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EDITORIAL

Watch and wait policy in advanced neuroendocrine tumors: What does it mean?

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Abstract

Neuroendocrine neoplasms (NENs) are a group of rare and heterogeneous malignancies, which can develop in various organs. The clinical course of NENs is quite heterogeneous, with different spontaneous growth rates after diagnosis, and different degrees of sensitivity to the same therapy even when they have similar characteristics. *Watch and wait* (W and W), is a term coined to indicate observation being conducted to assess the evolution of the tumor without administering any anti-tumor therapy. It has been applied to NENs since in extremely rare cases they tend to remain stable for a long time. Although W and W has been reported in several guidelines and recommendations it has never been validated, nor has it been specifically investigated. Furthermore it is not standardized. Therefore its application in clinical practice can differ in terms of tumor status assessment, type and timing of imaging or other exams utilized. In conclusion, while undertaking W and W to delay the first-line therapy by some weeks may be justified in good performance asymptomatic patients with low-grade NENs in order to usefully characterize the disease and patient and thereby choose the best therapy and therapeutic strategy, it seems to be far more difficult to justify W and W with the intent of avoiding an anti-tumor treatment. It should be considered that not only do NENs tend to grow even when they have very favorable biological characteristics but also that the alternative to W and W is most commonly a low toxic and effective treatment with somatostatin analogs.

Key words: Observation; Wait and see; *Watch and wait*; Surveillance; Neuroendocrine tumors

Core tip: *Watch and wait* (W and W) is a term coined to indicate observation without therapy assessing the evolution of the tumor. Given that neuroendocrine tumors sometimes are radiologically stable over months since they tend to grow slowly observation has been reported as an option to be considered in several guidelines and recommendations. However it has neither validated nor specifically investigated so far. Therefore its application

in clinical practice is arbitrary and it differs in terms of tumor status assessment, type and timing of imaging or other exams utilized. While undertaking W and W to delay the first-line therapy by some weeks may be justified in good performance asymptomatic patients with low-grade neuroendocrine neoplasms (NENs) in order to usefully characterize the disease and patient and thereby choose the best therapy and therapeutic strategy, it seems to be far more difficult to justify W and W with the intent of avoiding an anti-tumor treatment. It should be considered that not only do NENs tend to grow even when they have very favorable biological characteristics but also that the alternative to W and W is most commonly a low toxic and effective treatment with somatostatin analogs.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) represent a group of rare and heterogeneous malignancies, which can develop in various organ. They are classified on the basis of their level of aggressiveness into low, intermediate and high grades of malignancy.

Neuroendocrine neoplasms from the digestive tract, are classified on the basis of proliferation index as G1 (⩽ 2% Ki-67), G2 (3%-20% Ki-67) and G3 (> 20% Ki-67). Furthermore, based on their morphology they are named "tumors" (NETs) when they are well differentiated, whereas "carcinomas" (NECs) when they are poorly differentiated"[1]. Neuroendocrine neoplasms from the thoracic region are classified into typical carcinoid, TC (< 2 mitoses/2 mm^2 with absence of necrosis), atypical carcinoid, AC (2-10 mitoses/2 mm^2 with necrosis), large cell neuroendocrine carcinoma, LCNEC (> 10 mitoses with extensive necrosis) and small cell lung cancer, SCLC (> 10 mitoses with extensive necrosis)[2].

While high-grade NENs are treated with chemotherapy in the vast majority of cases when they are in advanced stage of disease, the therapeutic approach to advanced low-intermediate grade NENs varies. Somatostatin analogs (SSA), interferon (IFN), molecular targeted agents (MTAs), chemotherapy, peptide receptor radionuclide therapy (PRRT), and liver-directed treatments (LDTs), are all potentially effective therapies to propose, often in the same clinical setting. Although some of these therapies have been approved on the basis of positive regulatory phase Ⅲ trials[3-7] in specific settings and several guidelines about NENs do exist[8,9], no sequencing or priority criteria about the different therapies have been validated. Furthermore, the clinical course of NETs is quite heterogeneous, with different spontaneous growth rates after diagnosis, and different degrees of sensitivity to the same therapy even when they have similar characteristics.

"*Watch and wait* (W and W)", "watchful waiting", "wait and see", "observation" and "active surveillance" are all terms which are used to describe assessing the evolution of the tumor without an anti-tumor therapy. These terms have been applied synonymously to NETs as in rare cases they have a spontaneous very indolent clinical course. Sometimes they are also applied to a localized disease, as in the case of so-called pancreatic "incidentaloma", namely a < 2 cm isolated nodule in the pancreas. European Neuroendocrine Tumor Society (ENETS) 2016 guidelines recommend W and W for a < 2 cm pancreatic NET, "G1 or low G2, asymptomatic, mainly in the head, with no radiological signs suspicious for malignancy", and suggest that one also consider the patient's attitude, age and comorbidity. It is specified that the follow-up should be performed with endoscopic ultrasound (EUS), magnetic resonance imaging (MRI) (or computed tomography, CT) "every 6 to 12 mo". However, the length of follow-up is not specified[10].

In the ENETS guidelines W and W is also recommended for advanced disease, for instance in NETs from the midgut when they are "non-functional, G1, low tumor burden, no symptoms, stable disease". This policy is advised even for pancreatic NETs, when they are "non-functional, G1, ⩽ 10% Ki-67, low tumor burden, stable disease or initial diagnosis, no symptoms"[11].

In both midgut and pancreatic NETs the W and W policy is a possible alternative to SSA. However, SSA compared with placebo resulted effective in two phase III randomised controlled trials, with octreotide long-acting repeatable (LAR) producing a longer time to progression (TTP) in midgut NETs in the *PROMID* trial and lanreotide autogel significantly prolonging progression free survival (PFS) in enteropancreatic NETs in the *CLARINET* trial, respectively[5,6]. Notably, time to progression (TTP) was quite short in the placebo arm of the *PROMID* trial demonstrating that also NETs with < 3% Ki-67, as were the vast majority of the tumors included in the PROMID, will progress eventually. Interestingly, NETs included in the *CLARINET* trial, which resulted as having a stable disease in 96% of cases in accordance with RECIST criteria, in fact were progressing at baseline, as showed with the so-called tumor growth rate (TGR)[12].

Another report indicating that NETs tend to grow early spontaneously, is a retrospective analysis of more than 200 patients with advanced pancreatic NETs showing that those patients who did not receive antitumor treatment during follow-up had a significantly shorter PFS compared to treated patients, thus confirming that anti-tumor therapy can favorably impact on the clinical course of the disease[13].

In the ENETS 2016 guidelines it is not specified whether radiological or functional imaging or both are recommended to monitor the tumor status of a low-grade NET; it is not clear whether some biochemical tests, such as chromogranin-A, should be performed periodically;

timing of follow-up imaging is not specified.

Furthermore no data exist about the impact of the W and W on the patient's quality of life and costs.

The W and W policy is debated also in other fields of oncology. For instance in renal cancer it was investigated in a phase Ⅱ trial including medical anti-tumor treatment-naive patients with advanced disease[14]. The decision to choose W and W over immediate systemic therapy was made jointly by the patient and treating physician. Therefore patients underwent homogeneous radiological and clinical follow-up and also filled in quality of life questionnaires. Median time to radiological progression, RECIST-based, was 9.4 mo (95%CI: 7.4-13.4); at progression, patients received a first-line systemic therapy; no observed adverse effects on quality of life, anxiety and depression, were recorded during the observation period. Although this study seems to indicate that in some selected patients with metastatic renal carcinoma, active surveillance might be a good approach, homogeneous criteria for selection of patients to undergo W and W, type of follow-up and timing of first-line therapy remain debatable.

Further while in renal cancer one of the reasons for performing W and W instead of administering treatment to patients is to avoid therapies which may well be highly toxic, in NETs the choice is almost always between W and W and SSAs, which are a very low-toxic therapy.

Finally, in good-performance status asymptomatic patients with advanced NETs, the diagnostic work-up, morphological and functional staging and characterization of the disease require some weeks. Luckily in most cases this time without therapy is not detrimental for the patient and it allows an assessment to be made of clinical behavior and tumor growth, a thorough understanding of tumor and patient characteristics, and the discussion of the global therapeutic strategy within a dedicated multidisciplinary team. All of this may be very helpful to patients when compared with starting a single first-line therapy right from the time of diagnosis of an advanced NET. Proposing a W and W policy after completing this initial period of observation to a patient with a metastatic NET means waiting for a tumor growth or a clinical progression. On the one hand it is arbitrary to define whether morphological (radiological), functional (receptorial? metabolic?) or biochemical progression should be considered and with which threshold; on the other hand it could be detrimental to start therapy only when tumor-related symptoms arise. Nonetheless patients should be informed that no study has specifically investigated this topic comparing W and W and anti-tumor therapy, and therefore we have no evidence either for or against. Patients will need to understand that follow-up will be life-long even with stable disease, that there are data showing that the vast majority of advanced NETs tends to grow and that SSAs can be active even when the tumor is very indolent.

In conclusion, W and W policy in advanced NENs is yet to be well-defined. First of all it should be clarified whether W and W means delaying or avoiding an anti-tumor treatment. Delaying may be justified in an asymptomatic good performance status patient with a low-grade NETs over some weeks in order to thoroughly characterize both disease and patient and so make a well-informed choice as to the best therapy and therapeutic strategy to pursue. This is a quite common clinical scenario in the field of NETs. By contrast it is hard to justify W and W with the intent to avoid treatment considering that low-grade advanced NETs tend to grow even when they have very favorable biological characteristics. Therefore, also in that case, rather than avoiding, it would mean once again delaying the first-line therapy. Of course the first-line therapy and the therapeutic strategy depend on the specific clinical context and on the goal of treatment. In other words in a patient who is a good candidate for a future absolute debulking, then the first-line treatment even more than an SSA should be applied even with a stable disease without any delay. On the other hand, in a patient with a metastatic low grade, really stable NET, when absolute debulking is not possible and the goal of the treatment is the tumor growth control over time with a systemic medical therapy, then a thoughtful analysis needs to be made. It is important to bear in mind the cost- and risk-benefit of SSA, which is the most commonly proposed therapy in such a context, and also the cost, invasiveness, impact on quality of life and possible detrimental effect of W and W.

I would argue that given the absence of evidence and of clinical trials designed to specifically investigate this topic, as is currently the case, clinicians should consider administering treatment to all patients, whether their NETs are advanced.

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EDITORIAL

Translating new data to the daily practice in second line treatment of renal cell carcinoma: The role of tumor growth rate

Enrique Grande, Olga Martínez-Sáez, Pablo Gajate-Borau, Teresa Alonso-Gordoa

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Abstract

The therapeutic options for patients with metastatic renal cell carcinoma (mRCC) have completely changed during the last ten years. With the sequential use of targeted therapies, median overall survival has increased in daily practice and now it is not uncommon to see patients surviving kidney cancer for more than four to five years. Once treatment fails with the first line targeted therapy, head to head comparisons have shown that cabozantinib, nivolumab and the combination of lenvatinib plus everolimus are more effective than everolimus alone and that axitinib is more active than sorafenib. Unfortunately, it is very unlikely that we will ever have prospective data comparing the activity of axitinib, cabozantinib, lenvatinib or nivolumab. It is frustrating to observe the lack of biomarkers that we have in this field, thus there is no firm recommendation about the optimal sequence of treatment in the second line. In the absence of reliable biomarkers, there are several clinical endpoints that can help physicians to make decisions for an individual patient, such as the tumor burden, the expected response rate and the time to achieve the response to each agent, the prior response to the agent administered, the toxicity profile of the different compounds and patient preference. Here, we propose the introduction of the tumor-growth rate (TGR) during first-line treatment as a new tool to be used to select the second line strategy in mRCC. The rapidness of TGR before the onset of the treatment reflects the variability between patients in terms of tumor growth kinetics and it could be a surrogate marker of tumor aggressiveness that may guide treatment decisions.

Key words: Axitinib; Everolimus; Cabozantinib; Kidney cancer; Nivolumab; Renal cell; Sequence; Second line; Sorafenib; Tumor-growth rate

Core tip: The landscape of renal cell carcinoma has dramatically changed in the last decade. Today, at least 6 agents are approved after failure with cytokines,

sunitinib or pazopanib in first line treatment. Lack of reliable biomarkers to select the best treatment in daily practice is somewhat frustrating. Therefore, our decisions in real practice are based on safety profiles, patient' co-morbidities and physician experience or preference. Here we debate the pros and cons of the tumor-growth rate as a tool to select second line systemic treatment after failure to a prior tyrosine kinase-inhibitor in patients with advanced renal cell carcinoma.

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INTRODUCTION

The increased knowledge about the underlying pathogenesis of the metastatic renal cell carcinoma (mRCC) has led to the development of new therapeutic drugs that have completely changed patient prognosis. These drugs are targeting the vascular endothelial growth factor receptor (VEGFR) axis, the mammalian target of rapamycin (mTOR) pathway or the immune system and tumor cell interactions (PD1/PDL1). The number of patients that are candidates for a second line therapy after progressing on a first line varies from 43% to 79%[1]. The second line treatment is determinant in mRCC as patients can also benefit from an improvement in overall survival (OS) already achieved with first line choice and expand their chances for a longer therapeutic sequence. In this regard, a large registry-based experience in the United Kingdom has shown that those patients who received a second line treatment lived longer (33 mo; ranging from 30.8-35.2) than those who did not receive further treatment after first line (20.9 mo; ranging from 16.4-25.3)[2]. Fortunately, options for second line therapy have multiplied with the recent approval of nivolumab, cabozantinib and the combination of everolimus with lenvatinib[3-6]. However, there are no head-to-head comparisons between them and no predictive biomarker has been validated for the second line treatment decision making[7]. Besides, the uncertainty regarding the optimal therapeutic sequence, there is an urgent need for developing prognostic and predictive variables, in order to select patients who will benefit from a specific second line treatment[8].

There are some clinical and economic-derived factors coming from the pivotal trials of each agent that could be considered at the time of second line treatment decisions (Table 1). The patient's tumor burden has been suggested from retrospective data as being strongly correlated with the progression free survival (PFS) and OS in patients with mRCC[9-12]. The expected response rate from the approved drugs has been reported to be different between cabozantinib, nivolumab and axitinib that achieve an overall response rate (ORR) of 17% to 22%, unlike the combination of everolimus with lenvatinib that has been reported to be of 35% in the phase II pivotal trial[3-6]. Moreover, the time required to achieve a tumor response is a major concern for heavily symptomatic patients that need an early tumor control. Prior tolerance and duration of response to first line treatment may identify those patients harboring a kidney tumor that greatly benefits from the angiogenic blockade (angiogenesis addiction), but may limit the decision in primary refractory patients[13,14]. Finally, we also propose the assessment of the tumor-growth rate (TGR), as a novel outcome measure that could help in the therapeutic sequence decision in the mRCC setting.

$$TGR = 100 \times [\exp(TG) - 1]$$

$$TG = \frac{3 \times \log (D2/D1)}{\text{Time (months)}}$$

D1 = tumor size at date 1; D2 = tumor size at date 2; and time (months) = (date2 - date1 + 1)/30.44

Figure 1 Tumor growth rate calculation formula. TGR: Tumor-growth rate.

Several authors have discussed that the Response Evaluation Criteria in Solid Tumors (RECIST) may be inadequate to completely evaluate the response of targeted therapies in mRCC as often induce long-lasting stable disease rather than tumor shrinkage[15-18]. In addition, these criteria do not take into account tumor growth kinetics, and might not be relevant in slow-growing diseases[19,20]. Therefore, alternate modalities to assess the drug response have been proposed to overcome the limitations of the RECIST criteria, such as Choi, SACT, MASS, ETPIC or iRECIST. These approaches include the tumor perfusion evaluation, *via* the use of CT response assessment combining reduction in both, size and arterial phase density, changes in tumor CT texture or metabolism or the immune component evaluation. However, none of them appear to be an adequate surrogate of response or clinical outcome for its application in routine clinical practice[16,18,21,22].

TGR provides a dynamic and quantitative evaluation of tumor kinetics; it estimates the percentage of change in the tumor volume over one month. TGR is usually defined as the ratio between the slope of tumor growth before the initiation of treatment and the slope of tumor growth during treatment, and between the nadir and disease progression[9,23]. We can calculate TGR according to the formula shown in Figure 1[24]. The tumor size is defined using the sum of the longest diameters (SLD) of target lesions only, without considering non-target and new lesions. However, the assessment of the TGR in clinical practice is easier as there are internet tools available (http://ec2-54-218-32-173.us-west-2.compute.amazonaws.com:3838/tgrShiny/ or http://www.gustaveroussy.fr/doc/tgr_calculator/index_en.html).

Table 1 Phase Ⅲ clinical trials evaluating approved drugs in second and subsequent treatment lines for metastatic renal cell carcinoma

	Axitinib	Cabozantinib	Lenvatinib + Everolimus	Nivolumab
Trial design	Phase Ⅲ	Phase Ⅲ	Phase Ⅱ	Phase Ⅲ
Size	361	330	51	410
Patient population	2nd Line (100%)	2L- 71% 3L- 29%	2nd Line (100%)	2L- 72% 3L- 28%
MSKCC risk % (Good/int/poor)	28/37/33	45/42/12	24/37/39	35/49/16
Comparator	Sorafenib	Everolimus	Everolimus	Everolimus
ORR% (ICR)	19%	17%	35%	22%
Progression disease (%)	22%	12%	4%	35%
PFS (m)	6.7 (HR 0.66)	7.4 (HR 0.51)	12.8 (HR 0.40)	4.6 (HR 0.88)
PFS (m) in pts with bone mets	NR	7.4 (HR 0.33)	NR	NR
OS (m)	20.1 (HR 0.96)	21.4 (HR 0.66)	25.5 (HR 0.59)	25.0 (HR 0.73)
Dose reductions	30%	60%	71%	N/A
Discontinuations due to AEs	7%	9%	25%	8%
Toxicity G3/4 (%)	56%	68%	71%	19%
Average monthly cost (US basis)	9580$	10229$	22461$	12435$

MSKCC: Memorial Sloan Kettering Cancer Center Criteria; ORR: Overall response rate; OS: Overall survival; PFS: Progression free survival; AE: Adverse events.

