorectal carcinoma (CRC) is chromosomal instability, in which gain or loss of chromosomal regions affects levels of gene expression. Thus, loss of one or both alleles could explain the reduced expression of tumor suppressor genes, such as *NDRG2*, that is observed in CRC. Alternatively, *NDRG2* down-regulation could be due to transcriptional and epigenetic mechanisms.

Research frontiers

In order to understand the origin of CRC, it is important to investigate changes at the epigenetic, genetic and transcriptional level. This study investigated regulation of *NDRG2* gene expression using bisulfite-sequencing to study gene methylation, quantitative polymerase chain reaction to study gene copy number as well as chromatin immunoprecipitation to study DNA-binding of the endogenous gene-regulatory protein MYC.

Innovations and breakthroughs

This study shows for the first time that gene copy number for *NDRG2* is unaltered in CRC cell lines and clinical samples.

Applications

The authors describe a validated approach to determine gene copy number, relative to a control gene, using the comparative (ddCt) approach. Future approaches could focus on re-activating expression of *NDRG2* in CRC.

Terminology

NDRG2 is a newly described tumor suppressor gene that is down-regulated in a large range of cancers, including CRC. Interest in *NDRG2* as a therapeutic target is supported by studies showing a better prognosis in patients having higher *NDRG2* expression in tumor tissues.

Peer-review

The paper is very good.

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ORIGINAL ARTICLE

Prospective Study

Salient concerns in using analgesia for cancer pain among outpatients: A cluster analysis study

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Abstract

AIM

To identify unique clusters of patients based on their concerns in using analgesia for cancer pain and predictors of the cluster membership.

METHODS

This was a 3-mo prospective observational study (n = 207). Patients were included if they were adults ($\geqslant$ 18 years), diagnosed with solid tumors or multiple myelomas, and had at least one prescription of around the clock pain medication for cancer or cancer-treatment-related pain. Patients were recruited from two outpatient medical oncology clinics within a large health system in Philadelphia. A choice-based conjoint (CBC) analysis experiment was used to elicit analgesic treatment preferences (utilities). Patients employed trade-offs based on five analgesic attributes (percent relief from analgesics, type of analgesic, type of side-effects, severity of side-effects, out of pocket cost). Patients were clustered based on CBC utilities using novel adaptive statistical methods. Multiple logistic regression was used to identify predictors of cluster

membership.

RESULTS

The analyses found 4 unique clusters: Most patients made trade-offs based on the expectation of pain relief (cluster 1, 41%). For a subset, the main underlying concern was type of analgesic prescribed, *i.e.*, opioid *vs* non-opioid (cluster 2, 11%) and type of analgesic side effects (cluster 4, 21%), respectively. About one in four made trade-offs based on multiple concerns simultaneously including pain relief, type of side effects, and severity of side effects (cluster 3, 27.5%). In multivariable analysis, to identify predictors of cluster membership, clinical and socioeconomic factors (education, health literacy, income, social support) rather than analgesic attitudes and beliefs were found important; only the belief, *i.e.*, pain medications can mask changes in health or keep you from knowing what is going on in your body was found significant in predicting two of the four clusters [cluster 1 (-); cluster 4 (+)].

CONCLUSION

Most patients appear to be driven by a single salient concern in using analgesia for cancer pain. Addressing these concerns, perhaps through real time clinical assessments, may improve patients' analgesic adherence patterns and cancer pain outcomes.

Key words: Cancer pain; Analgesia; Opioids; Preferences; Conjoint analysis; Side-effects

Core tip: Lack of adherence to analgesia for cancer pain is a prevalent clinical problem. The 2016 Centers for Disease Control and Prevention guidelines provide recommendations to clinicians for opioid prescription. However, this focus will be incomplete without understanding what concerns anchor patients' decisions to use analgesia for cancer pain. We used a trade-off analysis technique and novel adaptive methods to first show that unique clusters of patients exist based on the main concerns that anchor their preferences for analgesia for cancer pain. We then identified factors that predict membership in each preference cluster. We found that socioeconomic factors, including education, health literacy, income (rather than attitudes and beliefs about analgesics) played a role in predicting three out of four clusters. Most analgesic beliefs and concerns, including the widely indicated addiction concerns, did not predict cluster membership.

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INTRODUCTION

In the early part of 2016, the Centers for Disease Control and Prevention (CDC) released guidelines for prescribing opioids in chronic pain, including cancer pain beyond active cancer treatment[1]. While the guidelines are shaping a conversation and debate among professionals and policy makers on opioid prescription[2-4], little is known about the other side of the coin-patients' preferences that shape their analgesic taking behaviors. Cancer pain in the United States is mainly managed using analgesics[5]. Non-pharmacological pain treatment approaches are either not consistently offered to patients by their clinicians/covered by health insurance or lack data on clinical effectiveness[6-10]. For the treatments that have demonstrated clinical effectiveness, the cost burden for the patients may be excessive[11,12]. Thus, clinicians and oncologists rely on analgesics as well as opioid medications to help patients whose daily lives and function are affected by significant pain[11]. Unfortunately, patients with unrelieved chronic pain have some of the lowest quality of life observed for any medical condition[13].

Despite widespread use of analgesics in managing cancer pain, there is serious paucity of literature to understand the heuristics cancer patients may employ in making decisions to use analgesics. The few extant studies had methodological aims, that is to investigate the predictive validity of a trade-off analysis technique in eliciting analgesic preferences with diverse subgroups of patients with cancer pain[14]. Others investigating analgesic trade-offs included patients with cancer as part of the broader category of chronic pain sufferers[15]. Also, to our knowledge, no studies have investigated the sociodemographic and clinical predictors of patients' analgesic preferences. Thus, the purpose of this study was to investigate if unique clusters exist with regard to cancer patients' preference to use analgesics for cancer pain and factors predicting cluster membership.

MATERIALS AND METHODS

This was a prospective study conducted with a cohort of adult (18 years or older) patients who were diagnosed with solid tumors or multiple myelomas and had at least one prescription of around-the-clock pain medication for cancer or cancer-treatment-related pain. Patients were self-identified African-Americans and Whites and were recruited from two outpatient medical oncology clinics within a large health system in Philadelphia, United States. Data were collected at baseline and at 3-mo. This study was approved by the Institutional Review Board of the University of Pennsylvania. All patients provided written informed consent.

Measures

Analgesic concern: Analgesic preferences (utilities) for cancer pain was derived from a choice-based con-

joint (CBC) analysis experiment, which is a valuation technique based on the Random Utility Theory[16] and mathematical psychology[17]. The goal of CBC is to elicit what people value and what really drives them to choose one set of alternatives over another when facing competing choices[18]. CBC proposes that the overall utility or desirability of any good can be described based on the value of its separate, but, conjoined parts[19], which are termed "attributes". Each attribute may have multiple levels. Individuals are asked to make trade-offs between attributes and attribute levels generating a unique set of values called part-worth utilities. A higher part-worth utility represents a higher level of value or importance individuals assign to that attribute. The design of CBC experiments is tailored based on the needs of an individual study.

We used a systematic approach to designing the CBC study to elicit analgesic utilities reported in the present study. The procedures are detailed in a previously published manuscript[14]. Trade-offs were elicited on five analgesic attributes: (1) type of analgesic, (2) percentage pain relief with analgesics; (3) type of side-effects; (4) severity of side-effects; and (5) out-of-pocket cost of analgesics. In addition to the design components, we also investigated the internal, external predictive validity and temporal stability of the CBC experiment over the study period[14].

Analgesic attitudes and barriers: Barriers Questionnaire-II[20,21] was used to assess patients' attitudes and beliefs about the management of cancer pain. It is a 27-item measure which elicits patients' pain management concerns in eight domains: (1) fear of addiction; (2) fear of tolerance; (3) fear of side effects; (4) fatalism about cancer pain; (5) desire to be a good patient; (6) fear of distracting health provider from treating cancer; (7) fear that the analgesics impair the immune system; and (8) concern that analgesics may mask ability to monitor illness symptoms. The response range is from 0 (do not agree) to 5 (agree very much). The scores are based on sums for items for the total scale and four subscales (physiological, fatalism, communication, and harmful effects). The internal consistency reliability of the scale is excellent at 0.89[20].

Analgesic side-effects: Side-effects resulting from taking analgesics were assessed using the Medication Side-effects Checklist (MSEC). MSEC elicits information on the presence and severity of eight common analgesic side-effects (*i.e.*, constipation, drowsiness, nausea, vomiting, confusion, dry mouth, stomach irritation, itching) on a scale of 0-10 (no severity-extreme severity). The internal consistency reliability is 0.80[21].

Pain severity and pain-related function: The Brief Pain Inventory (BPI) was used to assess pain severity. The BPI has two subscales; pain intensity (4-items) and pain-related functional interference (7-items: General activity, mood, walking ability, normal work, relationships, sleep and enjoyment of life)[22]. Each item is scored on a 0-10 scale (0 = no pain and 10 = pain as bad as you can imagine; and 0 = no interference and 10). The psychometric properties of the BPI are well-established with cancer patients with a Cronbach's alpha that ranges from 0.77 to 0.91[23,24].

Pain management index: Pain management index (PMI) is a measure of adequacy of pain treatment based on the World Health Organization's (WHO) guidelines for managing cancer-related pain[25,26]. The measure takes into account the most potent analgesic prescribed to patients relative to the level of their reported pain. PMI is calculated by subtracting patient's "pain worst" score (from BPI coded as mild, moderate, or severe) from the most potent analgesia prescribed based on the 3-step WHO analgesic ladder. A negative PMI means inadequate analgesic prescription relative to the pain level.

Social support questionnaire: A 6-item instrument was used to measure participants' perceptions of social support and satisfaction with social support[27]. The first part of the question asks participants to list individuals who provide social support and the second part asks them to indicate the level of satisfaction with this support. This questionnaire is an abridged version of the original 27-item Social Support Questionnaire[27].

Prescribed analgesics: Prescribed analgesics were coded according to the WHO analgesic ladder[25,26]. This included step 1 (non-opioid analgesics); step 2 (weak opioid analgesics such as codeine); and step 3 (strong opioids such as morphine, oxycodone, methadone).

Sociodemographic and clinical variables: Sociodemographic data were gathered on age, gender, self-identified race, marital status, education, health insurance, household income, job status and health literacy. Health literacy was assessed using three brief screening questions that were previously validated[28] and performs well against the widely used Test of Functional Health Literacy in Adults[28]. The brief questions were also found to be effective in identifying inadequate health literacy (areas under the receiver operating characteristic curve of 0.87, 0.80 and 0.76, respectively for the three questions).

Clinical variables (collected from patients' medical records) included stage of cancer, time since cancer diagnosis, past history of drug or substance abuse, comorbidities to compute the Charlson Comorbidity Index[29], presence of chronic kidney disease, and presence of depression. Pain and treatment related variables included total number and types of analgesics and co-analgesics, most potent analgesic prescribed, hours pain medications are effective, and pain relief with analgesics.

Statistical analysis

Descriptive statistics were generated for available baseline variables. A wide variety of variables were considered within the four categories of sociodemographic; illness; pain, function and pain treatment; and analgesic attitudes and barriers. Patients were clustered on their responses to the five analgesic attributes determined by the CBC analysis using the adaptive statistical methods of Knafl *et al*[30]. A variety of clustering procedures and numbers of clusters were considered, but restricted to alternatives with each cluster containing at least 10% of the patients, thereby avoiding sparse clusters. A clustering alternative was selected using likelihood cross-validation (LCV) scores with likelihoods based on mixtures of multivariate normal distributions as commonly used in cluster analysis.

Models were evaluated and compared using 10-fold LCV scores. These were computed by first randomly partitioning the data into 10 disjoint subsets, called folds. Likelihoods were then computed for the data in each fold using parameter estimates computed from the data in the other folds. These deleted fold likelihoods were combined over all the folds into a LCV score.

A larger LCV score indicates a better model for the data but not necessarily a distinctly better model. This issue was addressed using LCV ratio tests, based on the χ^2 distribution (and so analogous to standard likelihood ratio tests). These tests were expressed in terms of a threshold for a distinct (or substantial or significant) percent change in the LCV scores. A percent decrease larger than the threshold indicates that the model with the larger LCV score provides a distinct improvement over the model with the smaller score. Otherwise, the model with the smaller score is a competitive alternative, and if also simpler then preferable as a parsimonious, competitive alternative. The threshold changes with the sample size.

The indicators for being in each of the CBC clusters were modeled separately using logistic regression. This approach allows for identification of a different set of predictors for each cluster and so was considered preferable to multinomial regression modeling of membership in all four clusters combined since that would use the same predictors for all clusters. Each available baseline variable was used to adaptively identify an associated binary characteristic for predicting being in a CBC cluster by dichotomizing the associated variable's values and choosing the dichotomization that maximized the LCV score (with likelihoods based on the Bernouilli distribution as appropriate for logistic regression). Only dichotomizations with both sets of values having at least 10% of the data were considered to avoid sparse cases. The binary characteristic was defined using the indicator variable with value 1 for the set of values generating an odds ratio (OR) > 1. This indicator was conservatively set to 0 for missing variable values if there were any. The total BQ-Ⅱ along with each of its subscales and items were considered as predictors to provide a broad assessment of the impact of analgesic attributes and barriers on the analgesic preferences (CBC types or clusters).

Dichotomization can sometimes result in loss of predictive capability compared to using the associated variable as an unadjusted predictor. This can be assessed for ordinal and continuous variables by comparing LCV scores for models based on those variables to the models based on the associate binary characteristics, but only when there are no missing values. LCV ratio tests can be used to assess whether binary characteristics provide a distinct improvement or not by comparing their LCV scores to the score for the constant model (*i.e*., with only an intercept).

An adaptive multiple binary characteristics model was generated for each CBC-cluster indicator based on the binary characteristics that were individually significantly ($P < 0.05$) related to it in bivariate models using standard Wald χ^2 tests. The adaptive modeling process[31] is based on a heuristic search guided by LCV scores through alternative models. First, the model is systematically expanded adding in predictors, in this case binary characteristics, to the model. The expanded model is then contracted to remove extraneous predictors. LCV ratio tests are used to decide when to stop the contraction, leaving the adaptively generated model. This modeling process is implemented in a SAS® (SAS Institute Inc., Cary, NC) macro available upon request from G. Knafl. All results were computed in SAS Version 9.4.

Biostatistics statement

The statistical methods of this study were reviewed by Dr. George Knafl, Biostatistician and Professor in the School of Nursing at the University of North Carolina at Chapel Hill.

RESULTS

Complete data were available for 207 patients (Figure 1). The baseline demographic and illness related data are presented in Tables 1 and 2, respectively. The mean age of the respondents was 54 years (SD = 11). More than half were married (53%) and had college or more than college education (64%). About one-third (35%) reported a household income of less than $30000 year. None of the patients had any missing CBC analgesic attribute values. Only three of all these variables had any missing values. The threshold for a distinct percent change in LCV score for data with 207 observations is 0.92% (in contrast, the percent decrease is 2.00% for 95 observations and 1.00% for 190 observations).

Unique analgesic preference clusters

Using methods described (see data analysis), a 4-cluster solution was chosen. Figure 2 contains plots of the four cluster centroids, that is, the vectors with entries equal to averages of the five CBC analgesic attributes for patients in the clusters. Based on these plots, the clusters were characterized in terms of the more

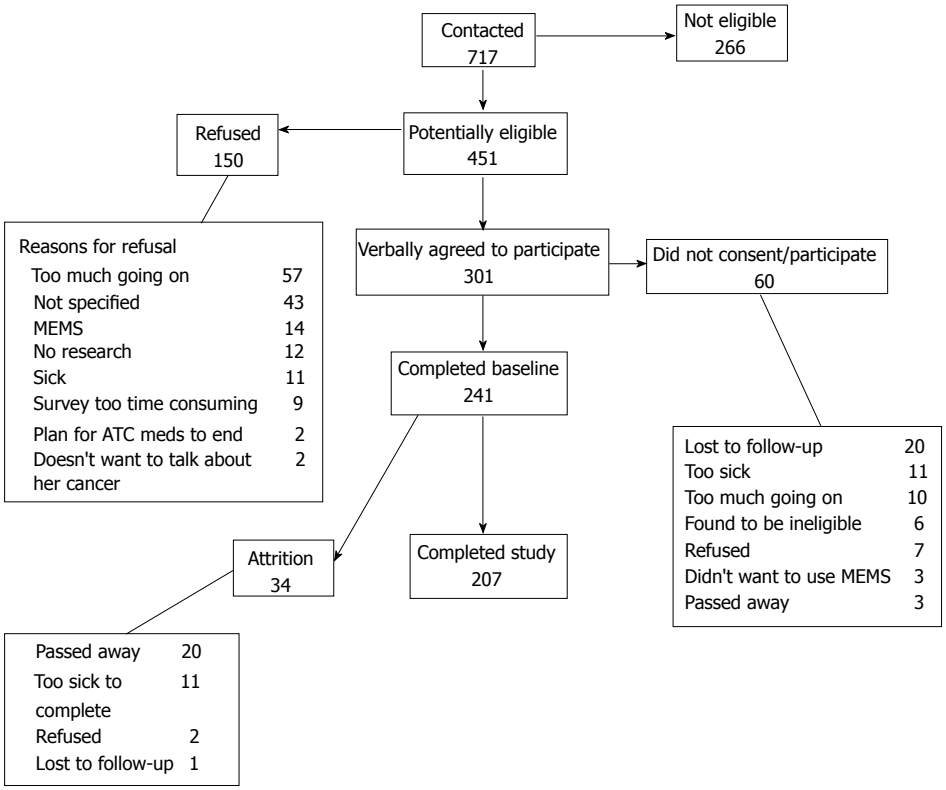

Figure 1 Participant recruitment flow diagram. MEMS: Medication Event Monitoring; ATC: Around-the-clock.