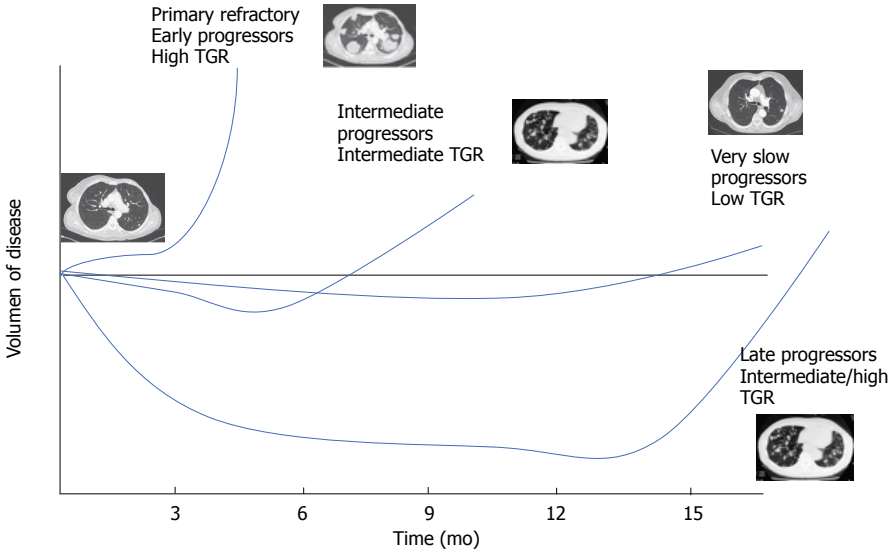

Figure 2 Hypothetical representation of different groups of patients and their patterns of response to first line treatment: Primary refractory patients with early progression and high tumor growth rate, intermediate progressors with intermediate tumor growth rate, very slow progressors with low tumor growth rate and late progressors with high tumor growth rate. TGR: Tumor-growth rate.

Current evidence from phase Ⅰ studies in solid tumors and from phase Ⅲ studies in mRCC (TARGET and RECORD trials) and metastatic neuroendocrine tumors (NETs) (CLARINET trial), although retrospective, show a significant association between prior TGR before the onset of the second line approach with the expected PFS and OS with the later systemic treatment administered[9,24-28]. Moreover, TGR could be an important tool in the evaluation of prognosis during treatment and after the discontinuation of VEGFR targeted agents. Iacovelli *et al*[29] showed that those patients with a higher than median TGR during treatment had a significantly shorter OS and, indeed, those patients with lower than the median TGR after discontinuation had longer OS, as compared to TGR after discontinuation greater than or equal to the median. Therefore, it would be possible to use TGR as a possible surrogate for tumor aggressiveness and survival in mRCC patients while on VEGFR-directed TKI in the first line. In the post hoc analysis from the CLARINET trial, TGR seemed to provide more precise information to predict pretreatment progression regarding actively growing tumors, but considered as stable disease by RECIST criteria, and more sensitive to detect early antitumor activity from treatment compared with RECIST criteria[28]. We consider that the addition of TGR in the assessment of individual patients undergoing targeted therapies may help clinicians to know if a given agent is modifying or not the course of the disease and guide the decision of which agent would be preferred in the subsequent line. However, for the use of TGR in the clinical setting, a prospective clinical trial for its validation would be needed[23].

Considering all aspects previously discussed, patients with mRCC that are candidate for a second line treatment could be differentiated into four main subgroups (Figure 2). Patients with florid symptoms, high tumor burden, short time to response to the first line (PFS less than 6 mo, so called, early progressors) and high TGR, in which we would need an early and high response, the

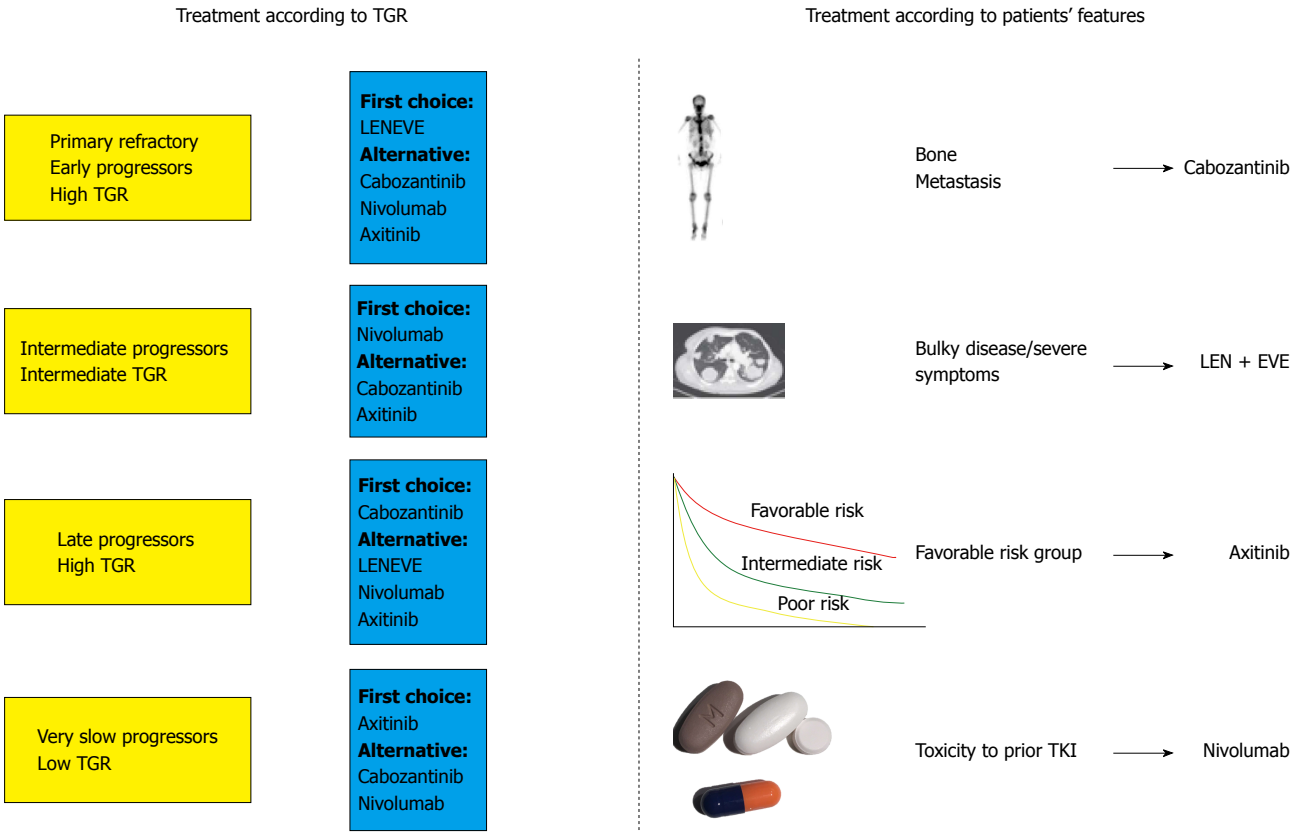

Figure 3 Adapting the study data to our clinic. A proposed algorithm to treat second line metastatic renal cell carcinoma patients according to tumor growth rate and patients' characteristics. TGR: Tumor-growth rate; TKI: Tyrosine kinase inhibitor; LEN: Lenvatinib; EVE: Everolimus.

combination of everolimus with lenvatinib should be considered, as we will target several mechanisms of action (VEGFR, fibroblast growing factor receptor, FGFR, and m-TOR pathways). In such patients, the expected benefit outweighs the increased toxicity of the combination therapy. In those patients with a long response to first antiangiogenic drug (PFS more than 18 mo, so called angiogenesis addicts) and low or intermediate TGR, the use of cabozantinib may be considered. Regarding those patients that are not responding radiographically but are stable for the advanced disease for a long period with a very low TGR (increase of less than 4% in the sum of the longest diameters per month) and have an adequate tolerability, we propose that axitinib could be a reliable option to prolong the clinical benefit. Finally, for patients with an interval free of progression with first line treatment between 6 and 18 mo, as considered intermediate-progressors, nivolumab may be the treatment of choice as an inhibitor of an actionable immune target by introducing a different mechanism of action against tumor growth.

Lastly, we highlight the upcoming availability of novel immune agents such as ipilimumab, atezolizumab, pembrolizumab either as single agent or in combination that might impact in the first line setting of patients with advanced RCC. Therefore, it is very likely that second line landscape of metastatic RCC may change shortly. Adaptation to the clinic of the amount of new data that are expected in a short term promises to be challenge.

In conclusion, patients with mRCC receiving a second line treatment achieve a median OS of more than 2 years with novel agents. Thus, the optimal treatment selection in this setting allows us to provide the maximal clinical benefit to our patients, but with no definitive biomarker to guide our decision. In this setting, we have considered some relevant clinical parameters before choosing a certain agent such as the patient's tumor burden, the expected response rate to the different drugs and the time to achieve this response, the prior response to previous VEGFR-TKIs, the toxicity profile of each agent and the patient preference. Thus, we propose the employment of the TGR as a new tool that could provide useful information in the management of mRCC patients in addition to clinical features that could better fit with one of the therapeutic alternatives (Figure 3). TGR may represent a surrogate of tumor aggressiveness, a relevant parameter before choosing a treatment and an early biomarker for treatment response and evaluation of the ability to interfere in the natural history of the tumor growth. TGR could be a valuable endpoint for clinical use in treatment decision-making favoring patients with mRCC, with more reliable information about prognosis and evaluation of response to molecular targeted agents.

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FRONTIER

Leptin signaling and cancer chemoresistance: Perspectives

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Abstract

Obesity is a major health problem and currently is endemic around the world. Obesity is a risk factor for several different types of cancer, significantly promoting cancer incidence, progression, poor prognosis and resistance to anti-cancer therapies. The study of this resistance is critical as development of chemoresistance is a serious drawback for the successful and effective drug-based treatments of cancer. There is increasing evidence that augmented adiposity can impact on chemotherapeutic treatment of cancer and the development of resistance to these treatments, particularly through one of its signature mediators, the adipokine leptin. Leptin is a pro-inflammatory, pro-angiogenic and pro-tumorigenic adipokine that has been implicated in many cancers promoting processes such as angiogenesis, metastasis, tumorigenesis and survival/resistance to apoptosis. Several possible mechanisms that could potentially be developed by cancer cells to elicit drug resistance have been suggested in the literature. Here, we summarize and discuss the current state of the literature on the role of obesity and leptin on chemoresistance, particularly as it relates to breast and pancreatic cancers. We focus on the role of leptin and its significance in possibly driving these proposed chemoresistance mechanisms, and examine its effects on cancer cell survival signals and expansion of the cancer stem cell sub-populations.

Key words: Obesity-related cancer; Cancer stem cells; Leptin; Chemoresistance; Breast cancer; Pancreatic cancer

Core tip: Obesity and its main mediator leptin, are implicated in many protumorigenic processes, with

emerging evidence from both the literature and our work pointing to a significant role in the development of resistance to chemotherapies. Chemoresistance is a major concern in the field of cancer therapy as some cancers have no targeted therapies available. As obesity reaches epidemic proportions around the world, its impact on diseases like cancer and its treatment becomes more relevant. In this paper, we will discuss the current state of the literature regarding the influence of obesity and leptin on cancer treatment and the development of chemoresistance.

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INTRODUCTION

Obesity is the state of having excessive adipose tissue reserves, commonly defined as having a body mass index (BMI) of 30 or more. The global prevalence of obesity is high, with 37% of men and 38% of women being either overweight or obese[1]. There are significant health consequences for being overweight or obese. Obesity is closely associated to high rates of morbidity and mortality. It is considered responsible for an estimated 3.4 million deaths and 4% of years spent with a disability. There is a well-documented increased risk in obese and overweight people for numerous cancers, including thyroid, esophageal, kidney, colon, rectal, melanoma, leukemia, endometrial, gallbladder, pancreas and breast cancer[2-6]. In addition, weight gain before 50 has been associated with greater risk of breast cancer, especially estrogen negative breast cancer[7-9]. A contributing factor could be complications related to therapy, as obesity is correlated with breast cancer recurrence, with increasing BMI being correlated with increased risk of breast cancer relapse. Obesity impacts on life expectancy, with premenopausal and postmenopausal obese women being 1.75 and 1.34 times, respectively, at increased risk of death from breast cancer[10].

A distinctive characteristic of obesity and overweight conditions is the high serum level of the main adipokine, leptin secreted by adipose tissue. Leptin, from the Greek work "leptos", thin, is a 16 kDa protein, composed of 167 aminoacids, its gene, *Ob*, is in humans on chromosome 7q32. *Ob* gene is composed by three exons and 2 introns, spanning 20 kb. Leptin is the first discovered adipokine, a cytokine secreted by adipocytes, both from the white adipose tissue and brown adipose tissue. Placenta, ovaries, skeletal muscle, bone marrow, stomach, pituitary gland, and mammary epithelial cells have been shown to express leptin[11]. Several cancer cell types and tumor stroma also express leptin[12].

OBESITY, LEPTIN/OB-R AND CANCER

The main role of leptin is to regulate energy balance by inhibiting hunger. Leptin levels correlate to adiposity. Under physiological conditions leptin binds and activates receptors in the arcuate nucleus of the hypothalamus, which regulate appetite[13]. In obese people a decreased sensitivity to leptin was observed resulting in a decreased capacity to feel satiety[14]. A result of this resistance is overeating that results in obesity and the concomitant high serum levels of leptin. In obese individuals serum leptin levels are 10 times higher (*i.e.*, 40 ng/mL) than normal weight people (*i.e.*, 4 ng/mL)[15]. The upregulation of leptin has an important role in carcinogenesis[16].

Leptin receptor (Ob-R) is predominantly found in the hypothalamus[17], but is expressed at lower level in the whole body, including pancreas[18] and mammary epithelial cells[19]. Remarkably, cancer cells overexpress Ob-R, which enable them to respond to leptin that is more prominent in obese individuals showing high levels of the adipokine. Ob-R belongs to Class Ⅰ superfamily cytokine receptors. It is a transmembrane protein composed by an extra-cellular domain, responsible for binding leptin, a transmembrane domain and a cytoplasmic domain for signaling[20]. Currently six different isoforms of the leptin receptor have been identified, Ob-Ra-f, generated by mRNA splicing or proteolytic processing, Ob-R isoforms are divided in three classes, short and long (which are bound to the cellular membrane) and secreted (a soluble protein that binds leptin in blood). The long isoform Ob-Rb (or l) is the predominant one, expressed at high levels in different cell types. Ob-Rb has full signaling capabilities in contrast to short Ob-R isoforms. It is generally accepted that leptin binding to Ob-R provokes the formation of a homodimer that is responsible for leptin-mediated signals. Leptin and Ob-R have absolute specificity for binding. Once leptin binds to Ob-Rb, it activates several signaling pathways. Because Class Ⅰ cytokine receptors lack auto phosphorylation function they need auxiliary kinases to initiate signaling upon ligand binding. The first signaling event after leptin binding to Ob-Rb is the activation of janus kinase/signaling transducer and activation of transcription factor pathway (JAK/STAT)[21]. JAK2 recruitment to Ob-R intracytoplasmatic tail leads to the phosphorylation of the kinase, subsequent phosphorylation of Ob-R in several intracytoplasmatic sites and recruitment and phosphorylation of tyrosine residue on STATs. Phosphorylated STATs, then form hetero or homodimers and translocate to the nucleus to induce the transcription of specific genes[22].

Leptin plays roles in other physiological functions, as indicated by the presence of its receptor in different organs and tissues types besides the hypothalamus[23]. Leptin is involved in immunity, proliferation, differentiation, apoptosis, angiogenesis, inflammation, fertility and oncogenesis[12,16,22]. Leptin is known to inhibit bone formation[24]. It can also regulate the ovulatory cycle by

stimulating GnRH from the hypothalamus[25,26] and is an important factor in embryo implantation[27-29]. Leptin is involved in the onset of puberty[30], regulates glucose homeostasis[31], hematopoiesis[32], and modulate immunity like T cell activity in response to atherosclerosis[33]. Leptin has been speculated to be an inflammatory marker that responds specifically to adipose-derived inflammatory cytokines[34].

Obesity is a significant risk factor for cancer incidence and mortality. The effects of obesity on cancer could be due in part to leptin's elevated levels and Ob-R over expression in cancer cells, which enable leptin-deregulated pleiotropic signals in cancer. Leptin has been shown to have several pro-tumorigenic effects, such as increasing cancer cell proliferation, anti-apoptosis, angiogenesis, self-renewal and possibly resistance to chemotherapeutic treatment[12,16].

Several studies linked the effects of leptin on the proliferation of cancer both *in vivo* and *in vitro* experimental models, and from patient data. Leptin signaling has been consistently linked to the development of breast, endometrial, pancreatic, colon, prostatic, hepatic, skin, brain, oesophagus, stomach, thyroid gland, and ovarian cancers, and leukemia and chondrosarcoma[35-43].

Leptin induces breast cancer cell growth *in vitro* and *in vivo*. Several leptin-induced signaling pathways and factors have been linked to the proliferation of breast, endometrial and pancreatic cancer cells[12,16,36,37]. Leptin induced tumor cell growth and inhibited apoptosis in papillary thyroid cancer (PTC) cells. Serum levels of leptin were shown to be higher in patients with PTC than in negative controls[42]. An increase in the expression of leptin receptor Ob-R was observed in PTC specimens[44]. Leptin can induce the development of non-alcoholic fatty liver disease (NAFLD), one of the major cause of hepatocellular carcinoma, by promoting insulin resistance, steatosis and hepatic inflammation by increasing transforming growth factor beta (TGF-β) expression[43]. Leptin is overexpressed in colon cancer, Ob-R mRNA was found in cancer cell lines and colon tumors[45] and Ob-R protein expression was confirmed by western blot[46]. Serum leptin levels were significantly high in patients with lung cancer, compared to healthy individuals. Lung cancer tissues showed higher expression of leptin compared to normal lung tissue[47]. Leptin was shown to stimulate the proliferation of human myeloid leukemia cell lines[48], and it might play a role in the development of prostate cancer, it can increase growth and survival of prostate cancer cells and Ob-R mRNAs has been found in prostate cancer cells through RT-PCR[49]. Epithelial ovarian cancer (EOC) is one of the principal cause of death in gynecological malignancies, but the role of leptin in this disease still needs further investigation as Ob-R mRNA was found in several immortalized EOC cell lines[50]. Limited data suggested also a link between leptin and adrenal cancer[51].