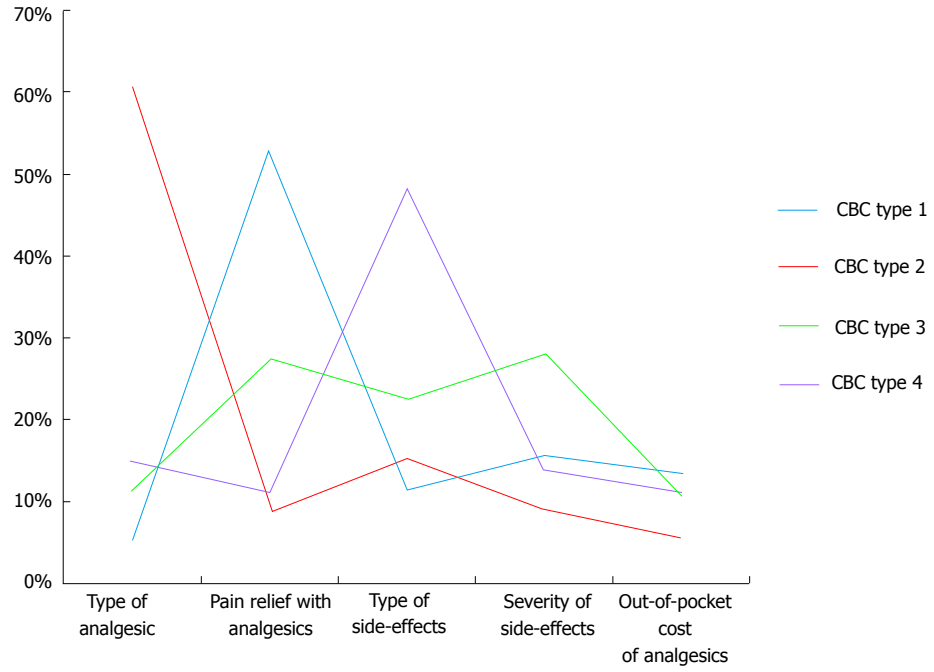

Figure 2 Choice-based conjoint analgesic attribute types. CBC: Choice-based conjoint.

strongly rated analgesic attributes (Table 3).

Cluster 1 (pain relief)

For less than half the patients (41%) in this study, expectation of pain relief was the main anchor in making analgesic related trade-offs for cancer pain. A total of 16 individually significant binary characteristics were identified for patients in this cluster (Supplemental Table 1). Patients in cluster 1 were more likely be White/Caucasians, carried a private health insurance, had higher education and health literacy, and reported less analgesic-related barriers in general. The strongest of

Table 1 Baseline sociodemographic variables (*n* = 207)

Variable	Range	*n* (%)[1]	Mean (SD)
Age	23-75		53.8 (11.1)
Education	Elementary	3 (1.5)	
	High school	70 (33.8)	
	College/Trade school	101 (48.8)	
	More than college	33 (15.9)	
Employment status	Employed outside home (full-time)	43 (20.8)	
	Employed outside home (part-time)	12 (5.8)	
	Employed at home (full-time)	4 (1.9)	
	Employed at home (part-time)	4 (1.8)	
	Retired	44 (21.3)	
	Unemployed	25 (12.1)	
	Other	75 (36.2)	
Health literacy	3-15		13.1 (2.6)
Income	< $10000	28 (13.5)	
	$10000-$20000	26 (12.6)	
	$20000-$30000	19 (9.2)	
	$30000-$50000	36 (17.4)	
	$50000-$70000	37 (17.9)	
	$70000-$90000	24 (11.6)	
	> $90000	37 (17.9)	
Primary insurance (1 missing)	Private	107 (51.9)	
	Medicare	41 (19.9)	
	Medicaid	27 (13.1)	
	Multiple	25 (12.1)	
	VA/other	6 (2.9)	
Marital status	Married	110 (53.1)	
	Separated/Divorced	48 (23.2)	
	Widowed	8 (3.9)	
	Never married	41 (19.8)	
Race	Black/African American	86 (41.5)	
	White/Caucasian	121 (58.5)	
Social support	0.17-9.00		3.7 (2.1)

[1]No missing values unless otherwise indicated. SD: Standard deviation; VA: Veterans Administration.

Table 2 Baseline illness and pain variables (*n* = 207)

Variable	Range	*n* (%)[1]	Mean (SD)
Cancer stage	I	20 (9.7)	
	II	33 (15.9)	
	III	37 (17.9)	
	IV	64 (30.9)	
	Unknown or unsure	53 (25.6)	
Time since cancer diagnosis	1-120 mo		36.7 (35.5)
Charlson comorbidity index	0-13		4.3 (2.6)
General health	Excellent	9 (4.3)	
	Very good	23 (11.1)	
	Good	63 (30.4)	
	Fair	77 (37.2)	
	Poor	35 (16.9)	
Physical health not good (number of days within last 30 d)	0-30		14.7 (10.7)
Mental health not good (number of days within last 30 d)	0-30		9.5 (10.7)
Past history of substance abuse	No	172 (83.1)	
	Yes	35 (16.9)	
Presence of depression	No	120 (58.0)	
	Yes	87 (42.0)	
Worst pain (last week)	0-10 (no pain - pain as bad as you can imagine)		6.9 (2.4)
Average pain (last week)	0-10 (no pain - pain as bad as you can imagine)		4.9 (2.1)
Least pain (last week)	0-10 (no pain - pain as bad as you can imagine)		3.4 (2.0)
Pain-related functional interference score	7-70 (does not interfere-completely interferes)		35.2 (15.9)
Pain relief with medications (last week)	1-10 (10%-100%)		7.2 (2.1)
Pain management index	-2	5 (2.4)	
	-1	13 (6.3)	
	0	92 (44.4)	
	1	63 (30.4)	
	2	31 (15.0)	
	3	3 (1.4)	
Number of analgesic side effects (MSEC)	0-8		3.8 (2.4)
Severity of analgesic side effects (MSEC)	8-80 (not severe-extremely severe)		25.2 (15.0)
BQ-II analgesic barriers (total)	0-96		39.8 (20.1)
No. of complementary alternative modalities used	0-8		2.1 (1.7)

[1]No missing values unless otherwise indicated. BQ-II: Barriers questionnaire; MSEC: Medication Side-effects Checklist; SD: Standard deviation.

these predictors, that is, the one generating the best (largest) LCV score, was lower endorsement of the belief that pain medicine can mask changes in your health with LCV score 0.51908 (LCV scores not reported).

The individually significant binary characteristics were adaptively combined into a multiple logistic regression model (Table 4). The three factors that remained in the multiple risk factor model and predicted membership in cluster 1 included, higher education, poor physical health and a lower endorsement of the belief that pain medications can mask changes in health. The most important of these (*i.e.*, the one whose removal generated the lowest LCV score) was BQ-II item, pain medicine can mask changes in your health. The LCV score was 0.53503, and so this model provided a distinct improvement over the best individual binary characteristic model with percent decrease 2.98% (since this was larger than the threshold of 0.92%).

Cluster 2 (type of analgesic)

For only 11% of patients in this study, the main anchor for analgesic trade-offs was "type of analgesic". A total of 15 individually significant binary characteristics were identified for patients in cluster type 2 (Supplemental Table 2). Patients in cluster 2 were more likely to have lower income, lower social support, greater burden of comorbidities and pain, and lower relief from taking pain medications. Patients in this cluster were more likely to hold beliefs such as pain medications can harm immune system, or make you addicted. However, the strongest

Table 3 Description of analgesic preference clusters (n = 207)

Cluster	n (%)	Salient concern(s)
1	84 (40.6)	Pain relief
2	23 (11.1)	Type of analgesic
3	57 (27.5)	Pain relief, type of side-effects and severity of side-effects
4	43 (20.8)	Type of side-effects

of these predictors was lower (≤ $50000) income with LCV score 0.71212 (LCV scores not reported).

In the multiple logistic regression model, lower social support, health literacy and income levels were predictive of membership in this cluster (Table 5). The most important of these was health literacy (LCV score was 0.72894), and so this model provided a distinct improvement over the best individual binary characteristic model with percent decrease 2.31%.

Cluster 3 (pain relief, type of side-effects and severity of side-effects)

More than one in four patients (28%) made trade-offs based on multiple factors including expectation of pain relief, type of side-effects, and severity of side-effects. A total of 18 individually significant binary characteristics were identified for patients in cluster 3 (Supplemental Table 3). Patients in this cluster were more likely to be married, had greater social support, reported lower pain and pain related functional impairment, and greater pain relief with analgesics. They were less likely to report analgesic side-effects and had lower endorsement for BQ items indicating lower attitudinal barriers. The strongest of these predictors was lower average pain (≤ 6) in the last week with LCV score 0.56530 (LCV scores not reported). In the multiple logistic regression model, being married, having greater social support, having lower average pain, lower side-effects predicted membership in cluster 3 (Table 6).

Cluster 4 (type of side-effects)

For one in five patients (21%), type of side-effects experienced was the main factor driving analgesic trade-offs. A total of 21 individually significant binary characteristics were identified for patients in cluster type 4 (Supplemental Table 4). Patients in this cluster had lower education and health literacy, were more likely to be Blacks/African Americans, reported lower relief with medications and reported shorter duration of relief with pain medications. Patients in this cluster were more likely to report greater severity of analgesic side-effects and past history of substance abuse but fewer number of days when mental health was not good. Patients in this cluster had the highest number of BQ barriers than any other cluster.

In the multiple logistic regression model, four factors including, lower health literacy, mental health, more analgesic side effects, and belief that pain medications keep you from knowing what is going on in your body predicted membership in this cluster (Table 7).

DISCUSSION

This is the first study to identify the sociodemographic and clinical predictors of unique clusters based on what may drive patients' preference for analgesic treatment for cancer pain. Lack of adherence to analgesia for cancer pain is a prevalent clinical problem[32-35]. Studies in cancer[35] and non-cancer[36-43] pain settings suggest that patterns of analgesic adherence are consequential in explaining clinical and health services outcomes. The 2016 CDC guidelines provide recommendations to clinicians for opioid prescription[1]. However, this focus will be incomplete without an understanding of how patients take prescribed analgesics and what salient concerns anchor their decisions. Previous studies have documented correlates of non-adherence to analgesia for cancer pain[44-47]. These studies do not allow discerning how risk factors and predictors may be distributed dissimilarly across subgroups of cancer patients. Using a well-established trade-off analysis technique (CBC) and more novel adaptive methods, we first showed that unique clusters of patients exist based on the main concern(s) anchoring their preferences for analgesia for cancer pain. We then identified sociodemographic and clinical factors that predict membership in each preference cluster.

Importantly, for an overwhelming majority in this study, analgesic preference for cancer pain was driven by a single salient underlying concern (see cluster 1, 2 and 4). In multivariable analysis to identify predictors of these clusters, "clinical" and "socioeconomic factors" (rather than attitudes and beliefs) were found important. Of note, at least one socioeconomic factor (including education, health literacy, income) played a role in predicting three out of four preference clusters. Furthermore, most analgesic beliefs and concerns, including the widely implicated addiction concerns, did not play a role as predictors of cluster membership. Only the belief that pain medications can mask changes in health or keep you from knowing what is going on in your body was found significant in predicting two of the four clusters. This is a common clinical concern among cancer patients and relates to the fear of disease progression[48-50].

An interesting finding was the contrast between cluster 1 and 4. Unlike cluster 1 (pain relief), those in the side-effects cluster (cluster 4) had lower health literacy and greater analgesic barriers using BQ-II questionnaire. Patients in this cluster were more likely to report greater burden of analgesic side-effects. Of note, there is a stark difference in the identified correlates of these two clusters. The correlates of cluster 1 included being white/Caucasian and having higher education, income and health literacy and lower analgesic barriers. Cluster 4, however was predicted by being African Americans and having lower education, literacy, and

Table 4 Multiple binary characteristics model for cluster 1 (pain relief)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Education	College/trade school or more than college *vs* Elementary or High school	134 (64.7)	0.001	3.88	1.75-8.59
Illness	Physical health not good (number of days within last 30 d)	⩾ 22 *vs* < 22	59 (28.5)	0.002	2.81	1.47-5.38
Pain, function and pain treatment	NS					
Analgesic attitudes and barriers	BQ-II item - pain medicine can mask changes in your health	⩽ 3 *vs* > 3	158 (76.3)	0.016	2.26	1.17-4.36

BQ-II: Barriers questionnaire II; CI: Confidence interval; OR: Odds ratio; NS: None significant.

Table 5 Multiple binary characteristics model for cluster 2 (type of analgesic)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Health literacy	= 15 *vs* < 15	93 (44.9)	0.006	3.86	1.46-10.2
	Income	⩽ $50000 *vs* < $50000	109 (52.7)	0.017	3.64	1.26-10.5
	Social support	⩽ 4.17 *vs* > 4.17	137 (66.2)	0.027	4.25	1.18-15.4
Illness	NS					
Pain, function and pain treatment	NS					
Analgesic attitudes and barriers	NS					

CI: Confidence interval; OR: Odds ratio; NS: None significant.

Table 6 Multiple binary characteristics model for cluster 3 (pain relief, type of side-effects and severity of side-effects)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Marital status	Married *vs* Separated, Divorced, Widowed or Never married	110 (53.1)	0.023	2.26	1.12-4.56
	Social support	⩾ 1.83 *vs* < 1.83	177 (85.5)	0.022	4.55	1.24-16.7
Illness	Mental health not good (number of days within last 30 d)	⩾ 2 *vs* < 2	140 (67.6)	0.002	3.46	1.55-772
Pain, function and pain treatment	Average pain (last week)	⩽ 6 *vs* > 6	163 (78.7)	0.01	4.41	1.42-6.86
	Severity of analgesic side effects (MSEC)	⩽ 28 *vs* > 28	133 (64.3)	0.005	3.11	1.41-6.86
Analgesic attitudes and barriers	NS					

CI: Confidence interval; OR: Odds ratio; MSEC: Medication Side-effects Checklist; NS: None significant.

Table 7 Multiple binary characteristics model for cluster 4 (type of side-effects)

Variable domain	Variable	Characteristic	*n* (% out of 207)	*P* value	OR	95%CI
Sociodemographic	Health literacy	⩽ 13 *vs* > 13	84 (40.6)	0.004	3.11	1.43-6.76
Illness	Mental health not good (number of days within last 30 d)	⩽ 12 *vs* > 12	144 (69.6)	0.001	6.18	2.06-18.5
Pain, function and pain treatment	Severity of analgesic side effects (MSEC)	⩾ 40 *vs* < 40	37 (17.9)	0.002	4.19	1.68-10.5
Analgesic attitudes and barriers	BQ-II item - pain medicine can keep you from knowing what's going on in your body	⩾ 4 *vs* < 4	42 (20.3)	< 0.001	5.25	2.32-11.9

BQ-II: Barriers questionnaire; MSEC: Medication Side-effects Checklist; CI: Confidence interval; OR: Odds ratio.

more analgesic barriers. Another interesting noteworthy contrast between the two clusters (1 and 4) was that in the multiple logistic regression models, individuals in cluster 1 (pain relief) were less likely to believe that pain medications can mask changes in your health whereas patients in cluster 4 were more likely to endorse pain can keep you from knowing what is going on in your body. Thus, literacy and analgesic beliefs appear to be

at play in different ways in the two clusters.

Previous studies have investigated and found racial and socioeconomic disparities in pain management in general, including cancer pain management[51-54]. Our findings indicate that analgesic side-effects are also poorly treated in cancer patients with lower health literacy. These patients will benefit from meticulous assessment of pain and symptoms and accessible interventions that promote self-advocacy and negotiation of pain and side-effects management with their clinicians and oncologists.

In the last few decades, significant resources have been devoted towards psychoeducational interventions that have a major focus on dismantling analgesic beliefs and barriers[20,55,56]. Unfortunately, a number of systematic reviews show that these interventions do not improve adherence to analgesia for cancer pain or cancer pain outcomes[57,58]. Our findings imply that meticulous assessment of clinical factors such as pain levels, analgesic side-effects, and addressing SES factors (such as health literacy) may play a role in improving cancer pain outcomes. Also, the finding that decision-making for most patients was driven by single salient underlying factor raises an exciting possibility of designing two-part interventions focused on eliciting real-time trade-offs and linking real-time preferences sequentially to brief, tailored, and patient-centered clinical interventions.