Leptin induced pleiotropic effects in cancer cells. Leptin increased breast cancer cell proliferation, which was linked to the up regulation of cyclin D[52] and increased expression of anti-apoptotic proteins like Bcl-2 in breast cancer[53]. Additionally, leptin can down regulate pro-apoptotic Bax[54]. Leptin induces tumor angiogenesis that has a pivotal role in solid tumor growth and metastasis. Leptin not only promotes the expression of angiogenic factors like vascular endothelial growth factor (VEGF)[55], VEGFR-2[52,56], and fibroblast growth factor 2 (FGF-2), but also itself induces vascular endothelial cell proliferation *in vitro* with similar effects than VEGF[57]. Moreover, in the absence of VEGF, leptin induced Notch signaling pathway in endothelial cells that was linked to leptin-induced transphosphorylation of VEGFR-1 and VEGFR-2[58]. Leptin induces two angiogenic factors: Interleukin (IL)-1[59] and Notch[60] that can increase VEGF expression. Moreover, leptin induces the secretion and synthesis of proteases and adhesion molecules needed for the development of angiogenesis. Leptin induces expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) that are involved in tissue remodeling, specifically the breakdown of extracellular matrix proteins[61,62]. Additionally, leptin induces the expression of avB3 integrin that is also involved in angiogenesis[37,63]. Leptin induces production of inflammatory cytokines like IL-1, IL-6 and tumor necrosis factor (TNF)-α, which like leptin can induce the expression of metalloproteinases, promoting tumor invasion and metastasis. TNF-α acts on adipocytes increasing leptin expression[34].

LEPTIN-INDUCED NOTCH AND RBP-JK AFFECT CANCER PROGRESSION

Gonzalez-Perez's lab earlier reported that leptin signaling crosstalk to Notch in breast cancer[60]. Notch signaling is an embryonic conserved pathway involved in proliferation, angiogenesis, cell fate and development. Notch system is composed by transmembrane proteins: Receptors (Notch1-4) and ligands expressed in adjacent cells (Delta-like, Dll1-3, and Jagged-like, JAG1-2), and molecular targets hairy enhancer of split (Hes1-7), hairy/enhancer-of-split related with YRPW motif subfamilies (Hey1, Hey2, HeyL, HesL/HelT, Dec1/BHLHB2, Dec2/BHLHB3) and survivin. Notch receptors are all composed of an extracellular domain (NECD) where ligands bind, a transmembrane domain (TM) and an intracellular domain (NICD). Notch is activated upon binding to a ligand that triggers a proteolytic cascade producing activated NICD, which is transported to the nucleus where it binds to a tumor repressor, DNA-binding protein, recombination signal binding protein for immunoglobulin kappa J (RBP-Jk) or CBF1/Su(H)/Lag-1 (CSL) family of transcription factors[64].

RBP-Jk is a DNA binding factor, which mediate either transcriptional repression or transcriptional activation. RBP-Jk binds to the ubiquitous corepressor proteins (Co-R: Silencing mediator of retinoid and thyroid hormone receptors, SMRT and Ski-interacting protein, SKIP)[65], histone deacetylases (HDACs), CBF1 interacting corepressors (CIR), and SAP30 (a linker between CBF1 and the HDAC complex)[66], which repress transcription of some genes. Thus, RBP-Jk is a transcription factor

that acts as a repressor in complex with SMRT and SKIP when it is not associated with Notch. In contrast, activated NICD-RBP-Jk complex displaces co-repressors and recruits coactivator (Co-A). When RBP-Jk is associated with NICD it acts as a transcriptional activator in complex with mastermind-like proteins, MAML[67]. This process is required for Notch-induced canonical signals that increase the transcription of target genes such as Hes, Hey, nuclear factor-kappa B (NF-κB), cyclin D, c-Myc and others[64]. Additionally, Notch signaling is linked to expansion of cancer stem cell populations (CSC), which show self-renewal capabilities and can recapitulate tumor heterogeneity and are believed to be responsible for recurrence and drug resistance[68,69].

Notch signaling is deregulated in many cancers. Indeed, deregulation of Notch signaling is a hallmark of breast cancer[64]. In breast and pancreatic cancer cells leptin upregulates Notch receptors, ligands and targets[16,60]. Moreover, latest reports show a positive correlation between leptin, Ob-R and Notch components in endometrial cancer tissues from obese patients[70]. Leptin induces RBP-Jk and Notch that impacts on CSC and self-renewal[16,60,71]. Moreover, a novel crosstalk between Notch, IL-1 and leptin (NILCO) was found in breast cancer[53,60,72]. NILCO induces proliferation/migration and upregulation of VEGF/VEGFR-2, and could represent the integration of developmental, pro-inflammatory and pro-angiogenic signals critical for leptin effects in breast cancer[60]. Paradoxically, low expression of RBP-Jk has been reported in several solid tumors that was associated with increase aggressiveness[73]. Our preliminary data indicate that knockdown of RBP-Jk in breast cancer cells induces a dramatic increase of Notch 3 and Notch 4 expression, CSC population ($CD24^-/CD44^+$) and N-cadherin (epithelial-mesenchymal-transformation marker)[74]. These data may suggest that tumor repressor activities of RBP-Jk could overcome the oncogenic actions of NICD-RBP-Jk complex upon activation of Notch, thus, cancer cells downregulate RBP-Jk expression in order to proliferate and develop tumors. However, this topic deserves follow up and more deep mechanistic investigation.

LEPTIN SIGNALING INDUCES BREAST CANCER PROGRESSION

Leptin and Ob-R are low expressed in human mammary glands, yet they play a role in the normal development[75]. In contrast, leptin and Ob-R expression is upregulated in breast cancer[76]. Obese patients with breast cancer show tumoral leptin overexpression that correlated to larger and more advanced tumors[77]. The molecular mechanisms involved in obesity-related breast carcinogenesis are not very clear. The binding of leptin to its receptor on breast cancer cells induces the activation of multiple oncogenic pathways, including Jak/STAT3, ERK1/2, and phosphoinositide 3-kinase (PI-3K) pathways, cyclin D1 expression and retinoblastoma protein hyperphosphorylation[78]. Triple negative breast cancer (TNBC) showed high level of molecules correlated with metastasis and lower survival of patients of leptin (*i.e.*, IL-1, Notch and VEGF/VEGFR2). Notch, IL-1 and leptin crosstalk outcome (NILCO) seems to play essential roles in the regulation of leptin-mediated induction of proliferation/migration and expression of pro-angiogenic molecules in breast cancer[64].

Breast adipose tissue is a source of estrogen, which is involved in tumorigenesis. Estrogens promote cell proliferation by inhibiting apoptosis and inducing angiogenesis[79]. Therefore, these molecules are breast cancer markers and therapeutic targets. A functional crosstalk between estrogen and leptin exists and may act to promote tumorigenesis[80]. The aromatization of androstenedione in adipose tissue is the main source of estrogen[81], a reaction catalysed by the enzyme aromatase, whose expression is increased by leptin in ER positive breast cancer cells[82]. Leptin has been shown to induce resistance in ER positive cancer cells to Faslodex[83] and Tamoxifen[84]. Leptin binding to ObR was also shown to transactivate HER2/neu[85], which is an important oncogenic protein involved in breast cancer growth. All these data indicate that leptin is involved in the development of breast cancer. Therefore, the use of leptin-signaling targeting drugs could be a novel strategy in breast cancer management.

LEPTIN SIGNALING PROMOTES THE EXPANSION OF CANCER STEM CELLS

Breast cancer stem cells

The cancer stem cell (CSC) theory postulates the existence of a sub-population of cancer cells with the ability to undergo self-renewal and also tumor differentiation[86]. The presence of these cells is a risk factor for carcinogenesis. CSC can recreate the bulk of the tumor, and are believed to be responsible for tumor initiation, cancer recurrence and metastatic progression[87]. CSC in breast cancer (BCSC) initiate and drive carcinogenesis and tumor differentiation[88]. BCSC can be identified by several molecular phenotypic markers. Networks of cytokines and growth factors, including leptin, have been implicated in BCSC interaction with the tumor micro-environment[89]. BCSC exhibit a high sensitized responses to leptin. It was reported that leptin mediates microenvironment effects on BCSC activity that establishes a self-reinforcing signaling circuit. Leptin upregulates several factors considered BCSC markers in several breast cancer cell lines like, including CD44, ALDH1[60], HER2[90], Oct-4 and Sox2[91]. Leptin is also involved in activation of transcriptional factors associated with BCSC, like STAT3[92] and NF-κB[93]. BCSC markers are shown in Table 1[60,90,91,94-105].

PANCREATIC CANCER STEM CELLS

Characterization of pancreatic cancer stem cells

Pancreatic cancer stem cells (PCSC) are characterized by the expression of cell markers, including $CD24^+CD44^+$, $CD133^+$, $CD24^+CD44^+$ and epithelial specific antigen (ESA^+ or $EpCAM^+$) and aldehyde dehydrogenase ($ALDH^+$)[106-108]. PCSC represent a rare cell population of 0.5%-1% of

Table 1 Breast cancer stem cells markers

Markers	Localization	Ref.	Markers	Localization	Ref.
CD44	Cell surface	Guo *et al*[60], 2011	MET	Cell surface	Baccelli *et al*[100], 2013
CD24	Cell surface	Kakarala *et al*[94], 2008	CD133	Cell surface	Tume *et al*[101], 2016
Epcam	Cell surface	Chiotaki *et al*[95], 2015	CD338	Cell surface	Leccia *et al*[102], 2014
CD49f	Cell surface	Chiotaki *et al*[95], 2015	ALDH1	Cytoplasm	Guo *et al*[60], 2011
MUC1	Cell surface	Nigam[96], 2013	Bmi I	Cytoplasm	Kim *et al*[103], 2015
CD29	Cell surface	Yeo *et al*[97], 2016	GLI I	Cytoplasm	Fernandez-Zapico[104], 2013
CD61	Cell surface	Yeo *et al*[97], 2016	Sox2	Cytoplasm	Feldman *et al*[91], 2012
CD47	Cell surface	Zhang *et al*[98], 2015	4-Oct	Cytoplasm	Feldman *et al*[91], 2012
HER2	Cell surface	Korkaya *et al*[90], 2008	NANOG	Cytoplasm	McClements *et al*[105], 2013
eHSP90	Cell surface	Stivarou *et al*[99], 2016			

total PC cells (Table 2). Remarkably, when isolated and inoculated into nude mice PCSC generate tumors, whereas implantation of PC cells negative for these markers could not. Rasheed *et al*[109] showed that a subpopulation of PCSC, $CD133^+CXCR4^+$ was found in patients with PC metastatic disease. Additionally, PC $ALDH^+$ cells showed enhanced clonogenic growth, migratory potential and affected negatively the overall survival of PC patients. In 2011, Li *et al*[106] described a new population of PCSC c-Met^+ involved in PC growth and metastasis. Recent preclinical data suggest PC c-Met^+ cells are involved in drug resistance. Indeed, the use of a c-Met inhibitor (Cabozantinib) in PC patient overcomes Gemcitabine resistance[110]. PCSC could also be identified by flow cytometry using Hoechst 33342 dye. PC side population that can exclude Hoechst 33342 dye correlated with chemoresistance and poor survival[111]. Wang *et al*[112] described a similar PC side population (Hoechst 33342 negative) showing high expression for $CD133^+$, $ABCG2^+$ and $Notch1^+$, which were more chemoresistant compared to non-side population cells. A PCSC population marked by the expression of Doublecortin and Ca/Calmodulin- Dependent Kinase-Like 1 (Dclk1) was described by Bailey *et al*[113] in 2014. PCSC $Dclk1^+$ were found in PanIN (pancreatic intraepithelial neoplasia) lesions, as well as in invasive stages of PC. These findings suggest that PCSC populations can be identified at the early stages of pancreatic tumorigenesis and may serve as a biomarker for early detection of this deadly disease.

PCSC show self-renewal and multipotency, and can initiate and propagate the parental tumor while serial passage into immunocompromised mice[114]. CSC including PCSC have retained the expression of at least three of the transcription factors that are characteristic to embryonic stem cells (ESC) (Oct-4, Sox-2 and Nanog). Increased levels of Oct-4 and Nanog are correlated with early stages of carcinogenesis and worse prognosis. Oct-4 and Nanog play important roles in embryonic development, and also in maintaining the stemness of PCSC. In contrast, PCSC double knockdown of Oct-4 and Nanog show reduced proliferation, migration, invasion and tumorigenesis[115]. Additionally, Oct-4 contributes to metastasis and cancer multidrug resistance[116]. *De novo* Sox2 expression alone in PC is sufficient to promote self-renewal, differentiation and stemness. Although ESC and PCSC share the property of self-renewal, ESC favors differentiation, while PCSC act more toward proliferation and inhibition of apoptosis. Targeting PCSC may be a viable therapeutic strategy against PC. A better understanding of Oct-4, Sox-2 and Nanog regulation could facilitate the design of individualized therapies for PC patients[117].

Current studies demonstrate that PCSC determine tumor relapse and metastasis following chemotherapy[118]. From a clinical perspective, targeting PCSC populations would ensure tumor eradication. However, PCSC possess escape mechanisms shared with normal stem cells, such as over-expression of multi-drug transporters. These transporters increase the efflux of anticancer drugs, thereby reducing their accumulation inside the cancer cells[118]. ABCB1 protein was significantly augmented in $CD44^+$ cells during acquisition of PC cells resistance to Gemcitabine. CD44 expression in PC was correlated with histologic grade and poor prognosis. These data indicate that cancer stem cells were expanded during the acquisition of Gemcitabine chemoresistance[119]. In line with these findings, the administration of anti-CD44 monoclonal antibody to a human PC xenograft mouse model increased Gemcitabine sensitivity[120]. Additionally, Metformin enhanced the capacity of Gemcitabine to inhibit the proliferation of PC cells by inhibiting the proliferation of $CD133^+$ cells[121]. Side population PCSC identified by Van der Broeck in 2012[111] are resistant to Gemcitabine. Side population PC cells isolated from Panc-1 cell line have been found to express both ABCB1 and ABCG2, which contribute to chemoresistance[122]. Identification of enhanced stem cell populations within PC tumors might be used as biomarkers for personalized therapy.

Pancreatic cancer stem cell regulators

Several factors could affect PCSC. Accumulated evidence suggested that microRNAs are involved in the regulation of PCSC. Specifically, miRNA34 affects the maintenance and survival of PCSC[123]. Obesity is associated with increased severity of acute pancreatitis[124] and decreased survival of PC patients. In obese mice, IL-6 contributes to prolonging inflammation and altering resolution from pancreatic damage, possibly contributing to a microenvironment favorable to tumorigenesis[125]. Cigarette smoking and nicotine, a major risk factor in PC, increase monocyte chemoattractant protein 1 (MCP-1)

Table 2 Pancreatic cancer stem cells markers

Stem cell population	Localization	Ref.
$CD24^+CD44^+$	Extracellular	Li *et al*[106], 2007
$CD24^+CD44^+ESA^+$	Extracellular	Li *et al*[106], 2007
$CD133^+CXCR4^+$	Extracellular	Hermann *et al*[107], 2007
$CD133^+CD44^+$	Extracellular	Ji *et al*[123], 2011
C-Met	Extracellular	Li *et al*[106], 2007
DCLK1	Intracellular	Bailey *et al*[113], 2014
ABCB1	Extracellular	Van den broeck *et al*[111], 2013
Sox2	Intracellular	Herreros-Villanueva *et al*[117], 2014

expression in PC cells. MCP-1 was found in 60% of invasive PC lesions, of whom 66% were smokers[126]. The concentration of six cytokines (IL-1β; IL-6, IL-8, VEGF, TGF, IL-10) were consistently reported to be increased in pancreatic ductal adenocarcinoma (PDAC) patients. These molecules were associated with the severity of PDAC (*i.e*., metastasis, tumor size, and advanced stage) that suggest these cytokines have prognostic biomarker for PC[127]. Additionally, IL-8/CXCR1 axis was associated with cancer stem cell properties in PC[128]. CXCR1 expression in PC patients correlates with lymph node metastasis and poor survival. MMP-13 has been shown to help mediate the effect of leptin on invasiveness and metastasis of PC. In addition, there was a positive correlation between the expression of PCSC markers CD133 and CD44, and CXCR1[129].

P300 is a tumor suppressor gene. However, this factor is also upregulated in various cancer types and associated with worse prognosis. In PC, P300 is associated with chemoresistance from apoptosis upon Gemcitabine-induced DNA damage[130]. TGF-β negatively regulates ALDH1 expression (a PCSC marker) in a SMAD dependent manner in PC cells. This regulatory mechanism might be disrupted by SMAD4 mutations and deletions in PC cells[131]. The binding of stem cell factor (SCF, a protein involved in PC progression) to its receptor, c-kit, determines an increase in HIF-1α synthesis that affects cancerous transformation, chemoradiotherapy resistance, and tumor progression[132].

Additionally, high levels of leptin receptor, Ob-R, are associated with PC stage and lymph node metastasis and overall shorter survival. Ob-R and HIF-1α expression was highly associated in PC tissues. HIF-1α regulated the expression of Ob-R in PC[133]. Leptin binding to Ob-R was earlier found to induce HIF-1α in breast cancer cells. Leptin-induced HIF-1α was involved in the upregulation of VEGFR2 in these cells[55]. Therefore, it is possible that a leptin-induced HIF-1α feedback regulating Ob-R is present in PC. Moreover, robust expression of Ob-R is a characteristic of tumor initiating stem cells and pluripotent stem cells that was mediated directly by Oct-4 and Sox2[91]. Furthermore, the expression of leptin in gastro-esophageal adenocarcinomas was associated with chemoresistance. The use of leptin receptor antagonist SHLA increased the sensitivity to Cisplatin in the resistant gastric cancer cell line, AGS Cis5, and the oesophageal adenocarcinoma cell line, OE33[134].

Chemoresistance and cancer stem cells

In the absence of targeted therapeutic options, chemotherapy, along with surgery and radiotherapy are usually the last and only options for cancer treatment. Thus, resistance to chemotherapy is a vital area of research. Investigations on the mechanisms involved in chemoresistance are essential to overcome this issue. There are several mechanisms related to chemoresistance that have been identified in cancer cells, which include reduction or inhibition of drug-induced apoptosis, overexpression of detoxification and efflux proteins, increased expression of survival factor and pathways as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and PI-3K/Akt, hypoxia and hypoxia inducible factor HIF, and expansion of chemoresistant CSC among others[135-138].

Inhibition of apoptosis

Numerous chemotherapies target the increased DNA synthesis that cancer cells undergo. Classes of chemotherapeutics such as platinum agents (Cisplatin), alkylating agents (Cytoxane) and anthracyclines (Adriamycin or Doxorubicin) inhibit DNA synthesis. A consequence of the action of these agents is increased apoptosis due to DNA damage. The p53 pathway plays an important role in cancer cell avoidance of apoptosis, with mutations in the p53 gene associated with increased drug resistance in cancer cell lines and poor survival in cancer patients[135,139]. In addition, cancer cells have been known to competitively inhibit Caspase 3, a central molecule in the apoptosis pathway. These cells show increased expression of B cell lymphoma 2 (BCL-2) and B cell lymphoma extra-large (BCL-xL) anti apoptotic proteins[140-143].