Study limitations

The clusters identified in this study are based on the CBC design. While CBC is a well-established method and we previously tested the validity of the CBC utilities used in this study, there is a notable consideration. About 1 in 3 patients used lexicographic decision rules (*i.e.*, unwillingness to trade more or less of one attribute in favor or detriment of the other)[14]. These processes may represent patients' actual preferences or mental shortcuts to get through the CBC exercise, potentially compromising the clinical validity of the data. Our confidence that the clusters represent actual preferences is enhanced by the study findings. For instance, patients in cluster 4 (side effects) were more likely to report greater burden of analgesic side-effects, which remained significant in the multivariable model. Similarly, patients in cluster 3 weighed multiple factors similarly (pain relief, type and severity of side-effects) possibly because of their experience of lower pain severity and lower burden of side-effects (*e.g.*, MSEC < 28 in cluster 3 *vs* > 40 in cluster 4). These findings increase confidence that the clusters identified in this study represent actual preferences rather than mental shortcuts. Also, we restricted our analysis to those patients who completed the study to avoid having missing data that may have affected the conclusions of the study. Excluded patients were with advanced illness who died or were too sick to complete the study (Figure 1), thus we caution against generalizing the findings to those with advanced illness. Nevertheless, our findings inform a scarce body of literature on what anchors cancer patients' preferences in using analgesia for cancer pain and a potential new path to brief, tailored, and accessible interventions to improve pain and functional outcomes among cancer patients.

COMMENTS

Background

The purpose of this study was to investigate if unique clusters exist with regard to patients' concerns in using analgesics for cancer pain and factors predicting cluster membership.

Research frontiers

The new Centers for Disease Control and Prevention opioid guidelines are shaping a national conversation among professionals and policy makers on opioid prescription. Little is known about the other side of the coin, *i.e.*, cancer patients' concerns in using analgesia and factors shaping these concerns and preferences that may relate to their analgesic taking patterns. This study fills this important gap.

Innovations and breakthroughs

The authors employed novel statistical methods to understand unique subgroups of patients based on their concerns in using analgesics for cancer pain and identified sociodemographic and clinical correlates of these unique clusters. In recent decades, significant resources have been committed to psychoeducational interventions that have a major focus on dismantling analgesic beliefs and barriers. However, recent systematic reviews show that psychoeducational interventions do not consistently improve adherence to analgesia for cancer pain or cancer pain outcomes. The authors' findings suggest that careful assessment of clinical factors such as analgesic side-effects and addressing social determinants, such as patients' health literacy, may play a role in improving cancer pain outcomes.

Applications

The authors' study finding that decision-making for most patients was driven by single salient underlying factor raise an exciting possibility of designing two-part interventions focused on eliciting real-time trade-offs and linking real-time preferences sequentially to brief, tailored, and patient-centered clinical interventions.

Terminology

Analgesic concerns and preferences in this study were elicited using choice-based conjoint (CBC) analysis, which is a trade-off analysis technique. Individuals are asked to make trade-offs between attributes (*e.g.*, pain relief, side-effects) and attribute levels (*e.g.*, percent pain relief, severity of side-effects) generating a unique set of values called part-worth utilities. A higher part-worth utility represents a higher level of value or importance an individual assign to that attribute.

Peer-review

The paper contributes important information.

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CASE REPORT

Intermittent facial spasms as the presenting sign of a recurrent pleomorphic adenoma

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Abstract

The intimate anatomical relationship of the facial nerve to the parotid parenchyma has a significant influence on the presenting signs and symptoms, diagnosis and treatment of parotid neoplasms. However, to our knowledge, hyperactivity of this nerve, presenting as facial spasm, has never been described as the presenting sign or symptom of a parotid malignancy. We report a case of carcinoma arising in a recurrent pleomorphic adenoma of the left parotid gland (*i.e.*, carcinoma *ex pleomorphic* adenoma) that presented with hemifacial spasms. We outline the differential diagnosis of hemifacial spasm as well as a proposed pathophysiology. Facial paralysis, lymph node enlargement, skin involvement, and pain have all been associated with parotid malignancies. To date the development of facial spasm has not been reported with parotid malignancies. The most common etiologies for hemifacial spasm are vascular compression of the ipsilateral facial nerve at the cerebellopontine angle (termed primary or idiopathic) (62%), hereditary (2%), secondary to Bell's palsy or facial nerve injury (17%), and hemifacial spasm mimickers (psychogenic, tics, dystonia, myoclonus, myokymia, myorthythmia, and hemimasticatory spasm) (17%). Hemifacial spasm has not been reported in association with a malignant parotid tumor but must be considered in the differential diagnosis of

this presenting symptom.

Key words: Facial spasm; Pleomorphic adenoma; Benign mixed parotid tumor; Reconstructive surgery; Salivary glands

Core tip: This report represents the first case of hemifacial spasm associated with transformation of a recurrent pleomorphic adenoma into a carcinoma *ex pleomorphic* adenoma. The causation of hemifacial spasms is discussed.

Machado RA, Moubayed SP, Khorsandi A, Hernandez-Prera JC, Urken ML. Intermittent facial spasms as the presenting sign of a recurrent pleomorphic adenoma. *World J Clin Oncol* 2017; 8(1): 86-90 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i1/86.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i1.86

INTRODUCTION

The intimate anatomical relationship of the facial nerve to the parotid gland has a significant influence on the symptoms/signs, diagnosis, and treatment of parotid neoplasms[1]. Involvement of the facial nerve by parotid malignancies usually results in partial or total hemifacial paralysis[2]. However, to our knowledge, hyperactivity of this nerve presenting as facial spasm has not been reported as the presenting feature of a malignant parotid tumor. Facial spasm has nonetheless been reported twice in the literature as a presenting feature of a benign parotid tumor[3]. We report a case of carcinoma arising in recurrent pleomorphic adenoma (*i.e.,* carcinoma *ex pleomorphic* adenoma) that presented with hemifacial spasms. We outline the differential diagnosis of hemifacial spasm as well as a proposed pathophysiology.

This is a single institutional case report in a tertiary referral hospital. Institutional Review Board was not required to report one case at our institution.

CASE REPORT

A 56-year-old female smoker had a history of a pleomorphic adenoma in the left parotid gland treated with a superficial parotidectomy at the age of 18. Nineteen years following that surgery, the patient presented with multifocal recurrence. Surgical exploration was undertaken and the tumor was found inseparable from the facial nerve. At that time, the resection was abandoned and the facial nerve was not sacrificed and gross disease was left in the parotid bed. The patient underwent external beam radiation therapy and the size of the tumor remained stable for 10 years on serial computed tomography (CT) and magnetic resonance imaging (MRI) monitoring. The patient had been clinically asymptomatic until she started developing intermittent ipsilateral hemifacial spasms occurring spontaneously and involving all portions of the left facial musculature, which prompted her to return for evaluation.

Repeat CT scan showed enlargement of avidly and uniformly enhancing solid tumor without areas of necrosis or extracapsular extension with extension into the left stylomastoid foramen, along with suspicious changes in enlarged (15 mm) left level IV lymph node (Figure 1A). Fine-needle aspiration biopsy of the tumor was suspicious for carcinoma *ex pleomorphic* adenoma. After a negative systemic metastatic work-up, the patient was brought to the operating room for a radical parotidectomy with facial nerve sacrifice, ipsilateral selective neck dissection (levels I-IV), and a de-epithelialized anterolateral thigh free flap for volume restoration and to enhance wound healing. The vertical segment of the facial nerve in the mastoid was exposed. Primary facial nerve repair was performed using sural nerve grafting from the main trunk to the temporal branch of the facial nerve, nerve to masseter grafting to the dominant midfacial branches of the facial nerve, together with construction of an oral commissure suspension with a fascia lata sling.

Final surgical pathology confirmed a 5.2 cm pleomorphic adenoma with a multinodular growth pattern. Well-circumscribed neoplastic nodules of variable sizes were embedded in densely fibrotic connective tissue (Figure 2). Nerve bundles were also entrapped in the scar tissue in-between the nodules, but no true perineural invasion was detected. Within the nodules, two foci of early non-invasive carcinoma were noted. Within one nodule a 4 mm focus of malignant cells surrounded by benign epithelial elements was identified. In a separate nodule, an intraductal malignant neoplastic proliferation with an intact benign myoepithelial cell rim was also noted. None of the malignant neoplastic foci showed invasion into adjacent fibroadipose tissue and nerves. Thirteen level II-V lymph nodes were negative for tumor involvement. The primary tumor was staged as rT4N0M0.

The hemifacial spasms subsided after surgery, and the patient remains disease free at 6 mo of follow-up. The patient has recovered facial tone but has yet to develop dynamic muscular activity.

DISCUSSION

Zbären *et al*[4] reported that pleomorphic adenomas comprised 60% of all of their benign and malignant parotid neoplasms**.** When left untreated, pleomorphic adenoma has a malignant transformation risk of 5% to 25% over a span of 15-20 years[5]. The risk of recurrence after primary superficial parotidectomy is 2%-5%[4], and malignant change in recurrent pleomorphic adenomas has an incidence of 2%-24%[6].

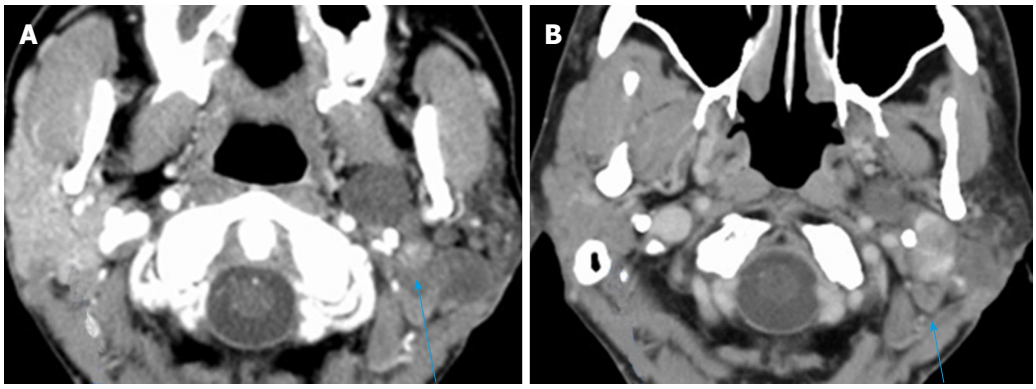

Figure 1 Axial computed tomography of the neck with contrast demonstrates oval shaped enhancing lesion of the left parotid gland deep to the left ramus of the mandible, centered at the left stylomandibular tunnel. A: The lesion measured 9 mm × 7 mm × 8 mm in 2007; B: The lesion measured 3.1 cm × 2.8 cm × 4.5 cm in 2015.

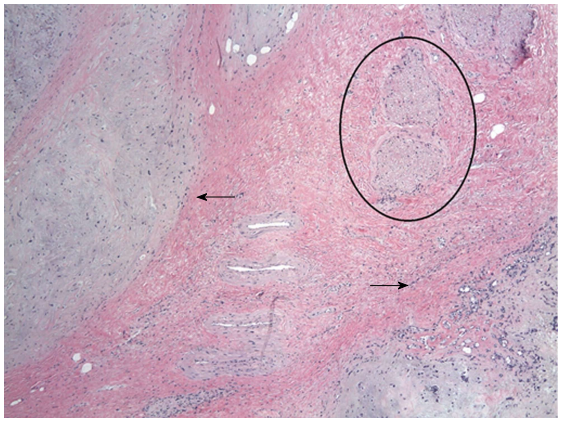

Figure 2 A 5.2 cm pleomorphic adenoma (circle) with a multinodular growth pattern and well-circumscribed neoplastic nodules with variable sizes were embedded in fibroadipose tissue (arrows).

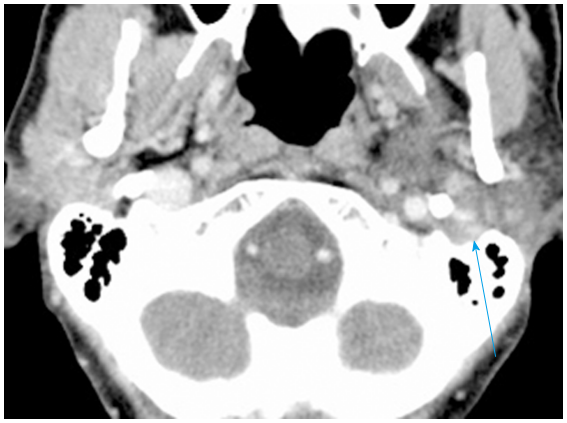

Figure 3 2015: Showing new extension into the left stylomastoid foramen not present on the examination of 2007.

Zbären *et al*[4] postulates that the risk of de novo malignant change increases with time from first presentation and the number of recurrent episodes of the tumor.

Treatment of recurrent pleomorphic adenoma involves primary surgery that can either be a superficial or total parotidectomy based on the site of the recurrence and the extent of previous facial nerve exploration[6]. Adjuvant radiotherapy is another treatment option that is suitable for patients whose tumor is not completely excised[6]. According to Witt et al, retrospective analysis provides evidence that radiotherapy improves local control of this tumor[6]. The risk of malignant change in salivary glands following radiation therapy to the neck in 11047 patients with Hodgkins Lymphoma was investigated by Boukheris *et al*[7]. They reported that 21 patients developed salivary gland carcinoma with an observed-to-expected ratio of 16.9 and a confidence interval of 95%[7]. The risk was highest in patients under 20 years of age and those who survived more than 10 years[7].

In a review of the literature, Gnepp reported that carcinoma *ex pleomorphic* adenoma was present in 3.6% of all salivary gland neoplasms, 6.2% of all mixed tumors, and 11.6% of all malignant salivary gland neoplasms[2]. The malignant tumor is mainly found between the sixth to eighth decades of life[2]. Carcinoma *ex pleomorphic* adenoma represents a malignant change in a primary or recurrent pleomorphic adenoma[2]. Nouraei *et al*[8] and Zbären *et al*[4] reported that 25% of their 28 patients and 21% of their 24 patients, respectively, had a previously treated parotid adenoma. Carcinoma *ex pleomorphic* adenoma predominantly affects the major salivary glands with a majority of cases noted in the parotid and submandibular glands[2]. Nouraei *et al*[8] and Olsen *et al*[9] reported that the carcinoma *ex pleomorphic* adenoma was located in the parotid gland in of 96% and 86% of their cases, respectively. The most common clinical presentation of carcinoma *ex pleomorphic* adenoma is as a firm mass in the parotid gland[2]. This tumor though typically non-invasive, confined to the capsule of the parotid adenoma and asymptomatic, has been reported to become invasive and involve local structures[2].

Carcinoma *ex pleomorphic* adenoma may present with pain when it is associated with invasion of local tissues[2]. Involvement of the facial nerve causes facial paresis or palsy[2]. Olsen *et al*[2] reported that

32% of the patients in their series had facial nerve involvement manifesting as partial or complete facial muscle weakness. Rarely, patients presented with skin ulceration, tumor fungation, skin fixation, palpable lymphadenopathy and dysphagia[2].

No case of hemifacial spasms or twitching associated with carcinoma *ex pleomorphic* adenoma or any other parotid or submandibular gland malignancies has been reported in the literature. The only malignant neoplasm presenting with facial spasm that we identified in the literature was a malignant astrocytoma located at the cerebellopontine angle[10]. Following resection of that tumor, the facial spasms resolved[10]. The two cases of hemifacial spasm have been reported with benign parotid tumors. Behbehani *et al*[11] reported the case of a 47-year-old man who presented with a right parotid mass and hemifacial spasm. The hemifacial spasms did not abate following surgery, but responded 8 mo later to botulinum toxin-A injections[11]. Destee *et al*[3] also reported a case of a pleomorphic adenoma in a 70 year-old man who presented with hemifacial spasms. During total parotidectomy, it was noted that the facial nerve was pale and appeared ischemic[3]. The hemifacial spasms reduced 8 days post operatively and had almost completely subsided within 6 mo[3].

The most common causes of hemifacial spasm are vascular compression of the ipsilateral facial nerve at the cerebellopontine angle (termed primary or idiopathic) (62%), hereditary (2%), secondary to Bell's palsy or facial nerve injury (17%), and hemifacial spasm mimickers (psychogenic, tics, dystonia, myoclonus, myokymia, myorthythmia, and hemimasticatory spasm) (17%)[12]. In addition to a thorough history and a complete neurological examination, some authors recommend magnetic resonance imaging and angiography of the cerebellopontine angle[12]. However, such imaging may not be cost-effective in all patients[13], as the presence of an ectatic artery on magnetic resonance imaging may not be specific for hemifacial spasms[12]. Therefore, this may be reserved for patients with atypical features such as numbness and weakness[13].

The authors postulate that in this patient the hemifacial spasm commenced with the onset of the malignant transformation in the recurrent pleomorphic adenoma in the parotid gland. In the absence of any evidence of perineural invasion, we believe that peri-tumoral inflammatory responses caused the neural stimulation that resulted in hemifacial spasm. This patient did not have any prior ear surgery or any other known etiology to account for this symptom. An alternative explanation to the patient's neurological symptoms is external compression to the facial nerve. This could be related to the dense fibrotic tissue surrounding both tumor nodules and nerves or to direct tumor extension into the left stylomastoid foramen[14] (Figure 3). The latter mechanism has been previously proposed by Blevins *et al*[14].