Detoxification and efflux proteins

Aldehyde dehydrogenases (ALDH) are a class of enzymes that oxidise aldehydes. ALDH isoforms have been implicated in CSC and resistance to chemotherapeutics. ALDH1 is a marker of CSC and progenitor cells[144], whose expression correlated with poor response to Docetaxel therapy in advanced breast cancer[145]. Increased expression of ALDH1A1 and ALDH3A1 lead to greater inactivation of Cyclophosphamide in breast cancer[136].

ATP binding cassette (ABC) transporters are a family of transmembrane proteins involved in the efflux of drugs from cancer cells. ABC (ABCB1, ABCC1 and ABCG2) family of proteins are mainly found on CSC side-population (SP, Hoechst negative). ABCB1, also known as p-glycoprotein, CD243 or MDR1, is an efflux pump protein with broad substrate specificity. It is known to pump out chemotherapeutics such as Doxorubicin and Paclitaxel. ABCC1 is known to give cancer cell resistance to anthracyclines such as Doxorubicin. ABCG2 also called the breast cancer resistance protein or CDw338, allows cancer cell resistance to Mitoxantrone and Doxorubicin[146].

NFκB pathway

NFκB signaling pathway is a survival mechanism that

controls DNA transcription of several genes. In non-malignant cells NFκB signaling plays a central role in immune response to infection. It is responsible for cellular responses to a wide range of stimuli which include reactive oxygen species, ionising radiation, bacterial lipopolysaccharide, IL-β and TNF-α. To drive oncogenesis, NFκB signaling cooperates or crosstalks with signaling pathways, oncogenic or cancer-related proteins such as STAT3, p53, ALDH1, glycogen synthase kinase (GSK-3β), PI-3K, MAPK, PKC, and others[147].

NFκB signaling is a critical mediator of chemoresistance in cancer. Glioblastoma multiforme's resistance to Gemcitabine involves NFκB, ALDH and ROS actions[148]. Anti-ovarian cancer effects of MK5108 compound relied on the inhibition of the Aurora-A kinase and NFkB signaling, which induced polyploidy and cell cycle arrest[149]. In breast cancer, targeting NFκB signaling increased apoptosis and reduced proliferation in drug resistant breast cancer cell lines[150]. In mesothelioma, the STAT3-NFκB signaling crosstalk is essential in ALDH-mediated chemoresistance[151]. Abnormal activation of NFκB signaling is also implicated in cancer resistance to Paclitaxel therapy[152].

HIF and tumor hypoxia

Hypoxia is a term which describes deficient oxygen supply to tissues due to poor vasculature, as it is in the case of obesity and cancer. Proliferation and expansion of adipose tissue induce tissue hypoxia and the expression of HIF. Hypoxia in cancer is associated with poor outcomes and chemoresistance[137,153]. In TNBC, chemotherapeutic treatment with Paclitaxel and Gemcitabine results in expression of HIF, and enrichment of CSC through IL-6 and IL-8 actions. Chemical inhibition of HIF results in the depletion of CSC and tumour abrogation *in vitro* and *in vivo*[154].

In addition, hypoxia promotes survival of TNBC MDA-MB231 from Paclitaxel-induced apoptosis *via* mTOR/JNK pathway[155].

CSC resistance to chemotherapy

The presence of CSC within tumors make them resistant to chemotherapy. CSC are commonly more resistant to chemotherapeutics which target the bulk of the tumour that allow the proliferation of CSC[156]. The CSC stemness phenotype and chemoresistance involve TGF-β signaling, which plays a prominent role in stem biology, facilitating epithelial to mesenchymal transition in mammary cancer cells, which is a property of CSC[138]. TNBC cell lines treated with Paclitaxel showed an enrichment of cancer cells with stem like properties and increased TGF-β signaling both *in vitro* and *in vivo*. Chemical inhibition of TGF-β signaling abrogates tumor formation[157]. CSC show higher expression of ABC family of proteins that increase their capability to efflux chemotherapeutics from cells. CSC also show diminished apoptosis rate, and over activation of detoxification proteins and survival pathways as NFκB and PI-3K[158].

OBESITY, LEPTIN AND DRUG RESISTANCE

Obesity and leptin signaling have been implicated in enhance capabilities of cancer cells to avoid apoptosis. Leptin expression was associated with higher expression of BCL-2 and BCL-xL expression in breast cancer cells[159]. Furthermore, leptin signaling has been reported to activate the PI-3K/Akt pathway that antagonizes apoptosis in various cancers such as colon cancer, liver cancers, endometrial cancers and lymphomas[44,160-163]. Additionally, obesity has been shown to influence breast cancer response to Doxorubicin therapy. Indeed, obese mice treated with Doxorubicin showed more proliferative tumors that also had more CSC as compared with non-obese mice[164]. Leptin increases the expression of ABC protein transporters in glioblastoma[165]. Our preliminary data further show that leptin increases the expression of ABCB1 in breast and pancreatic cancer cells.

Another mechanism involved in obesity-induced chemoresistant is NFκB signaling. It is known that NFκB is activated by leptin signaling and that can increase survival of cancer cells under chemotherapeutic treatment[55]. An additional link between obesity (*via* leptin signaling) and cancer chemoresistance is HIF, which correlates with activation of leptin signaling in several cancers including endometrial, pancreatic, breast and colon cancers[133,166-168]. A potential mechanism involved in obesity-mediated drug resistant is TGF-β signaling. Leptin and TGF-β are commonly co-expressed in breast cancer[34]. It is known that TGF-β signaling induces leptin expression. However, the connection between leptin and TGF-β signaling in breast cancer is still unclear[169].

Leptin increased proliferation and survival of breast cancer estrogen receptor positive cells, MCF-7 cells treated with Cisplatin. These data further assessed that leptin is a survival factor that induces drug resistant in breast cancer[170]. Moreover, leptin was found able to induce CSC expansion in breast[60] and pancreatic cancer[16]. Furthermore, our preliminary data suggest that leptin induces the expression of Oct-4 and Nanog in breast cancer cells. These factors are essential for the upregulation of Ob-R in cancer cells[91]. Thus, leptin can induce a feedback mechanism through Oct-4/Nanog to sustain Ob-R expression and its pro-oncogenic signals in breast cancer.

LEPTIN ANTAGONISTS AND CANCER PROGRESSION

Leptin signaling has numerous protumorigenic effects, including the increase chemoresistance found in several tumors. Therefore, leptin antagonism could be a new strategy to overcome drug resistance in cancer. Several molecules have been described as potential new agents to target leptin-induced cancer growth and drug

resistance. Majority of the leptin antagonists reported are mutated or truncated versions of leptin molecule: Leptin muteins, Allo-aca and D-ser, LDFI, and leptin peptide receptor antagonists (LPrA).

SMLA and SHLA

Leptin muteins or mutant proteins, were generated using random mutagenesis of the leptin sequence and screened for high affinity variants using a yeast surface display. This resulted in the creation and identification of high affinity muteins. Two mutein antagonists named superactive mouse leptin antagonist (SMLA) and superactive human leptin antagonist (SHLA) were made by the introduction of an *Asp23* mutation. These antagonists showing 4 aminoacid residue mutations (D23L/L39A/D40A/F41A) were reported to have 60-fold increased affinity for Ob-R and 14 fold greater antagonistic activity as compared with the original leptin antagonist showing 3 mutations (L39A/D40A/F41A)[171]. These muteins were pegylated at the N terminus to increase bioavailability and stability. However, the pegylated muteins increased BW in mice. Pegylated SMLA induced higher BW gain as compared with the pegylated SHLA[171]. No effects of muteins on leptin-induced chemoresistance in cancer have been reported to date.

Allo-aca and D-ser

Allo-aca is a non-toxic, 9-residue peptide leptin antagonist based on the C terminal Ob-R binding leptin site Ⅲ. Allo-aca was reported to increase survival of CD1 nude mouse hosting TNBC. The effective dose of the peptide was found after 9 to 13 d of treatment by injecting intraperiotoneally between 0.1 and 1 mg Allo-Aca/kg body weight (BW)/day. Allo-aca was nontoxic in C57Bl/6 and CD1 nude mice, but showed hepatotoxicity at 0.2 mg/kg BW/day in SCID mice[172]. Additionally, it induced weight gain of 6% to 10% of BW[172]. Treatment of TNBC MDAMB231 cell line with Allo-aca 50 pM inhibited leptin-induced proliferation *in vitro*[172]. D-ser, peptide inhibitor is an analogue of Allo-aca that at 1 nM concentration inhibited leptin-induced proliferation in Ob-R positive breast and colon cancer cells *in vitro* without exhibiting agonist activity[173]. However, no data on the effects of these antagonists on leptin-induced drug resistance and CSC are available.

LDFI

LDFI is a leptin peptide antagonist composed by amino acid 39 to 42 on the leptin binding site I (Leu-Asp-Phe-Ile). LDFI was reported to inhibit leptin-induced growth of breast cancer cells *in vitro* and *in vivo*[174]. This peptide antagonist inhibited proliferation, colony formation on soft agar and Boyden chamber transmigration of estrogen receptor positive as well as estrogen receptor negative breast cancer cells. LDFI effects correlated with reduced expression of key downstream leptin effectors such as JAK2, STAT3, AKT and MAPK. *In vivo*, the pegylated peptide (LDFI-PEG) was shown to inhibit tumour growth in a murine mammary xenograft model. LDFI-PEG showed no toxicity or effects on BW of mice[174]. No reports on potential effects of LDFI on drug resistance in breast or other cancer types are available.

LPrAs

LPrA1 and LPrA2 were earlier designed and tested *in vitro* and *in vivo* in mouse models[52,53,56,72,175,176]. LPrAs are composed by aminoacid sections of the binding site Ⅰ (LPrA1) and Ⅲ (LPrA2) of the leptin molecule[63]. LPrA2 was conjugate to polyethylene glycol 20 kDa (PEG-LPrA2) or to iron-oxide nanoparticles (IONP-LPrA2) to increase its bioavaibility and effectiveness to block leptin signaling in cancer cells. Unconjugated and conjugated LPrA2 effectively inhibited leptin-induced protumorigenic actions in breast and pancreatic cancer cells[52,53,56,72,175,176]. LPrA2 showed potent effects for the reduction of leptin-induced growth of tumors and expression of inflammatory (IL-1/IL-1R tI), proliferation (Ki67, PCNA), angiogenic factors (VEGF/VEGFR2) and Notch in tumors and endothelial cells[53,56,58,72]. The antagonist effects of LPrA2 on tumor growth and angiogenesis were more evident in obese than in lean mice[53,72]. However, unconjugated or conjugated LPrA2 showed no toxicity and did not affect energy balance (BW or food intake) or general health when it was applied (0.1 mM/i.v. per twice weekly) to many lean and obese mice for two months. Remarkably, LPrA2 negatively impact on leptin-induced expansion of CSC and Notch expression in breast and pancreatic cancer cells, derived tumorspheres and xenografts[16,74]. Moreover, LPrA2 significantly reduced the leptin-induced effects on drug resistance (Cisplatin, Sunitinib, Paclitaxel, Doxorubicin) in breast cancer cells[16,176].

CONCLUSION

Combination of poor dietary habits and low physical activity, which are reinforced by accessibility of low-cost high caloric and fat foods have led to the obesity pandemic. Accumulated evidence supports a negative role of obesity on cancer risk, progression and management. Despite many efforts and social programs to tackle obesity, its effects on morbidity and mortality and its influences on cancer incidence and treatment are in crescendo[1-5]. It is known that obesity and leptin signaling not only affect cancer cells, but also tumor stroma. Moreover, leptin and paracrine factors secreted from cancer and stroma cells (adipocytes, fibroblasts, endothelial cells and inflammatory cells) could affect tumor progression, CSC and chemoresistance[16,176]. In this regards, the use of nontoxic leptin antagonists that do not affect energy balance could be a novel adjuvant therapy for cancer drugs. These compounds can increase chemotherapeutic effectiveness and allow reducing their dosage and undesired side effects in cancer patients.

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REVIEW

Targeted therapies in breast cancer: New challenges to fight against resistance

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Abstract

Breast cancer is the most common type of cancer found in women and today represents a significant challenge to public health. With the latest breakthroughs in molecular biology and immunotherapy, very specific targeted therapies have been tailored to the specific pathophysiology of different types of breast cancers. These recent developments have contributed to a more efficient and specific treatment protocol in breast cancer patients. However, the main challenge to be further investigated still remains the emergence of therapeutic resistance mechanisms, which develop soon after the onset of therapy and need urgent attention and further elucidation. What are the recent emerging molecular resistance mechanisms in breast cancer targeted therapy and what are the best strategies to apply in order to circumvent this important obstacle? The main scope of this review is to provide a thorough update of recent developments in the field and discuss future prospects for preventing resistance mechanisms in the quest to increase overall survival of patients suffering from the disease.

Key words: Breast cancers; Resistance; Human epidermal growth factor receptor 2; Angiogenesis; Triple negative; Immune tolerance

Core tip: There are several reviews in the literature dedicated to breasts cancers. However, our manuscript is an updated review on the current knowledge and particularly on the molecular mechanisms involved in the relapse of patients on the current treatments. A summary of ongoing clinical trials gives a perspective for future therapeutic strategies. Our manuscript represents a working document for researchers/oncologists in the field of breast cancers.

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INTRODUCTION

Breast cancer targeted therapies involve substances or drugs which block the growth of cancer by interfering with the function of specific molecules responsible for tumor cell proliferation and survival[1-21]. Breast cancer cells may overexpress specific receptors which, when activated can initiate downstream signaling resulting in the expression of genes for cancer cell proliferation, growth, survival, migration, angiogenesis and other vital cell cycle pathways[22,23].

There are various types of breast cancer, some have hormone receptors like estrogen or progesterone (some have both) and are called ER+ or PR+ breast cancer respectively.

The estrogen receptor ER is a major driver of the majority of breast cancers as it is expressed in 75% of breast cancers overall. It is more frequently related with postmenopausal women and there is a 99% survival rate at ten years. Hormone sensitive breast cancer has a strong correlation with lower tumor grade; lower levels of amplification of the human epidermal growth factor receptor 2 (*HER2*) oncogene and concomitant loss of *p53* tumor suppressor gene; expression of progesterone receptor (PR), soft tissue and bone metastases and slower rates of disease recurrence. In cases of hormone positive breast cancer along with the expression of ER, multigene tests may be carried out to make treatment decisions particularly for adjuvant therapy and screen those patients who would benefit more from combination of endocrine plus chemotherapy[24-26].

The most common receptors that are overexpressed in breast cancer cells are part of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases: EGFR and HER2 are overexpressed in approximately 40% and 25% of breast cancers respectively and are believed to be responsible for more aggressive tumor behavior and poor prognosis[27].

Triple negative breast cancer (TNBC) is defined by the lack of expression of both estrogen and progesterone as well as the HER2 protein and is often associated with an unfavorable prognosis as no treatment is yet available for this particular breast cancer subtype[28].

The rapid acquisition of resistance in breast cancer targeted therapies seems to limit the effectiveness of treatment and even though some of the genetic mutations and epigenetic changes in molecular pathways have been understood, it is sometimes necessary to combine several pathway blockades in order to achieve successful treatment results[29-35].

The identification of new target molecules in breast cancer and the use of combination therapies may have improved the understanding of compensatory pathways which lead to the emergence of resistance mechanisms, nevertheless, breast cancer subtypes like TNBCs seem to exploit alternative proliferative pathways which are not yet fully understood and need urgent attention and elucidation[11] (Figure 1).

TARGETED THERAPIES IN BREAST CANCER

Estrogen and estrogen receptors are key drivers in breast cancer progression. This is the reason why targeting estrogen has been used for many years to inhibit the estrogen signaling pathway in women with estrogen positive breast cancer. Selective estrogen receptor modulators or SERM have been used to suppress tumor growth in estrogen dependent breast cancers and tamoxifen was the first drug to be approved for estrogen positive metastatic breast cancer reducing recurrences by approximately 40%-50%[36].

Aromatase inhibitors (anastrozole, letrozole, exemestane) are also used as an alternative therapy to treat estrogen dependent breast cancers as they block the biosynthesis of androgens through inhibition of the aromatase enzyme resulting in reduction of estrogen levels in tumor cells[36].

Other therapies are available for other forms of breast cancer that are not hormone dependent. The HER2 protein represents the most common overexpressed receptor signature in breast cancer and is considered a relevant biomarker for treatment.

The recombinant antibody trastuzumab (Herceptin) targets HER2 and is the first drug that was approved by the FDA in 1998 for the treatment of HER2 positive breast cancers[37,38].

Other agents that followed such as pertuzumab and lapatinib have not shown immunity to the development of resistance mechanisms with significant side effects for the patients[7,39,40].

The conjugated monoclonal antibody TDM1 (trastuzumab emtansine) may be used in HER2 positive breast cancers as trastuzumab efficiently transports the DM1 drug, a microtubule inhibitor, directly into the breast cancer cells to inhibit growth.

Triple negative cancers lacking hormone receptors and HER2 may respond to agents like PARP1 inhibitors and may have HER1 as a potential target. The monoclonal antibody cetuximab combined with cisplatin chemotherapy has shown promising results in a Phase Ⅱ study, suggesting some subtypes of TNBC may be EGFR inhibition sensitive[41].

The conventional route to treat TNBC patients by taxol derivatives and anthracycline chemotherapy is still widely used until more "druggable" targets are identified[41]. Recent studies suggest that the microtubule-stabilizing agent ixabepilone in combination with capecitabine may

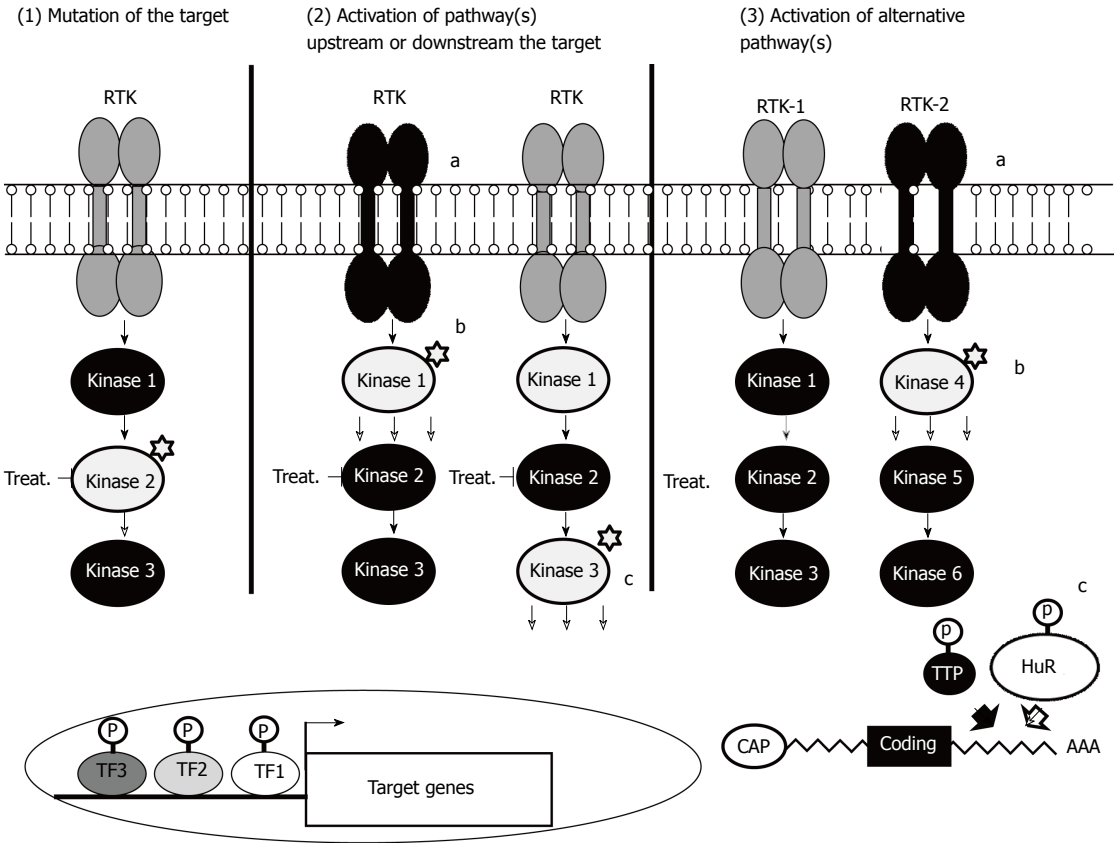

Figure 1 A schematic diagram of the most common resistance mechanisms to targeted therapies. (1) Alteration of the drug target (Treat.): This type of resistance involves mutations as well as amplifications of drug targets such as kinases; (2) Upstream and downstream pathway effect through the activation of receptor tyrosine kinase (RTK) (a) and/or the mutation/amplification of upstream (b) or downstream (c) components; (3) Bypass mechanisms occur as a result of a second receptor tyrosine kinase activation (a), through a mutation of a parallel kinase (b) or modulation of mRNA binding proteins (c). These alternative mechanisms of resistance especially through kinases activation result in the modification of gene expression *via* the phosphorylation or transcription factors (TF).

be effective in TNBC that are resistant to anthracycline and taxane drugs and the PACS08 Phase Ⅲ trial is evaluating this possible treatment strategy[28].

Targeted therapies have also been approved against the vascular endothelial growth factor (VEGF) and the drug bevacizumab has proven effective in the treatment of advanced metastatic breast cancer when used in association with paclitaxel or docetaxel[42,43].

Inhibitors of downstream pathways like PI3K/AKT/mTOR and RAS/MEK/ERK are also available for therapeutic purpose as well as agents directed against other tyrosine kinases like SRC, insulin-like-growth-factor [IGF/IGF-receptor (IGFR)], poly-ADP ribose polymerase (PARP) Inhibitors and also matrix metalloproteases (MMPs) which are involved in cancer cell invasion and metastasis[8,29,31,44-48].

Compensatory survival pathways, increased phosphatydil-inositol-3-kinase (PI3K)[49-52] signaling, receptor tyrosine kinase signaling outside the ErbB/HER family and involvement of other HER receptors[53], may all play a key role in the development of alternative molecular pathways responsible for the development of therapeutic resistance in breast cancer cells.

Indications of breast cancer targeted therapies

Breast cancer targeted therapies are used to treat patients whose breast cancer cells overexpress certain characteristic proteins on their surface allowing an abnormal growth pattern. Antibodies are sometimes used as they work in a similar way as the human immune system.

The most efficient breast cancer targeted therapy today is the one targeting the HER2 protein overexpression on the surface of breast cancer cells. At present, there are seven widely used breast cancer targeted therapies which are effective in blocking several molecular pathways: Afinitor or everolimus, an m-TOR inhibitor, stops cancer cells from getting energy supplies[54-57]; Avastin or bevacizumab inhibits the growth of new blood vessels which supply oxygen and nutrients to cancer cells for growth and function[14,58]; Herceptin or trastuzumab blocks the ability of cancer cells to receive signals which tell them to grow[12,59]; Kadcyla or T-DM1 is a combination of Herceptin and emtansine[7,60]. In this case Herceptin is used as a transport method to deliver the emtansine chemotherapy to cancer cells; Perjita or pertuzumab works by stopping cancer cells from receiving growth signals[12,61]; Tykerb or lapatinib is a HER2 inhibitor that blocks signals of cell growth[4,42].

The HER2 protein

The HER2 proto-oncogene is overexpressed in 10%-12% of over 2500 cases of human breast cancers and this

is associated with malignant transformation and poorer overall survival rates particularly in breast tumors with lymph node metastasis[62].

The HER2 or neu oncogene (erbB2) is part of the EGFR family of tyrosine kinases and is located on chromosome 17 (17q12). It represents the most common overexpressed receptor in breast cancers and is considered a relevant therapeutic target[59,63-69].

The EGFR family is composed of four receptors: EGFR/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. These receptors share common domains: an extracellular region characterized by leucine-rich repeats; cysteine rich repeats in the intracellular domain; a single transmembrane spanning region; a short juxtamembrane region; a kinase region and a cytoplasmic tail with various tyrosine phosphorylation sites[5,10]. Binding of ligands to the extracellular domain of EGFR, HER3 and HER4 allows for the formation of kinase active homo- and heterodimers to which HER2 is recruited as a preferential partner. Heterodimer formation between HER2/HER3 is the most common occurrence in these receptors' preferences. HER3 is often responsible for the activation of the PI3K/AKT signaling pathway *via* six docking sites for the p85 adaptor subunit of PI3K. The HER3/PI3K axis plays a key role in the survival of HER2-dependent cells as the loss of HER3 inhibits the survival of HER2-overexpressing breast cancer cells[70,71].

Trastuzumab resistance mechanisms

The first recombinant antibody approved by the FDA to target HER2-positive breast cancers was trastuzumab or Herceptin followed by other agents like pertuzumab and lapatinib.

Trastuzumab binds to the juxtamembrane region of the HER2 receptor tyrosine kinase resulting in the uncoupling of the HER2/HER3 heterodimers and consequent inhibition of downstream signaling and cytotoxicity.

Resistance mechanisms to trastuzumab develop often as a result of HER2 gene amplification and RNA/protein overexpression. HER2 overexpressing tumor cells continue to depend on the HER2 oncogene even after bypassing trastuzumab action possibly due to signaling from receptor tyrosine kinases (RTK) outside the ErbB family, increased PI3K signaling and the presence of alternative forms of HER2 which are not detected by trastuzumab. Also, the modulation of Cdk inhibitor p27 by IGF-1 may be a key player in resistance to trastuzumab as overexpressed IGF-1 is responsible for the activation of the PI3K downstream signaling pathway and further effects on Akt[72,73]. One of the key players in trastuzumab-resistance in HER2 positive breast cancer was the inhibition of expression of miR-375, a tumor suppressor gene which targets IGF1R[74]. Also, molecular pathway crosstalk may have resulted in increased cell survival and division by interference with HER2 accessibility, independent downstream signaling activation as well as HER2 mutations, particularly the expression of p95HER2, an active truncated form of HER2. Blocking IGFR1 completely resulted in restored sensitivity of HER2 positive cancer cells to trastuzumab *in vitro*. The loss of miR-375 with consequent epigenetic changes such as DNA methylation and histone deacetylation may drive the upregulation of IGFR1 and hence the development of trastuzumab-resistant cancer cells; in this case, targeting miR-375 may prove to be worthy of further investigation as a potential therapeutic target to restore trastuzumab sensitivity in HER2 positive breast cancer cells[74].

The new antibody-drug conjugate trastuzumab-DM1 (TDM1) which has been recently developed for the treatment of HER2 positive cancer has proved to be effective in inhibiting trastuzumab sensitive and resistant HER2 positive breast cancer cell lines *in vitro.* TDM1 drives both apoptosis and mitotic catastrophe in the trastuzumab resistant breast cancer cell line Jimt-1, acting as a potent inhibitor of microtubule assembly. These cells are characterized for having several co-existing trastuzumab resistance mechanisms like a mutation in the PIK3CA gene, low PTEN expression, overexpression of NRG1 and a moderate expression of the HER2 receptor. Interestingly, in the T-DM1 treated Jimt-1 cell line model, an accumulation of HER2 was observed in organelles which resembled enlarged lysosomes, suggesting sequestration of the protein in these intracellular granules[75].

The integrin αvβ6, involved in promoting migration, invasion and cancer cell survival, seems to play a significant role in the development of trastuzumab resistance mechanisms. Targeting αvβ6 with the 264RAD antibody in HER2 positive breast cancer cell lines expressing both HER2 and the integrin seems to slow down the growth of trastuzumab resistant tumors[62].

Resistance of breast cancer cells to trastuzumab mediated cytotoxicity has been implicated in the secretion of soluble factors by adipocytes and preadipocytes in adipose tissue proximal to breast cancer cells. The development of resistance mechanisms in this case occurs by inhibition of trastuzumab-mediated tumor lysis by natural killer cells *in vitro* and by adipose tissue *in vivo*. A reduced antitumor effect was observed in mice which had tumors in close proximity to a lipoma, while in another group of mice which had tumors located distant to the lipoma, the trastuzumab anti-tumor effects were enhanced. The inhibition of antitumor activity was enhanced when the adipocytes were in hypoxic conditions, these factors might suggest a link between patient obesity and development of trastuzumab resistance mechanisms[76].

The dual targeting of HER family receptors with antibody therapy may prove to be a strategy to overcome acquired resistance mechanisms by cancer cells to cetuximab. When both HER3 and EGFR were neutralized by cetuximab and the anti HER3 monoclonal antibody U3-1287, cetuximab sensitive tumor cells showed a significant decrease in proliferation possibly due to inhibition of both MAPK and AKT pathways

and a diminished signaling from all three HER family receptors[77].

The efficacy of trastuzumab in inhibiting proliferation of breast cancer cells might be dependent on the presence of endogenous HER-receptor activating ligands EGF and heregulin-β1; the receptor density of HER-family members and growth ligands are key players in the development of resistance mechanisms to trastuzumab therapy, which interfers with cell cycle kinetics by inducing a G1 accumulation in HER-2 positive breast adenocarcinomas[78].

An unexpected mechanism of resistance is associated with down-stream mutations especially those targeting the mRNA binding protein tristetraprolin (TTP). *ttp* gene germinal mutation generates a form of TTP mRNA which is inefficiently translated in protein. The lack of TTP and the general increase of the TTP competitor the ELAV-like protein 1 (HuR) results in the increase of the half-life of mRNAs encoding oncogenes, inflammatory and angiogenic factors. The mutation of TTP is predictive of trastuzumab resistance[79]. Hence TTP is considered as a tumor suppressor for breast cancers[80-82]. TTP and HuR are phosphorylated by the same kinases and phosphorylation has antagonistic effects on both proteins (inactivation/degradation for TTP and activation/stabilization for HuR). Hence, activation of intracellular signaling pathways results in a general increase of proteins associated with oncogenic properties[83] (Figure 1).

The main drawback in trastuzumab therapy is represented by the emergence of serious cardiac side effects resulting from administration of this monoclonal antibody. Analysis of HER2 specific mutation may predict cardiac toxic effect[84].

HER-2 is expressed in the adult human myocardium and trastuzumab therapy unfortunately carries the risk of inducing cardiac dysfunction and congestive heart failure. When adjacent chemotherapy is applied in addition to trastuzumab, one has to take into consideration anthracyclin-associated cardiotoxicity following the inhibition of the HER-2/erbB2 receptor to ensure safety for patients. Some of the cardiotoxicity side effects of trastuzumab may be reversible over time and in some cases, administering the monoclonal antibody after chemotherapy or radiotherapy may decrease the risk of potential cardiac side effects. Trastuzumab therapy seems to represent clear overall benefits for patients in the long run, therefore, should be still considered as an appropriate standard choice of treatment as a HER-2/erbB2 inhibitor as long as care is taken to minimize its side effects[85].

Endocrine therapy resistance mechanisms

Resistance to hormone therapy is a major challenge within hormone sensitive breast cancers even though ER and PR targeted therapy has proven to be very effective, improving the quality of life of hormone sensitive breast cancer patients. The major pathways responsible for endocrine resistance mechanisms might be several: The HER tyrosine kinase receptor family; receptors for insulin/IGF1, FGF and VEGF, Src, AKT, stress related kinases, might each play a pivotal role in contributing to endocrine therapy resistance when their cognate ligands are amplified or overexpressed.

Cross-talk between the estrogen receptor (ER) and growth factor receptor signaling with hyperactivation of the PI3K pathway have also been associated the development of endocrine resistance[86].

Nuclear receptors and the androgen receptors may also act as alternative growth stimulators by post translational modification, enabling the bypass of ER inhibition. Co-targeting the EGFR and HER2 pathway simultaneously seems to be the most promising way forward in circumventing endocrine resistance as these two seem to be the most important factors responsible in this particular resistance scenario[26].

The mTOR pathway

The mTOR pathway seems to be a master regulator of cell physiology and may be a key player in the targeted therapy of cancer[87]. When the natural product rapamycin was discovered in the early 1970's as an antifungal agent, it emerged in later studies that the molecule could halt growth in many types of eukaryotic cells and have a powerful immunosuppressive function. In 1999 the FDA approved sirolimus as a drug used against rejection of transplanted organs particularly the kidneys. Rapamycin binds to another molecule, FKBP12 and once this complex is formed it associates with a protein called mTOR[88]; a serine/threonine kinase, resembling the kinase domain of PI3 kinase and its related enzymes. The circuitry of the mTOR pathway is of interest as it represents a key element of the mammalian cell cycle integrating incoming signals and vital mechanisms such as glucose import and protein synthesis, as well as phosphorylating two kinases involved in translation: S6 kinase (S6KI) and 4E-BP1[89,90]. The activation of S6KI is followed by the activation of the small 40-S ribosomal subunit which can initiate protein synthesis after associating to the large ribosomal subunit. mTOR is also a key upstream regulator which controls the AKT signaling pathway for the regulation of apoptosis and proliferation; inhibiting the mTOR complex results in a shutdown of the AKT signaling stream resulting in an hyperactivated PI3K/loss of PTEN expression[91,92].

The PI3K/AKT/mTOR pathway is overactivated in 70% of breast cancers and the protein kinases found along these pathways may be potential drug targets for breast cancer therapy. Due to the large scale involvement of this pathway the cell cycle regulation, selectively silencing of the PI3K/AKT/mTOR pathway represents an attractive approach for patients who might have shown resistance mechanisms to previous types of therapy. The combination of mTOR inhibitors with other targeted therapies might be a winning formula to circumvent resistance mechanisms of breast cancer patients.

Inhibition of the mTOR pathway by the drug everolimus in combination with HER-2 or estrogen receptor

inhibitors may be a promising future strategy to apply, in order to reinstate sensitivity of breast cancer cells to traditional therapies and overcome resistance mechanisms which seem to emerge when the mTOR pathway is functioning in hyperactive mode[93]. Molecular alterations like mutations in EGFR, BRAF, AKT, or PI3K are associated with activation of downstream signaling pathways resulting in unrestricted proliferation in cancer cells.

Glaysher *et al*[94] have shown that targeting a breast epithelial cell line after having knocked-in mutations and using EGFR and mTOR inhibitors, there was an increased sensitivity to therapeutic drugs. As development of resistance in breast cancer cells may be a result of the activation of the PI3K/AKT/mTOR pathway, Glaysher *et al*[94] studied the effects of inhibiting both mTOR and EGFR by combined drug action of ZSTK474/sirolimus and erlotinib/gefitinib, observing a more effective signaling blockade, as opposed to use of single agents on the parental cell line and irrespective of the knocked-in mutations in EGFR,KRAS,PI3K,BRAF or AKT[94].

Receptor tyrosine kinase inhibitors resistance mechanisms

Lapatinib is a dual EGFR/HER2 tyrosine kinase inhibitor which acts as an ATP competitor. It is used as a first line monotherapy in patients with HER2 positive metastatic breast cancer in addition to conventional chemotherapy like paclitaxel.

Unfortunately, the activation of compensatory pathways after onset of therapy with lapatinib seems to be responsible for the emergence of resistance mechanisms, particularly when inhibition of AKT phosphorylation leads to increased estrogen receptor-α transcription and estrogen receptor signaling. This mechanism of resistance can be circumvented by administering an ER-down-regulator fulvestrant, which can prevent the proliferation of lapatinib resistant cells. In addition, mutations in the HER2 protein, particularly a YVMA insertion at G776 in exon 20, seems to be responsible for mechanisms of *de novo* resistance to lapatinib as well as trastuzumab[73].

The inhibitory effects of lapatinib may be bypassed as downstream signaling is amplified and upregulation of activated HER3 becomes responsible for compromising the inhibitory effects of tyrosine kinases.

Activation of the PI3K/AKT pathway results from HER3 upregulation with a subsequent nuclear increase in FoxO3A family of transcription factors responsible for control of cell cycle, neoplastic transformation and epithelial-to-mesenchymal transition[30].

Targeting erb-B3 (HER3) with an antibody has proven to be quite effective in both preclinical and clinical studies although tumor cells eventually develop resistance as the antibody is only active in inhibiting signaling without altering the actual expression of the erb-B3 receptors. New strategies which aim at reducing erb-B3 levels are being investigated such as the HDAC inhibitor entinostat and the antisense oligonucleotide EZN-3920[95].

The hepatocyte growth factor receptor HGFR/c-Met tyrosine kinase responsible for cell proliferation, protection from apoptosis and cell invasion, seems to be implicated in the emergence of resistance to targeted therapies particularly lapatinib and trastuzumab and recent preclinical studies suggested that inhibition of c-MET in gastric cancer cell lines circumvented resistance mechanisms as well as restored growth inhibition[96].

The overexpression of the receptor tyrosine kinase AXL is associated with poor prognosis and a more aggressive phenotype in ovarian, breast colon, esophageal, thyroid and lung cancers and may be implicated in the emergence of lapatinib acquired resistance in *in vitro* models of preclinical breast cancer studies.