In conclusion, we present the first case of hemifacial spasm in conjunction with transformation of a recurrent pleomorphic adenoma into a carcinoma *ex pleomorphic* adenoma. The pathophysiology of hemifacial spasms is discussed.

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COMMENTS

Case characteristics

A 56-year-old female with a history of recurrent pleomorphic adenoma of the left parotid gland treated with surgery and external beam radiation therapy presented with ipsilateral hemifacial spasm.

Clinical diagnosis

The clinical diagnosis is a malignant change in a parotid pleomorphic adenoma with involvement of the facial nerve.

Differential diagnosis

The differential diagnosis is the stimulation of facial nerve by perineural invasion or an inflammatory reaction caused by malignant parotid tumor.

Imaging diagnosis

Repeat CT scan showed enlargement of avidly and uniformly enhancing solid tumor without areas of necrosis or extracapsular extension with extension into the left stylomastoid foramen, along with suspicious changes in enlarged (15 mm) left level IV lymph node (Figure 1A).

Pathological diagnosis

Fine-needle aspiration biopsy of the tumor was suspicious for carcinoma *ex pleomorphic* adenoma. Final surgical pathology confirmed a 5.2 cm pleomorphic adenoma with a multinodular growth pattern with two foci of early non-invasive carcinoma and no malignant spread to adjacent fibroadipose tissue, nerves or thirteen level II-V lymph nodes.

Treatment

A radical parotidectomy with facial nerve sacrifice, ipsilateral selective neck dissection (levels I-IV), and a de-epithelialized anterolateral thigh free flap was performed. A sural nerve grafting from the main trunk of the facial nerve to its branches and an oral commissure suspension with a fascia lata sling was done.

Experiences and lessons

The authors postulate that the hemifacial spasm commenced with the onset of the malignant transformation in the recurrent pleomorphic adenoma in the ipsilateral parotid gland. In the absence of any evidence of perineural invasion, they believe that peri-tumoral inflammatory responses caused the neural stimulation that resulted in hemifacial spasm.

Peer-review

This is the first reported case of malignant transformation of a recurrent pleomorphic adenoma in a parotid gland presenting with ipsilateral hemifacial spasm. In the absence of evidence of perineural invasion of the ipsilateral facial nerve, it is postulated that peri-tumoral inflammatory responses were responsible for the excitation of this nerve and the resultant hemifacial spasm.

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CASE REPORT

Difficult endoscopic diagnosis of a pancreatic plasmacytoma: Case report and review of literature

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Author contributions: Williet N, Kassir R, Roblin X and Phelip JM designed this work; Williet N wrote the paper; Williet N performed the systematic review; Cuilleron M, Dumas O and Rinaldi L contributed equally to this work; Augeul-Meunier K edited the first version text and provided her onco-hematologic regard on this case; Cottier M wrote cytology part of the text and provided cytological pictures.

Institutional review board statement: This case report was exempt from the internal Review Board standards of the Hepato-gastroenterology department managed by Pr Jean-Marc Phelip, at University of Saint-Etienne in Saint-Priest en Jarez.

Informed consent statement: The patient who is involved in the present case report gave his verbal informed consent before his death, authorizing use and disclosure of his protected health information.

Conflict-of-interest statement: None.

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Abstract

A 71-year-old man, with history of plasmacytoma in relapse since one year, was hospitalized for a initial presentation of acute pancreatitis and hepatitis. Although there was a heterogeneous infiltration around the pancreas head, the diagnosis of an extramedullary localization of his plasmacytoma was not made until later. This delayed diagnosis was due to the lack of specific radiologic features and the lack of dilatation of biliary ducts at the admission. A diagnosis was made with a simple ultrasound guided paracentesis of the low abundance ascites after a transjugular hepatic biopsy, an endoscopic ultrasound-guided fine needle aspiration of the pancreatic mass, and a failed attempt of biliary drainage through endoscopic retrograde cholangiopancreatography. In order to document the difficulty of this diagnosis, characteristics of 63 patients suffering from this condition and diagnosis were

identified and discussed through a systematic literature search.

Key words: Plasmacytoma; Pancreas; Diagnosis; Ultrasound endoscopy; Review

Core tip: We wrote an interesting case report about a pancreatic plasmacytoma for which diagnosis, including endoscopic diagnosis, was a challenge. In a second part, a systematic pubmed search was performed from 1950 to June 2016, reporting characteristics and route to diagnosis of 63 similar cases reports! Strengths of our paper are the original route to diagnosis (by a simple ultrasound guided paracentesis, after failed of the endoscopic route) and our literature search which is particularly exhaustive: we are first to identify more 20 case similar reports (63!!) and their characteristics.

Williet N, Kassir R, Cuilleron M, Dumas O, Rinaldi L, Augeul-Meunier K, Cottier M, Roblin X, Phelip JM. Difficult endoscopic diagnosis of a pancreatic plasmacytoma: Case report and review of literature. *World J Clin Oncol* 2017; 8(1): 91-95 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i1/91.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i1.91

INTRODUCTION

Here we describe the case of a pancreatic plasmacytoma and difficulties to establish the diagnosis. Characteristics of patients and routes to diagnosis in this condition will be identified through a systematic literature search, in a second part.

CASE REPORT

A 71-year-old man was hospitalized for a clinical and biological presentation of acute pancreatitis. Pain occurred suddenly and was associated with an increased level of lipase above 2000 UI/L, a cholestatic icterus (bilirubin: 103 μmol/L) and a hepatic cytolysis (ALT: 154 UI/L; AST: 131 UI/L). An initial computerized tomography (CT) scan showed a significant but unspecific infiltration around the pancreas head, without dilatation of biliary ducts. A first endoscopic ultrasound (EUS) (Pentax, EG 3670 URK, France) showed similar data. The hypoechoic infiltration of the pancreas head was heterogeneous and extended to the hepatic hilum, in contact with portal vein. There was no biliary lithiasis, nor context of alcohol consumption during the last days before the admission. However, the patient was treated with Lenalidomide plus dexamethasone for a Immunoglobulin A (IgA) plasmacytoma diagnosed 3 years ago [t(4;14) positive, del(17p) negative; at baseline: LDH: 173 UI/L, monoclonal immunoglobulin peak: 40.5 g/L, Kappa and Lambda serum free light chain: 11.7 and 18.6 mg/L, respectively], without hypercalcemia nor kidney failure. He relapsed dramatically one year ago, with an extramedullar localization (L4 lumbar spine). Based on hematotoxicity (platelets: 41000 G/mm^3) and lake of specific radiologic features, the initial diagnosis suspected was a dual hepatic and pancreatic toxicity of Lenalidomide. Indeed, acute pancreatitis and hepatitis had been occasionally reported as a side effect of Lenalidomide[1,2]. Common hepatitis viral serologies were tested before carrying out a trans-jugular hepatic biopsy which showed a histological aspect compatible with the diagnosis of drug hepatitis or hepatitis related to a biliary obstruction (centrilobular and portal infiltrate of polymorphs inflammatory cells including eosinophils). Although an empirical treatment with 500 mg intravenous methylprednisolone daily was started, bilirubin level increased at 345.8 μmol/L within the following ten days. Hence, a new CT-scan was performed and showed the occurrence of a mild to moderate dilatation of biliary ducts and a low abundance ascites. At the moment of admission, the infiltration of the pancreas head significantly resembled a tumor (Figure 1) and the diagnosis of a pancreatic localization of the plasmacytoma was suspected. After platelets support, EUS (Pentax, EG 3670UTK, France) guided fine needle aspiration (FNA) was carried out with a 22-gauge needle. Tumor infiltration appeared to be growing due to portal vein invasion. Linear EUS passage through the pylorus was drastically limited, so that FNA was performed from the gastric antrum. Then, an endoscopic retrograde cholangiopancreatography was attempted to place a biliary stent for palliative treatment, but the cannulation of the bile duct had failed due to a major parietal oedema of the duodenum which was easily bleeding due to the contact of the sphincterotome. A percutaneous biliary drainage was considered, but an ultrasound-guided paracentesis was preferred, taking into account technical difficulties of the biliary drainage. Cytology of the FNA was not contributory (epithelial cells of pancreas without malignity signs) while the analysis of ascites showed plasmacytosis with severe atypia enabling the diagnosis of pancreatic plasmacytoma (Figure 2). Bone marrow was exempted from dystrophic plasma cells, proving an extramedullar relapse. The increase of the monoclonal spike (from 2.3 g/L to 8.1 g/L within 4 mo) and LDH (259 UI/L) was compatible with this diagnosis. Kappa and Lambda free light chain, at this time of the disease, were 0.4 mg/L and 24.8 mg/L, respectively, without hypercalcemia, Bence Jones proteinuria, nor kidney failure. Hence, after contacting the referral hematologist of the patient, a cure of 40 mg dexamethasone daily was started inciting a drastic decrease of bilirubin level within the next three days (183.1 μmol/L). Then, a second line of chemotherapy (Bortezomib + Cyclophosphamide) was started with a good short-term safety. Although