Lapatinib resistance has been also associated with SRC tyrosine kinase activity; overexpression of SRC in breast cancer cell lines seems to result in an increased interaction with EGFR rather than HER2. According to Formisano *et al*[97], when EGFR was inhibited with the monoclonal antibody cetuximab and SRC was inhibited by the small molecule saracatinib, lapatinib resistant breast cancer cells would not survive and sensitivity was restored. The combined treatment of lapatinib with cetuximab both *in vitro* and *in vivo* resulted in the reduction of EGFR/HER2 signaling and proved to be effective[97].

As observed by Wilson *et al*[98], autocrine tumor cell production might be responsible for increased levels of receptor tyrosine kinase-ligand levels and in breast cancer cell lines the HER3 ligand neuregulin-1 seems to induce complete rescue from lapatinib.

An additional mechanism of resistance to lapatinib may occur as a result of crosstalk between the estrogen receptor and the HER2 pathway. Lapatinib induced upregulation of ER by inhibition of the PI3K/AKT signaling pathway results in overexpression of the anti-apoptotic protein Bcl-2 leading to the emergence of lapatinib resistance and cell death escape[99].

The VEGF

The VEGF and its cell surface receptors represent the main modulators in the emergence of tumor angiogenesis. Avastin or bevacizumab, a humanized anti-VEGF antibody, has played a key role in anti-angiogenic therapy for cancer treatment in concomitance with small molecule VEGF receptor kinase inhibitors[43].

The VEGF ligand presents itself as an antiparallel homodimeric structure in which each monomer is made mostly of β strands stabilized by a disulfide knot and two symmetrically disposed intermolecular disulfide bridges that are responsible for linking the monomers together. On the extracellular domain of each of the three VEGF receptors (VEGF-1, VEGF-2, VEGF-3) there are seven immunoglobulin-like structures (Ig domain)[100,101].

All four members of the VEGF family and the placenta growth factor bind to three endothelial cell tyrosine kinase receptors which have each different functions. VEGFR1 is responsible for promoting differentiation and vascular maintenance, VEGFR2 induction of endothelial

cell proliferation and vascular permeability, VEGFR3 stimulation of lynphangiogenesis. Isoform specific receptors neuropilin-1 and neuropilin-2 may bind to class 3 semaphorins involved in axonal growth and also to some isoforms of VEGF1 as co-receptors which results in additional VEGF binding to VEGFR2[102].

Several other pathways are implicated by the function of VEGF as proteolytic and heparin activation further modulates receptor sites resulting in various cellular effects like the increase of vascular permeability, endothelial cell proliferation, survival and tubular formation. The VEGFR are usually endothelial in origin but in some instances they may be located in the stroma as macrophages and tumor cells themselves. Under abnormally low oxygen conditions (hypoxia), the hypoxia-inducible factor (HIF) plays a central role in transcription of genes like VEGF.

In normoxic conditions, the alpha-subunit of the HIF heterodimer (alpha, beta) is degraded by ubiquitylation as HIF-alpha binds to the von Hippel-Lindau tumor suppressor protein (p-VHL) forming the E3 ubiquitin ligase complex, a recognition component leading to proteasome-dependent degradation. In hypoxic conditions, as the HIF-alpha subunit is stabilized by heterodimerization with HIF-beta and hypoxia response elements (HRE), regulatory elements of HIF target genes are activated including VEGF, genes controlling cell proliferation and cell metabolism[103,104].

VEGF is one of the genes that is upregulated in hypoxia microenvironments eliciting a particular vascular phenotype; the high expression of VEGF is a common prognostic factor in human breast cancer malignancies representing an important therapeutic target. Other family members though play a role in angiogenesis even when VEGF is not expressed, in addition to the function of these homologues, the switching of angiogenic pathways may represent an area for further investigation to be possibly circumvented by multiple pathway inhibition[105].

Emerging patient data suggests that the combination of the anti-angiogenic drug bevacizumab with chemotherapy agents such as paclitaxel has proven to be a very dangerous therapeutic choice in terms of fatal side effects including hemorrhage, neutropenia, perforations of the gastrointestinal tract, blockage of arteries and stroke[106].

VEGF resistance mechanisms

Several mechanisms are implicated in the emergence of resistance mechanisms to anti-angiogenic therapy (Figure 2). The most prominent one relates to the promiscuity of cancer cells to produce many types of alternative angiogenic signals which limit drug efficacy. The rescue of tumor vascularization may occur as escape mechanisms are induced by anti-angiogenic therapy and hypoxia of tumor tissue.

Cancer cells may amplify angiogenic genes which in return do not respond to low doses of anti-angiogenic drugs; they may switch from vessel sprouting to vessel co-option, vasculogenesis or vascular mimicry in order to ensure tumor nutrients. The recruitment of bone-marrow derived cells by cancer cells may result in the secretion of pro-angiogenic factors like angiopoietin, fibroblast growth factor or ephrins. The VEGF receptors may induce the release of a cytokine cascade which results in an inflamed microenvironment allowing for the emergence of tumor extravasation and metastasis.

Some of the alternative targets to overcome drug resistance to anti-angiogenesis therapies might be to target the placental growth factor and Bv8 (Bombina variegata) to reduce tumor inflammation, reduce leakiness of vessels, moderate hypoxia and reduce angiogenesis; the Notch pathway by anti-delta like ligands 4 (DII4) and secretase inhibitors to reduce excessive sprouting and reduce leaky dysfunctional vessels. Vessel normalization may be achieved by PHD2 inhibition improving vessel function and reducing metastasis and hypoxia. Lymphangiogenesis may be targeted by inhibiting neuropilin-2 (Npn2) and by targeting neuropilin-1, tumor growth and angiogenesis can be significantly reduced[107].

Several alternative pathways may take over as resistance develops to anti-angiogenic therapy through intrinsic tumor resistance or acquired resistance: angiogenic redundancy involves the production of redundant pro-angiogenic factors like the fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs), placenta growth factor (PlGF), tumor necrosis factor-α (TNF-α). As these pro-angiogenic factors allow for the growth of tumor vasculature despite the VEGF pathway being inhibited it would be appropriate to target several of them synergistically.

The increase of tumor hypoxia as a result of anti-angiogenic therapy is often implicated in angiogenic redundancy: The overexpression of the hypoxia-induced factor-1 (HIF-1) is correlated with chemotherapy resistance and selection of aggressive cancer cells as it is directly involved in the induction of transcription of genes involved in angiogenesis. The important role of activating the membrane tyrosine kinase receptor c-MET by the hepatocyte growth factor during angiogenesis, allows for downstream activation of SRC, AKT, MEK, STAT3 with an increased expression of VEGF and its receptor by endothelial cells. The HGF/c-MET collaboration is often associated with invasive cancer phenotypes and increased metastasis. In these cases, the selection of more invasive tumor cells may occur as hypoxic environments pressure cancer cells to move rapidly toward normoxic locations. The recruitment of bone marrow derived pro-angiogenic cells and inflammatory cell invasion may contribute to adaptive mechanisms of resistance as low oxygen concentrations induce these cells to release large amounts of pro-angiogenic factors. As alterations in endothelial cells and pericytes may be responsible for crosstalk between angiogenic pathways resulting in the emergence of anti-angiogenesis therapy resistance, inhibiting the VEGF pathway and the platelet derived growth factor receptor with a tyrosine kinase inhibitor simultaneously might be a promising strategy to enhance treatment efficacy. The process of vessel co-

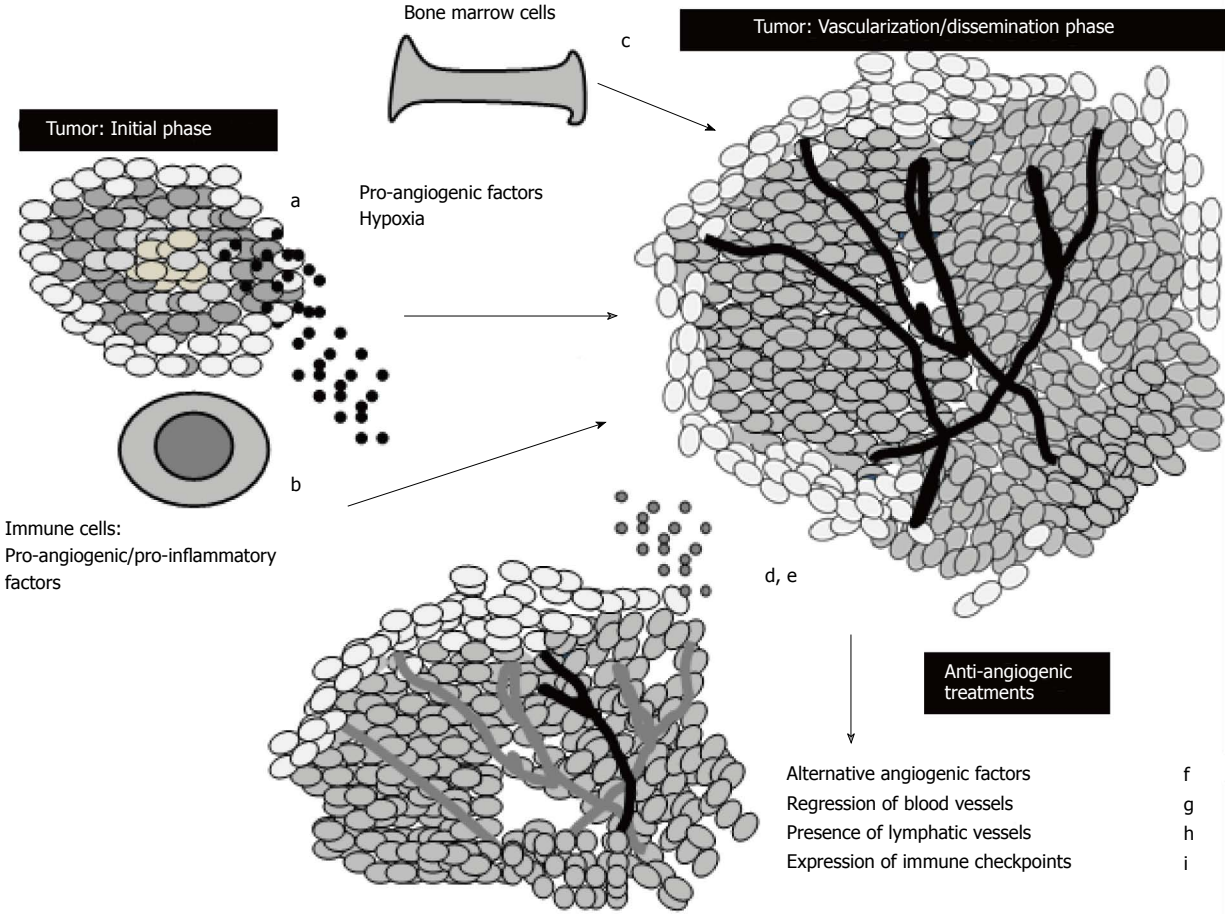

Figure 2 Resistance mechanisms to anti-angiogenic therapy. During the initial development, tumor cells that are in the core of the tumor, become hypoxic and secrete pro-angiogenic factors (a); Proangiogenic factors are also produced by immune cells (b) and bone marrow cell participate in tumor vascularization (c); The amplification of cancer cell genome stimulates high gene expression levels, consequently, requiring an increased anti-angiogenic drug concentration (d); Tumors have evolved to switch from various modes of vascularization, in order to ensure a sufficient supply of nutrients, such as sprouting angiogenesis, vasculogenesis, vessel co-option as well as vascular mimicry (e); Various pro-angiogenic factors that are redundant of VEGF are secreted by tumor and stromal cells in malignant cancers (f); In response to the treatments, blood vessels regress (g) and tumor cell produced alternative proangiogenic prolymphangiogenic factors with the development of a lymphatic network (h); Tumor cells also express immune checkpoints proteins resulting in immune tolerance (i).

option may result in cancer cells displaying a normal looking vasculature which is less sensitive to anti-angiogenic therapy and early stage tumors may escape inhibition as they grow in an angiogenesis independent fashion[108].

The future of anti-angiogenic therapy seems to depend on how different tumors become vascularized and by what alternative pathways these manage to escape therapeutic effects. Elucidation of the complexity of the biology of angiogenesis, coupled with the function of key biomarkers, may prove to be a promising way forward to enhance the function of anti-angiogenic therapy to achieve vascular normalization and increase the effects of complementary chemotherapy.

TNBC and PARP inhibitors

TNBC represent 10%-20% of invasive breast cancers in the general population and have been associated with the African-American ethnic group where a clear prevalence of the disease affects up to 28% of all patients within that group[109].

About 80% of breast tumors which lack the over-expression of the HER-2/erbB2 protein, the estrogen receptor (ER) and the progesterone receptor (PgR) fall under the category of TNBC. They may be characterized by elevated levels of PARP enzymes and often originate from basal-like cell types. TNBC represent the most aggressive phenotype of the disease with no specific targeted therapies available for treatment. Twelve percent of TNBC are characterized by a claudin-low subtype; these can be identified by DNA microarray expression profiling, a method slowly emerging in clinical practice for the detection of this rare form of breast cancer. These tumors seem to respond to molecules which target DNA repair systems to induce synthetic lethality if used in combination with other drugs. PARP inhibitors are an example of therapeutic choice when one of the genes in a synthetic lethal pair, with one gene already defective, is targeted resulting in cell death. PARP iso-enzymes include a group of 18 molecules which are central to base-excision repair pathways of single strand DNA breaks. An example is the BRCA1-2 mutation in breast cancer, this

scenario allows for PARP inhibitors to target and block the only functioning DNA repair system, hence, the selective killing of tumor cells while sparing healthy ones and limiting toxicity for the patient[110]. Nuclear basic fibroblast growth factor (bFGF) is a protein found in a subset of TNBC which contributes to the emergence of resistance following chemotherapy[111]. *In vitro* studies have shown that a residual TNBC subpopulation remains after short-term chemotherapy and this resumes proliferation over time. When bFGF was knocked down in these residual cancer cells using short hairpin RNA, the number of residual TNBC cells decreased. This phenomenon is linked to a down-regulation of DNA-dependent protein kinase (DNA-PK) responsible for accelerated DNA repair. This study might suggest that expression of bFGF in TNBC cells could be a prognostic predictor of incomplete chemotherapy response and future tumor recurrence in TNBC patients[111].

The main challenge of circumventing treatment induced resistance mechanisms and the emergence of alternative escape pathways, significantly lowers the overall survival rate of breast cancer patients belonging to this particular subtype as they often exhibit an incomplete pathological response[93].

Sunitinib seems to suppress angiogenesis, tumor proliferation, migration and growth of basal like breast cancer cells; xenograft models indicate that tumor volumes decrease under sunitinib action but due to its effects on the Notch-1 protein expression and hypoxia through HIF-1, there was an increase in proliferation of breast cancer stem cells. The use of a γ-secretase inhibitor in addition to sunitinib may represent a promising treatment option for TNBC while simultaneously targeting cancer stem cells and angiogenesis[112].

Sunitinib may prove to be an effective treatment choice for patients with TNBC as this breast cancer subtype may express increased levels of VEGF. High levels of VEGF may act as a potential prognostic factor in TNBC as the vascular pathway is a key component when targeting this particularly rare subtype of breast cancer[113].

As targeted therapies have not yet been discovered for TNBC, the conventional route is to treat patients with chemotherapy particularly anthracycline and taxane. The multitude of pathways which drive proliferation of this particular breast cancer subtype need to be further investigated in order to isolate potential therapeutic targets. Patients with BRCA1 and BRCA2 gene mutations which are present in 20% of TNBC, may be sensitive to the function of PARP inhibitors in addition to chemotherapy[6].

In a Phase II clinical trial carried out to evaluate the combined administration of the PARP1 inhibitor iniparib with cisplatin and gemcitabine on patients with TNBC, iniparib seemed to show significant anticancer activity enhancing the antiproliferative and cytotoxic effects of cisplatin and gemcitabine[114]. Combination therapy of cisplatin, gemcitabine and iniparib is currently under Phase Ⅲ clinical trial to see if this association could represent the new standard of care for the treatment of TNBC (ClinicalTrials.gov No.NCT00938652).

Immunotherapy for breast cancer

Breast cancer has been considered non-immunogenic for quite a long time and only recently, data has suggested that TNBC and HER2 positive types are characterized by an immune infiltrate, which might prove to be a promising target to complement the function of other synergistic drugs. Solid tumors like melanoma and lung cancer have already responded to immunotherapeutic agents like ipilimumab and sipuleucel-T has proven a successful vaccine against castration-resistant prostate cancers. Ongoing studies are also evaluating to what extent immune response is correlated to prognosis in breast cancer (Table 1).

The aim of immunotherapy is that of activating the human immune response to recognize tumors as a foreign entity and eventually kill the tumor cells. The tumor microenvironment (TME) including T-regulatory cells (T-Reg) involves a complex structure of intercellular communication which represent a very promising area of research aiming at the isolation of key immunogenic targets which may enhance the function of existing therapies[115].

The immune-checkpoint receptor PD-1 is expressed on tumor-infiltrating lymphocytes (TILs) with the role of inhibiting the activity of effector T-cells, preventing autoimmunity and inflammatory response; it is often upregulated on tumor cell surface in many types of solid tumors. The PD-1 ligand PDL1 engages with T-cells resulting in upregulation of the receptor followed by an immunosuppressive signal, which inhibits kinases involved in the activation of the immune response[116]. Clinical blockade of the PD-1/PDL1 axis should enhance antibody function in cancer patients underlining the importance of further investigation in this particular area of breast cancer research (Table 2). Pro-inflammatory cytokines and the overexpression of PDL1 inhibitory ligand may play a key role in the development of cancer immune resistance mechanisms, resulting in a state of exhausted or tolerant immune T-cell response hence the importance of studying the possible role of PDL1 expression as a resistance biomarker. Overall, main role of PD-1 blockade results in the reversal of chronic antigen response which is often found in cancer and viral infection scenarios[117]. The anti PD-1 antibody nivolumab has shown successful activity in melanoma and lung cancer patients targeting these immunoregulatory proteins and enhancing tumor response. There are several other ligands being investigated at present which might be potential targets like: CD80, CD86, PDL2, ICOS-L, B7-H3, B7-H4 and B7-H6 and future directions in cancer immunotherapy research point towards the effects of combined checkpoint blockade to maximize clinical response[118].

Future direction: Breast cancer combination therapy

Over the last few years, new agents have been intro-

Table 1 Recapitulative breast cancer targeted therapy scheme cited in this article

Target pathway	Current therapy	Combination therapy
HER2 (HER2-positive breast cancer)	Trastuzumab/herceptin Pertuzumab lapatinib	Combination trastuzumab/lapatinib (EPHOS-B trial) trastuzumab/264RAD
m-TOR pathway	Everolimus	Possible combination everolimus/HER2 inhibitor
Angiogenesis (VEGF)	Bevacizumab paclitaxel Docetaxel	Targeting the placental growth factor and Bv8/Targeting the Notch pathway by anti-delta like ligands 4 and secretase inhibitors inhibiting simultaneously the VEGF pathway and the platelet derived growth factor receptor with a TK inhibitor
DNA repair mechanisms (TNBC)	Parp inhibitors/anthracyclins and taxanes	Possible combination cisplatin/gemcitabine/iniparib
Notch-1 protein over-expression/ breast cancer stem cells proliferation (TNBC)		Possible combination of g-secretase inhibitor in addition to sunitinib
Immune system response Cell cycle checkpoints	Immunotherapeutic agents Antibodies against PD-1 T-cell inhibitory molecule or its ligand PD-L1	Nelipepimut-S(human leukocyte antigen)/GM-CSF Pembrolizumab in TNBC/PD-L1 positive (KEYNOTE-086 trial)

HER2: Human epidermal growth factor receptor 2; DII4: Delta like ligands 4; TNBC: Triple negative breast cancer; GM-CSF: Granulocyte-macrophage colony stimulating factor; VEGF: Vascular endothelial growth factor.

Table 2 Some of the current clinical trials in breast cancer targeted immunotherapy (http://www.cancerresearch.org./cancer-immunotherapy/impacting-all-cancers/breast-cancer)

Title of clinical trial	Type of breast cancer
Phase Ⅲ clinical trial: NEUVAX: nelipepimut-S or E75NCT01479244	HER1+ HER2+
Phase Ⅱ clinical trial: NEUVAX NCTO1570036	Node positive or TNBC
Phase Ⅰ clinical trial: Pembrolizumab PD1 antibody + dendritic cell vaccine NCTO2479230	Metastatic breast cancer
Phase Ⅱ trial: Pembrolizumab PD1 antibody + HDAC inhibitor and anti-estrogen therapy NCT02395627	Breast cancer
Phase Ⅱ first line neo adjuvant trial: Atezolizumab + chemotherapy NCTO2530489	TNBC
Phase Ⅰ clinical trial: Atezolizumab and HER2 inhibitors NCTO2605915	HER2+
Phase Ⅰ / Ⅱ clinical trial: PDR001(PD1 antibody)	Advanced breast cancer, TNBC
Phase Ⅰ / Ⅱ clinical trial: MEDI6469 anti OX40 antibody NCTO1642290	Stage 4 breast cancer (patients with prior failure of hormone or chemotherapy)
Pilot study of QBX258 targeting IL-4 and IL-13 NTCO2494206	Advanced TNBC whose cancer cells make a protein called glycoprotein NMB to which CDX-011 binds

IL: Interleukin; HER2: Human epidermal growth factor receptor 2; TNBC: Triple negative breast cancer.

duced in breast cancer targeted therapy resulting in overall improved treatments and greater patient overall survival rates. Some of the most widely used combination therapies involve the use of agents which target the PI3K/AKT/mTOR pathways such as everolimus combined to exemestane. The everolimus-FKBP12 complex that forms when the m-TOR inhibitor binds with high affinity to the intracellular receptor FKBP12, is very effective in inhibiting down strem signaling in cancer cells. The BOLERO study has demonstrated the efficacy of the m-TOR inhibitor everolimus used in combination with exemestane (endocrine therapy) to restore hormonal sensitivity in breast cancer patients[6]. Palbocib has been combined with letrozole in treating women with ER positive (estrogen positive), HER2 negative, advanced breast cancers as a first line endocrine therapy in metastatic cases. Trastuzumab and lapatinib have been used successfully in combination to treat metastatic breast cancers that overexpress HER2[6]. Trastuzumab and pertuzumab have been used in combination for the treatment of HER2 positive metastatic breast cancers and have shown a statistically significant increase in overall survival of patients[6]. The trastuzumab/lapatinib/hormonal therapy combination has proven to be effective in cases of hormonal receptor positive and overexpressed HER2 protein breast cancers like the luminal B/HER2 enriched type. Iniparib, a PARP1 inhibitor, in combination with gemcitabine and carboplatin chemotherapy have been evaluated in a Phase Ⅰ clinical trial for the treatment of metastatic TNBCs and a clinical benefit of 56% was observed in the combined therapy arm, compared to the gemcitabine/carboplatin arm which had a 34% clinical

Table 3 Some of the current clinical trials in breast cancer targeted therapy (http://www.breastcancertrials.org)

Title of clinical trial	Type of cancer
Randomized open label Phase Ⅱ trial: Kadcyla, tykerb and abraxane *vs* herceptin, tykerb and taxol before Surgery for HER2+ tumors NCT02073487	HER2+
Phase Ⅲ randomised, placebo controlled clinical trial: Chemotherapy and a PARP-inhibitor for BRCA1/2+, HER2- advanced breast cancer NCTO2163694	HER2-, BRCA1/2+ metastatic or locally advanced unresectable breast cancer
Phase Ⅱ, multicenter, randomized clinical trial: Alisertib with taxol for advanced ER+/HER2- or TNBC NCTO2187991	ER+/HER2- TNBC
Phase Ⅱ Clinical trial: Gemzar, herceptin and perjeta for HER2+ metastatic breast cancer NCTO2252887	HER2+ metastatic breast cancer
Phase Ⅰ clinical trial: CD-839 for advanced breast tumors NCTO2071862	Advanced breast cancer and solid tumors
Phase Ⅰ clinical trial: Saracatinib and anastrozole for ER-positive disease NCTO1216176	ER+
Randomised Phase Ⅲ clinical trial: Hormone therapy with or without ibrance for HR+, HER2- stage Ⅱ-Ⅲ breast cancer NCTO2513394	HR+, HER2-
Phase Ⅱ clinical trial: CDK-inhibitor for previously treated metastatic disease NCTO1037790	Previously treated metastatic breast cancer
Phase Ⅰ clinical trial: GS-5745 in metastatic HER2- breast cancer and other solid tumors NCTO1813282	Metastatic HER2- breast cancer not responding to other treatments

benefit[114].

A promising area of clinical research for breast cancer targeted therapy involves the use of immune checkpoints inhibitors or immune checkpoint stimulatory molecules. In order to unleash anti-cancer immune responses, inhibitory molecules are blocked or stimulatory molecules are activated to allow the immune system to attack directly cancer cells as foreign invaders. An example would be the anti PD1 antibody pembrolizumab (Keytruda), anti CTLA antibodies, the anti PD-L1 antibody atezolizumab, anti CD73 antibodies or anti OX40 antibodies being tested currently in Phase I/II clinical trials (Table 2).

As the importance of the TME is being discovered with its potential contribution to cancer therapy, novel agents are being developed to target the non-malignant tumor stroma like trabectedin which inhibits macrophage differentiation; other drugs target the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) pathway such as mapatumumab and dulanermin; immunomodulators used alone or in combination to cytotoxic agents should be also investigated as a strategy to decrease the immunosuppression caused by T-effector cell upregulation in the quest to increase the innate immune response against cancer cells, keeping the right balance as immune over-stimulation could be potentially harmful to patients[86].

The main future challenge for breast cancer combination therapy is to design a winning formula that is simultaneously effective against the many subtypes of breast cancers like luminal A, luminal B, basal-like and overexpressing HER2. This approach would represent a hopeful avenue to explore in the quest to inhibit the multitude of pathways being exploited by the various breast cancer subtypes. The phenotype of each breast cancer subtype should be thoroughly investigated as well, to allow researchers to gather a general picture describing in detail the different mechanisms of action for cell survival. Only then, more precise targets can be identified allowing for the discovery of more inclusive breast cancer combination therapies. A more precise and personalized characterization of each cancer as well as the identification of factors involved in resistance for each patient may provide useful improvements in current therapeutic approaches.

CONCLUSION

Decoding of the human genome has allowed for the isolation of key gene signatures for many types of known cancers; unfortunately, targeted therapies to inhibit the function of these genes have proven quite elusive as the quest to circumvent the emergence of resistance mechanisms continues. Breast cancer subtypes, particularly TNBCs, still represent a major challenge; future studies should revolve around the discovery of new prognostic biomarkers as no targets for these rare types of breast cancer have yet been identified.

The EPHOS-B trial carried out by researchers in The Institute of Cancer Research, London, the University of Manchester and University Hospital of South Manchester NHS Foundation Trust investigating the response of HER2 positive breast cancer to dual lapatinib and trastuzumab therapy shortly after diagnosis and surgery to remove the tumors, has released very promising data in which of 257 women who were administered the two drugs synergistically 11 d before surgery, 17% had only minimal residual disease with invasive tumor smaller than 5 mm in size, 11% had a pathological complete response with no biological invasive tumor present in the breast and 3% had a complete response. This dramatic response after only 11 d suggests that combination anti-HER2 targeted therapy prior surgery may reduce the number of breast cancer patients requiring chemotherapy in the future and significantly eliminate long term chemotherapy associated side effects[4,119].

Resistance mechanisms in breast cancer targeted therapies represent the main challenge to current research; the combination of different molecules used to target

different levels of signaling pathways by synergistically blocking cancer cell escape routes and minimizing the emergence of survival mechanisms, could prove to be a promising way forward, keeping in mind that specific molecular profiling particularly for metastatic relapses should be carried out to elucidate further resistance phenotypes and allow for the design of specific new targets. Several clinical trials are underway to try to improve survival of the worse cases (Table 3).

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MINIREVIEWS

How best to manage gastrointestinal stromal tumor

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Abstract

Gastrointestinal stromal tumors (GISTs) are rare but most common nonepithelial tumor of gastrointestinal tract. They are often found incidentally on computed tomography and endoscopic investigations. Increasing knowledge of the pathogenesis of GISTs and the advent of tyrosine kinase inhibitors revolutionized the management of GISTs. The newer advanced endoscopic techniques have challenged the conventional surgery although the true efficacy and safety of endoscopic approach is not clear at this time. This review article focuses on pathogenesis, diagnosis and management of GISTs.

Key words: Gastrointestinal stromal tumor; Endoscopy; Endoscopic ultrasound-fine-needle aspiration; Tyrosine kinase inhibitor; Imatinib

Core tip: Gastrointestinal stromal tumors (GISTs) are most common mesenchymal tumors in the gastrointestinal tract. The management of GISTs is revolutionized with the advent of tyrosine kinase inhibitors (TKIs) and newer advanced endoscopic techniques. Accurate identification and differentiation of GISTs from other submucosal tumors is achieved with the help of endoscopic ultrasound. The management of small to medium GISTs are feasible by newer advanced endoscopic and/or laparoscopic techniques. Team approach involving endoscopist, pathologist, radiologist, medical oncologist and surgeon is key in optimal management of GISTs. This article focuses on role of TKIs and endoscopist perspective in the management of GISTs.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal (sub epithelial) tumor, and are

frequently found in stomach and small intestine[1]. GISTS are hypothesized to originate from interstitial cells of cajal (ICC) which coordinate gut motility[2]. GISTs are rarely found in the peritoneum, mesentery and omentum[3]. GISTs have varied malignant potential, with about 40% of GISTs that are localized at initial diagnosis give rise to metastasis[4], and about 10%-20% of GISTs present with distant metastasis[5,6]. In Europe, the annual incidence of GISTs is about 10 cases per million[7]. In the United States, the annual incidence of GIST ranges from 4000 to 6000 new cases per year (7-20 cases per million population per year)[8]. The mean age at diagnosis is 63 years[9]; men and women are equally affected. The majority of GISTs are sporadic and may be associated with mutations like NF1, C-kit, platelet derived growth factor receptor-alpha (PDGFRA), succinate dehydrogenase (SDH) and deletions in chromosome 1 involving SDH c[10].

PATHOGENESIS OF GIST

Overall, GISTs are defined by the presence of *KIT* gene or PDGFRA mutation. Majority (80%) of GISTs have *KIT* gene mutations and biologic response of KIT receptor is produced without a bound ligand[11]. KIT receptor tyrosine kinase activity in normal cells is regulated by binding of endogenous KIT ligand or stem cell factor (SCF)[12]. In the majority of cases, spontaneous receptor dimerization and activation occurs when exon 11 is affected by *KIT* gene mutation. However, in few cases, a different mechanism results in uncontrolled KIT signaling if mutation occurs in Exon 9, 13 or 17. In cases with NF1, uncontrolled KIT activation may be present even in the absence of *KIT* gene mutation (wild type)[13]. A subset of GISTs which are negative for *KIT* gene mutations are positive for receptor tyrosine kinase PDGFRA mutations. GISTs expressing PDGFRA or *KIT* gene mutations have similar biologic consequences[14]. About 10% of adult GISTs have neither KIT gene nor PDGFRA mutation[15]. SDH-ubiquinone complex 2 is composed of subunits A, B, C and D which is part of Krebs cycle and respiratory chain[16]. In mutant SDH, dysfunction of electron transport chain in mitochondria leads to defective oxidative phosphorylation, which ultimately leads to abnormal stabilization of hypoxia inducible factors (HIF)[17]. Carney-Stratakis syndrome is caused by germline mutation in SDH subunits B, C or D which leads to GIST and paraganglioma[18].

Histologically GISTs are subdivided in to spindle cell (60%-70%), epithelioid (30%-40%) or both (10%). GISTs with spindle cells are compact, highly cellular, arranged in fascicular or whorled pattern with minimal amount of stroma and contain eosinophilic, basophilic or amphophilic cytoplasm. Epithelioid tumors have abundant cytoplasm which is amphophilic to clear and cellular borders are clearly defined[19]. Antibodies to CD34 and CD117 appear in most GISTs[20]. CD34 is a transmembrane glycoprotein present on vascular endothelium and human hematopoietic progenitor cells[21]. CD34 is expressed in a wide variety of tumors and it is detected in about 50%-80% of GISTs[2,11,20]. CD 117 is expressed in 80%-100% of GISTs and it is not expressed in smooth muscle or neural tumors which helps in distinguishing GISTs from other gastrointestinal mesenchymal tumors[20] (Figure 1).

CLINICAL PRESENTATION AND DIAGNOSTIC TOOLS

Clinical manifestations of GISTs are highly variable and it depends on tumor size and location. GISTs are usually asymptomatic and found incidentally by imaging or endoscopy[22]. Symptoms include melena, hematemesis, abdominal pain, discomfort, fullness, early satiety and palpable mass. GISTs in proximal stomach can cause dysphagia and tumors in pylorus can present as gastric outlet obstruction[23]. Rectal GISTs can present with hematochezia[24]. Rarely, they can present as intraperitoneal rupture of large tumor causing hemoperitoneum[25]. GISTs can occur as part of a syndrome; Carneys triad (gastric GIST, pulmonary chondroma, paraganglioma)[26], or neurofibromatosis type1 (mostly spindle cell GIST)[27]. Overall, about 50% of GISTs have local or distant metastasis at the time of presentation[28], with the liver being the most frequent site of metastasis. Other common sites of metastasis include the bone, peritoneum, retroperitoneum, lung, pleura, and subcutaneous (scar) tissue[29].

Computed tomography (CT) is the primary modality of choice for diagnosing GISTs[30,31]. CT tumor characteristics such as size greater than 10 cm, calcifications, irregular margins, heterogeneous, lobulated, regional lymphadenopathy, ulceration, extraluminal and mesenteric fat infiltration are more likely to be associated with metastasis[29]. CT enterography uses large volumes of oral contrast and it is superior to conventional CT. It has advantage of displaying the entire thickness of the small bowel, better visualization of deep ileal loops without superimposition and evaluation of surrounding mesentery[32]. MRI is more accurate than CT for delineating rectal GISTs and in detecting liver metastasis, hemorrhage and necrosis[33].

Esophagogastroduodenoscopy (EGD) shows most sub epithelial lesions as a bulge with a smooth, intact, normal appearing mucosa in the gastrointestinal tract. Hwang *et al*[34] did a prospective study and patients were referred for endoscopic ultrasound (EUS) to evaluate sub epithelial masses diagnosed previously by EGD, sigmoidoscopy or colonoscopy. The size of the mass during endoscopic exam was measured by open biopsy forceps for size reference. Results showed endoscopy was 98% sensitive and 64% specific in identifying intramural lesions. Intramural size measurement of endoscopy correlated with EUS ($r = 0.88$, $P < 0.001$) but, for extramural lesions, it was suboptimal ($r = 0.56$)[34]. Overall, the study concluded endoscopy had a high sensitivity but low specificity in identifying the location of sub epithelial lesions and histologic confirmation by EUS-fine-needle aspiration (FNA) should be obtained for masses originating from 3rd (submucosa) and 4th layer

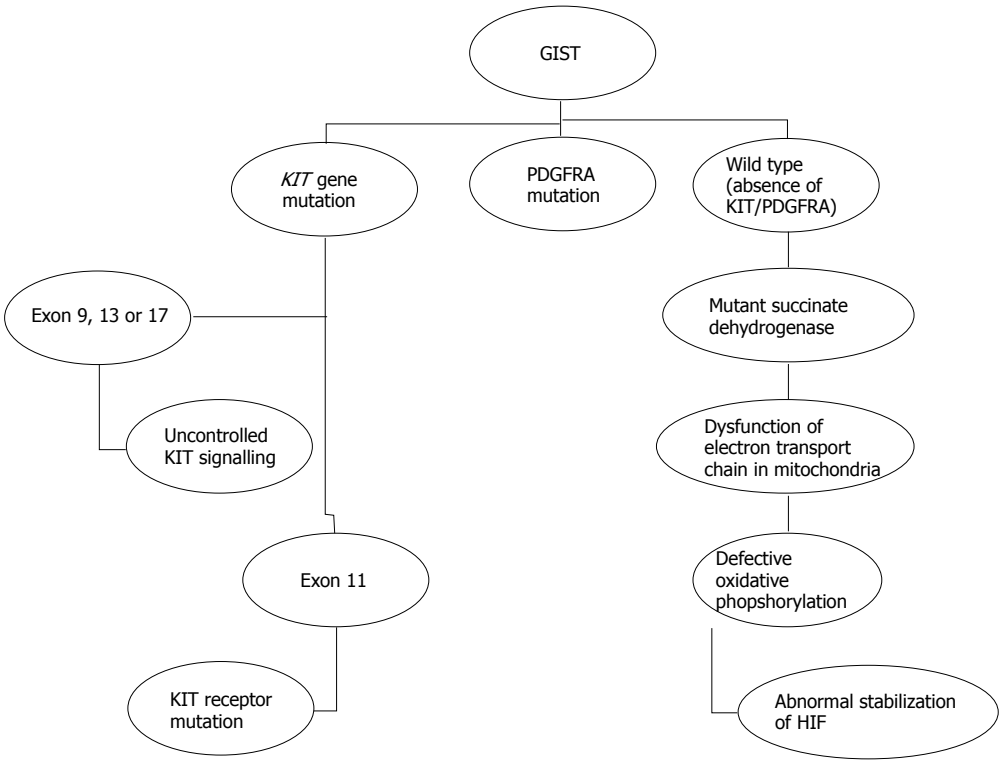

Figure 1 Pathogenesis. GIST: Gastrointestinal stromal tumor; PDGFRA: Platelet derived growth factor receptor-alpha; HIF: Hypoxia inducible factors.