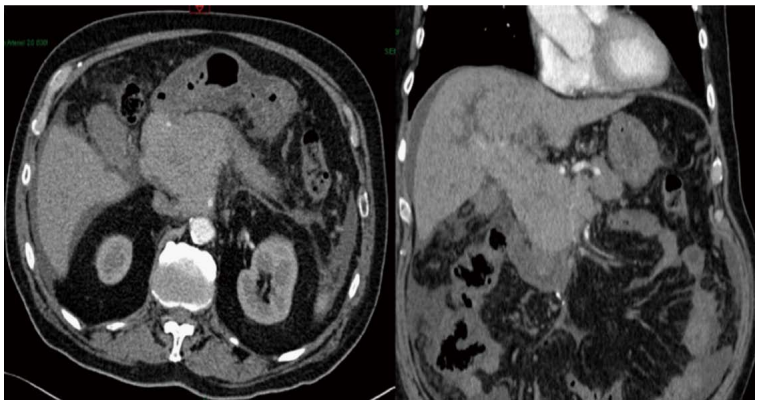

Figure 1 Abdominal computerized tomography scan showing a head pancreas mass extended to the hepatic hilum with mild to moderate dilatation of biliary ducts and a low abundance ascites.

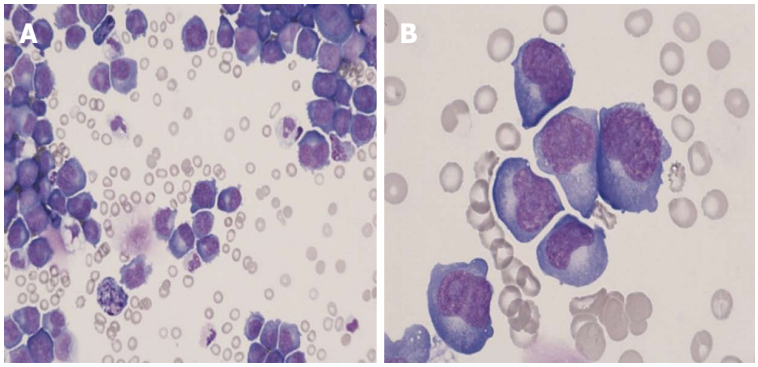

Figure 2 Peritoneal fluid Cytology, May-Grünwald-Giemsa stain. A: An almost pure population of myeloma cells (× 40); B: Malignant plasma cells exhibiting severe atypia (× 100).

a biological response, especially for monoclonal peak (2.1 g/L), at one month, the patient died 4 mo after the diagnosis of pancreatic plasmacytoma.

DISCUSSION

Extramedullary plasmacytoma involvement is not an uncommon presentation, occurring in 10%-15% of patients[3]. They are commonly identified after the diagnosis of multiple myeloma. The most commonly involved organs are those located around skeletal lesions, and less frequently, skin, liver, kidney, or central nervous system. Regarding the digestive system, liver and spleen are classically the organs which could be damaged by disease through deposits of amyloid proteins[4]. Extramedullary plasmacytomas involving the pancreas is a very rare condition with a prevalence rate estimated at 2.3%, based on autopsy studies[5].

After conducting a systematic Pubmed search, we identified 63 case reports of pancreatic plasmacytoma and collected a set of clinical and diagnostic data which were reported in Table 1. About half of them were male, with a median age of 58.5 years old, and presented jaundice in 70.0% with (36%) or without pain. About 2/3 of patients (68.4%) had a known history of plasmacytoma since 1 year (0-13) (median, interquartile ranges 25%-75%), before the involvement of the pancreas head. Only two cases involved the body or the tail of the pancreas[6,7]. Only 1/3 of patients (32.6%) were diagnosed by EUS-guided FNA *vs* 1/5 (20.9%) by CT-guided percutaneous FNA. About ¼ of patients (25.6%) have needed for a surgical biopsy, including situation involving bowel obstruction. A direct biopsy of the mass was possible in 16.3% during an upper gastrointestinal endoscopy. Most of patients were treated with chemotherapy (56.0%) and/or radiotherapy (52.0%), providing a 100% tumor response rate. A biliary stent was placed in half of patients with jaundice (46.7%).

Hence, to the best our knowledge, this is the first case report of a pancreatic plasmacytoma which was diagnosed by ascites analysis. Diagnosis by noninvasive procedures and rapid response to conservative therapy were important in this patient's care. It is very difficult to radiologically differentiate extramedullary plasmacytoma of the pancreas from other pancreatic tumors. EUS guided FNA provides the easiest and most safe route to diagnosis of pancreatic plasmacytoma. Studies have shown that the overall accuracy of EUS-guided FNA ranges between 71% and 90% in case of pancreatic tumor[8]. However, there is no corresponding data in case of pancreatic plasmacytoma.

In our case, the missed diagnosis of pancreas plasmacytoma through EUS-guided FNA may be due to a sampling bias. Furthermore, we made only one diagnostic EUS attempt while in few cases reported, authors specified the need for repeating EUS-guided FNA[9-13].

This case highlights that a pancreatic mass in patients with plasmacytoma should be systematically considered as an extramedullary extension of the disease until proven otherwise. Ascites analysis could

Table 1 Main characteristics of the 63 patients who had been reported to date with a pancreas plasmacytoma: Results of a PubMed search from 1950 to June 2016

Demographic characteristics	*n* (%)
Male	22 (56.4)
Age (years, median, IQR)	58.5 [51.2-82]
Symptom(s) at diagnosis	
Jaundice	35 (70.0)
Pain	18 (36.0)
Myeloma	
Known history of myeloma	26 (41.3)
Disease duration at diagnosis of pancreas plasmacytoma (years, median, IQR)	1 [0-13]
Type Kappa	13 (71.4)
Immunoglobulin	A (36%), G (52%), M (12%)
Diagnosis process of the pancreas plasmacytoma	
Endoscopic ultrasound FNA	14 (32.6)
Percutaneous FNA	9 (20.9)
Endoscopic biopsy	7 (16.3)
Surgical biopsy	11 (25.6)
Paracentesis	0 (0.0)
Postmortem biopsy	3 (7.0)
Management of the pancreas plasmacytoma	
Chemotherapy	14 (56.0)
Radiotherapy	13 (52.0)
Biliary stent in patients with jaundice	10 (40.0)
Surgery	8 (32.0)
Biliodigestive derivation	3 (37.5)
Duodenopancreatectomy cephalic	2 (25.0)

FNA: Fine needle aspiration; IQR: Interquartile range.

be a simple route to diagnosis, even in low abundance. Finally, in case of jaundice, excluding angiocholitis, potential risks of biliary stenting should be taken into account, regarding safety and the drastic efficacy of radiotherapy or medical treatment (dexamethasone and chemotherapy).

COMMENTS

Case characteristics

A 71-year-old man with history of plasmacytoma in relapse since one year, and treated with Lenalidomide.

Clinical diagnosis

The initial diagnosis suspected was a dual hepatic and pancreatic toxicity of Lenalidomide.

Differential diagnosis

An adenocarcinoma of the pancreas, or other less frequent pancreatic tumor such as a non Hodgkin's lymphoma, or endocrine tumor.

Laboratory diagnosis

An increased level of lipase above 2000 UI/L, a cholestatic icterus and a hepatic cytolysis.

Imaging diagnosis

Computerized tomography showed a significant but unspecific infiltration around the pancreas head, without dilatation of biliary ducts, extended to the hepatic hilum, and evolving as a pseudotumor within few days.

Cytological diagnosis

A (pancreatic) plasmacytoma.

Treatment

An empirical corticotherapy followed by a second line of chemotherapy (Bortezomib + Cyclophosphamide).

Related reports

Cytology of the mass was not contributory in contrast with the very low abundance ascites located around the liver.

Terms explanation

Extramedullary plasmacytoma involvement is not an uncommon presentation, and occurres preferentially in located around skeletal lesions, or less frequently in, skin, liver, kidney, or central nervous system.

Experiences and lessons

A pancreatic mass occurring in a patient with history of plasmacytoma and with an uncommon presentation should make suspecting an extramedullar site of the disease. No diagnostic way should be forgot, even a simple analysis of an ascites sample.

Peer-review

This is an interesting case about pancreas involvement in a case with relapsed myeloma.

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