(muscularis propria)[34].

Endosonographically GISTs appear as oval or hypoechoic mass arising from the muscularis propria. EUS features suggestive of malignancy include enlarged lymph nodes, size greater than 4 cm, irregular borders and cystic spaces with in the mass[35]. EUS has 92% sensitivity and 100% specificity in differentiating submucosal tumor from extrinsic compression[36]. Chen *et al*[37], retrospectively evaluated EUS characteristics to predict the malignant potential of GISTs. EUS features of GISTs were compared to National Institutes of Health (NIH) criteria for classification of malignant potential and were divided in to very low/low risk, intermediate/high risk. Results showed that GISTs at high risk for malignancy were associated with EUS characteristics like lesion size ($P < 0.0001$), cystic change ($P = 0.015$) and surface ulceration ($P = 0.036$)[37]. EUS-FNA cannot accurately differentiate benign from malignant GIST due to lack of mitotic activity on smears. The definitive method for assessment of GIST malignant potential requires surgical resection.

Dewitt *et al*[38] evaluated the diagnostic yield and complications of EUS-Trucut biopsy (EUS-TCB) for gastrointestinal mesenchymal tumor (GIMT). EUS-FNA was performed in 33/38 (87%), and was diagnostic on final cytology in 25/33 (76%) and by FNA-immunochemistry (FNA-IC) in 12/24 (50%). EUS-TCB obtained visible tissue specimen in 37/38 (97%), and diagnostic in the final TCB histology in 30/38 (79%) and TCB-IC in 30/31 (97%)[38]. Overall, the authors concluded that EUS-TCB should be considered as an alternative to EUS-FNA when technically feasible[38].

Na *et al*[39] evaluated the yield and utility of 19-gauge (G) TCB *vs* 22-G FNA for diagnosing gastric sub epithelial tumors (SETs). The diagnostic yield of TCB *vs* FNA were 77.8% *vs* 38.7% ($P < 0.0001$). The Accuracy of TCB *vs* FNA for diagnosing GISTs was 90.9% *vs* 68.8%; and for non-GIST SETs was 81.1% *vs* 14.3% respectively. There were 9 technical failures with TCB likely due to stiffness, poor maneuverability of the needle and location of the tumor[39]. The most common procedure associated adverse events were pain, hemorrhage (requiring endoscopic hemostasis) and fever[39]. Procedure related events in TCB *vs* FNA were [3/90 (3.3%) *vs* 5/62 (8.1%); $P = 0.27$] respectively[39].

Positron emission tomography (PET)-CT using ^{18}F-fluorodeoxy glucose (FDG) detects cancer based on changes in tissue metabolism[40,41]. PET-CT is used for initial staging and to monitor disease progression. A baseline ^{18} FDG-PET should be obtained before treatment so that the results can be used to compare with future studies[42]. Liver metastasis from GIST often appear as isodense lesions on CT, but may be detected by PET. Hence PET compliments CT in resolving ambiguity of liver lesions in patients with GISTs[42].

Gayed *et al*[43] showed that the sensitivity and positive predictive value of ^{18}F-FDG PET were 86% and 98% respectively and it is superior to CT in predicting early response to therapy in recurrent or metastatic GISTs[43]. Yoshikawa *et al*[40] evaluated the efficacy of PET-CT to predict the malignant potential of GIST. Standardized uptake value maximum (SUVmax) and GIST parameters

(Ki-67 labeling index and mitotic index) were compared. SUV max and Ki67 labeling index were significantly elevated in high risk group when compared to low/intermediate risk group[40]. Tumor response to treatment with imatinib mesylate may be detected by a decrease in CT attenuation units (Hounsfield units, HU)[44]. However, there may be delay in measurement of cellular and macroscopic changes after treatment with imatinib by CT. In contrast, PET using ^{18}F-FDG can detect early effects induced by imatinib and decrease in FDG uptake after the initiation of imatinib treatment indicates good prognosis[45].

The "Response Evaluation Criteria in Solid Tumors" (RECIST) classification was previously used, however, due to limitations in assessing malignant response to immunotherapy such as imatinib, RECIST has been replaced by the Choi criteria[46]. Limitations of RECIST were primarily because the response to therapy can occur not only in tumor size but also in structure like decreased tumor density and enhancement of intratumoral nodules[31,47]. The Choi criteria of contrast-enhanced CT is based on decrease in tumor size by 10% in any dimension or decrease in structure by 15%, and was found to be more predictive of time to tumor progression (TTP) than RECIST[48].

PROGNOSIS AND RISK STRATIFICATION

Mitotic index, tumor size, location (gastric *vs* non-gastric) and tumor rupture are independent risk factors for GIST metastases[4]. Joensuu *et al*[49] analyzed the association between KIT and PDGFRA mutation and RFS in GIST patients treated with surgery alone. The authors concluded that tumor mutation status should not be interpreted in isolation from other risk factors[49]. The American College of Surgeons Oncology trial (ACOSOG) Z90001 study found that tumor size, location and mitotic rate were important in RFS but not tumor mutation status[50]. Gold *et al*[51] developed a nomogram by calculating concordance probabilities and by comparing three commonly employed staging systems NIH-Miettinen[52], NIH-Fletcher[53] and Armed Forces Institute of Pathology (AFIP)-Miettinen[54]. The investigators concluded that the nomogram can accurately predict RFS after the resection of localized, primary GIST[51].

MANAGEMENT OF GIST

Surgery is the treatment of choice for primary and localized GISTs[55]. The goal of surgery is complete tumor resection (negative microscopic and macroscopic margins) with functional preservation (often accomplished by wedge resection), while avoiding tumor rupture and injury to the pseudo capsule[55]. McCarter *et al*[56] analyzed factors associated with R_0 (grossly and histologically negative margin), R_1 (grossly negative but histologically positive margins), R_2 resection (grossly positive margins) and assessed the risk of recurrence with and without imatinib[56]. Factors associated with R_1 resection included tumor size (> or = 10 cm), tumor rupture and location[56]. The authors concluded there was no significant difference in recurrence free survival (RFS) in patients who underwent R_1 *vs* R_0 resection of GIST with or without adjuvant imatinib[56]. Although the management of R1 resection after complete resection is not clear, options include careful observation (watchful waiting), re-excision and adjuvant imatinib treatment.

Laparoscopic wedge resection (LWR) is recommended for gastric GIST smaller than 5 cm. To prevent tumor seeding in laparoscopy, plastic bag is recommended to collect the tumor sample and direct handling of tumor with forceps is contraindicated. Wedge resection of gastric GIST is considered standard treatment[57] and lymphadenectomy is not indicated as nodal metastasis is rare[28]. LWR has the advantage of early resumption of diet, early return of bowel function, shorter hospital stay and decreased duration of parenteral or epidural analgesia[58]. Lee *et al*[59] study concluded that LWR can be safely performed and have better outcome in terms of recovery after surgery regardless of tumor size and location. Kim *et al*[60] study concluded that LWR is safe and feasible for small to medium sized gastroduodenal tumors irrespective of location in cardia or pylorus. However, they recommended careful consideration of direction of stapling for exogastric resection of submucosal tumors located in antrum, lesser curvature and pylorus to prevent gastric outlet obstruction.

Endoscopic enucleation and other related procedures are more feasible for GISTs less than 5 cm[61]. Complete resection of GIST is indicated with endoscopic enucleation in the presence of a pseudo capsule. According to location in the gastric wall, GISTs are classified in to several types such as type 1 [very narrow connection with muscularis propria (MP) layer which protrudes in to the lumen], type 2 (wide based connection with MP layer and protrudes in the luminal side at obtuse angle), type 3 (located in the middle of gastric wall) and type 4 (protrudes into the serosal surface of gastric wall)[61]. This classification is very important when considering endoscopic enucleation. Endoscopic enucleation is best suitable for type 1 because of narrow connection to the MP layer and can be attempted for type 2. Type 3 and type 4 cannot be completely resected by endoscopic enucleation and hence endoscopic full-thickness resection (EFTR), laparoscopic and endoscopic cooperative surgery (LECS), laparoscopic-assisted endoscopic full-thickness resection (LAEFR) and non-exposed wall-inversion surgery (NEWS) should be considered[61]. Endoscopic enucleation includes various techniques like endoscopic submucosal dissection (ESD)[62], endoscopic muscularis dissection (EMD)[63] and endoscopic submucosal tunnel dissection (ESTD)[64]. Bialek *et al*[62] evaluated the efficacy, safety and outcomes of ESD for gastric sub epithelial tumors. Results showed 47% (17/37) sub epithelial tumors were GISTs, overall rate of R_0 resection was 81.1% (30/37), and perforation rate was 5.4%[62]. Liu *et al*[63] evaluated the feasibility and safety of EMD. Results showed that 51.6% (16/31) were GISTs, 96.8% (30/31) were completely resected, perforation occurred in 12.9% (4/31, all of which were managed by

endoscopic methods)[63]. ESTD procedure involves creation of the submucosal tunnel, dissection of the submucosal tumor (SMT) and closure of mucosal entry with hemostatic clips[64]. Gong *et al*[64] evaluated the feasibility and safety of ESTD in upper gastrointestinal SMTs. Results showed that 58.3% (7/12) were GISTs, complete tumor resection was achieved in all patients, *en bloc* resection in 83.3% (10/12, other 2 lesions were resected in 2 pieces) and 2 patients had both pneumothorax and subcutaneous emphysema which were managed conservatively[64]. Disadvantages of endoscopic techniques include tumor recurrence and peritoneal seeding secondary to perforation. It is unclear whether there is remnant GIST tissue after dissection causing tumor recurrence, although the dissection site is usually ablated with electrical knife or snare. Perforation occurs due to pseudo capsule injury during difficult MP layer dissection which increases the chance of peritoneal seeding. Peritoneal seeding is associated with poor prognosis because of increased tumor recurrence.

EFTR without laparoscopic assistance procedure involves introducing a single-chamber gastroscope into the stomach with a transparent cap attached to its tip. Dots are marked around the lesion and submucosal injection is done using normal saline with 1% indigo carmine and epinephrine (1:100000). Hook knife and IT knife are used to incise superficial layers overlying the SMT and snare is used to remove the mucosal and submucosal layers of gastric wall. Hook knife and IT knife are used to make circumferential dissection around the border of SMT. To visualize the SMT clearly, submucosal injection can be done again in the lower border of the tumor as needed. After the MP layer is reached and root of the tumor is exposed, gastric fluid is extracted as much as possible. Active perforation is made with the help of hook knife. After the tumor is completely exposed, SMT is removed *en bloc* with the snare. Dual channel gastroscope can be used for tumors with a broad basement which has the advantage of passing two snares through the accessory channels in to the gastric cavity. Tumor body is grasped with one snare and the other snare is used to *en bloc* enucleate the tumor along with the attached serosal layer. Titanium clips are used to close the defect in gastric wall. Paracentesis can be performed if there are signs of pneumoperitoneum during the procedure. Feng *et al*[65] evaluated the efficacy and safety of EFTR in 48 patients with gastric SMTs. Results showed that 43/48 had GIST, no post-EFTR complication such as bleeding or peritonitis, 5 had moderate postoperative abdominal distension because of air filtration (3 had abdominal paracentesis and the other 2 were managed conservatively)[65]. Zhou *et al*[66] evaluated the efficacy, feasibility and safety of EFTR for gastric SMTs originating from MP layer. Results showed that 16/26 were GISTs, *en bloc* resection rate was 100% and no major complications[66]. In general, there is a risk of peritoneal seeding with EFTR because it involves creating an active large perforation and hence gentle handling of GIST is necessary to maintain an intact pseudo capsule to prevent peritoneal seeding.

LECS has advantage over LWR especially for gastric SMTs located near esophagogastric junction or pyloric region because SMTs can be located accurately using endoscope and the resection of healthy stomach can be minimized[67]. The best indication for LECS is for gastric GISTs originating from MP layer which are intraluminal[61]. First, Argon plasma coagulation (APC) can be used to mark the periphery of the tumor[67]. A small incision is made on the marked area using standard needle knife after injecting 10% glycerin into submucosal layer. Using the IT knife, three-fourth of the marked area is cut circumferentially. Next, laparoscopic dissection of seromuscular layer is performed by making an artificial perforation and seromuscular dissection is carried out with ultrasonically activated device[67]. The incision is closed with the help of laparoscopic stapling device[67]. Hiki *et al*[67] analyzed seven patients who underwent LECS for gastric GISTs. Results showed that 6/7 were GISTs, no postoperative complications like bleeding, stenosis or anastomotic leakage, and successful tumor resection was done irrespective of tumor location (esophagogastric junction or pyloric ring). Tsujimoto *et al*[68] evaluated the feasibility and surgical outcomes of LECS for gastric SMTs. The authors found 16/20 were GISTs, no postoperative complications like bleeding, stenosis or anastomotic leakage, and there was no recurrence of tumor[68].

NEWS is a new technique developed to prevent peritoneal seeding from large active perforation and minimize resected tissue volume of stomach[69]. Mitsui *et al*[69] evaluated the efficacy and safety of NEWS in 6 patients with suspected gastric GIST. Results showed that 5/6 were GIST, *en bloc* resection was achieved in all GISTs, perforation occurred in 2/6 cases (1 case had muscle injury leading to perforation during mucosal cutting by endoscopic knife and the other case had laparoscopic mucosal injury leading to perforation during seromuscular cutting), and no postoperative complications[69]. Future studies with large cohort are needed to validate the safety of NEWS before it is standardized for GISTs treatment.

IMATINIB AS ADJUVANT THERAPY

Tumor size, location, mitotic index and tumor rupture are the most important independent prognostic indicators to determine RFS[4]. Multiple stratification schema like National Institutes of Health (NIH) consensus criteria, Armed Forces Institute of Pathology (AFIP) criteria and the modified NIH consensus criteria were developed to predict risk of recurrence[4,70-72]. The most commonly used stratification method is AFIP criteria[73]. AFIP groups 3a and above are considered high risk for recurrence. This corresponds to 5-year recurrence rate of 30% based on nomogram evaluation[73]. DeMatteo *et al*[74] evaluated the overall survival (OS) in 106 patients who had undergone complete gross tumor removal but were considered high risk for recurrence. It was a phase II Z9000 trial lead by ACOSOG and all patients were treated with imatinib 400 mg per day for 1 year[74]. Results showed that OS for

1, 3 and 5-year was 99%, 97% and 83% respectively after a mean follow up of 7.7 years[74]. RFS rate for 1, 3 and 5-year was 96%, 60% and 40% respectively[74]. In the subsequent trial, patients were randomly assigned to receive imatinib 400 mg per day or placebo for one year[75]. RFS at the end of 1 year for imatinib *vs* placebo was 98% *vs* 83% respectively and OS for imatinib *vs* placebo was 99.2% *vs* 99.7% respectively[75]. Li *et al*[76] evaluated RFS in Chinese patients after complete tumor resection of GISTs. All patients in treatment group (56/105) were treated with imatinib 400 mg once a day for 3 years and 49/105 were not treated (control group)[76]. RFS for imatinib *vs* control group at the end of 1year, 2 year and 3 years were 100% *vs* 90%, 96% *vs* 57% and 89% *vs* 48% respectively[76]. All GISTs with size ⩾ 3 cm, small bowel site and high mitotic index were shown to benefit from adjuvant imatinib treatment[50,75]. Joensuu *et al*[77] evaluated the RFS and OS in KIT-positive GISTs treated with imatinib for 3 year *vs* 1 year who had undergone complete tumor resection but considered high risk for recurrence. Results showed that RFS for patients treated with imatinib for 3 year *vs* 1 year were 65.6% *vs* 47.9% respectively and OS for 3 year *vs* 1 year were 92% *vs* 81.7% respectively[77]. Kang *et al*[78] evaluated the efficacy of adjuvant imatinib for 2 years in high risk GISTs with KIT exon 11 mutation after complete resection at four South Korean centers. The results showed median RFS was 58.9 mo compared to 22.7 mo in pre-imatinib era[78]. They also concluded that imatinib is effective in GIST recurrence even after completion of adjuvant imatinib therapy[78].

NEOADJUVANT OR PREOPERATIVE IMATINIB THERAPY

National comprehensive cancer network (NCCN) guidelines recommend neoadjuvant imatinib therapy to reduce tumor size before surgery and minimize morbidity in patients with primary GISTs considered unresectable or resectable with high risk morbidity[73]. Eisenberg *et al*[79] evaluated the safety and efficacy of neoadjuvant imatinib (600 mg/d) in patients with KIT positive primary GIST (⩾ 5 cm, 32 patients) or with operable metastatic/recurrent GIST (⩾ 2 cm, 20 patients). It was a prospective nonrandomized trial and imatinib was continued postoperatively for 2 years[79]. In primary GIST group, preoperative response was partial in 2 patients (7%), stable in 25 (83%) and unknown in 3 (10%); in metastatic or recurrent group, partial in 1 (4.5%), stable in 20 (91%), and progression in 1 (4.5%)[79]. Only 7 (13%) patients did not have any surgery (5 inoperable or unresectable, 1 patient refusal and 1 physician refusal)[79]. The estimated 2-year rate of TTP, PFS, OS in primary *vs* metastatic/recurrent GIST was 13.9% *vs* 13.6%, 82.7% *vs* 77.3% and 93.3% *vs* 90.9% respectively[79].

Fiore *et al*[80] prospectively evaluated the PFS in locally advanced or unresectable primary GISTs treated with preoperative imatinib. All patients who were considered high risk or needed extensive surgery (3 considered unresectable underwent complete resection, 7 who were initially considered to undergo extensive surgery were conservatively operated, 4 who were considered high perioperative risk underwent safe surgery) improved after preoperative imatinib therapy. PFS after 3 years was 77% from the time of initial imatinib treatment[80].

IMATINIB IN METASTATIC GIST

The outcome of advanced GISTs treated with imatinib is not clear. Demetri *et al*[81] evaluated the efficacy of imatinib on antitumor response, safety and tolerability in advanced GISTs. Results showed that 79 patients (53.7%) had partial response, 41 patients (27.9%) had stable disease and in 7 patients (4.8%) response could not be evaluated[81]. Adverse effects related to imatinib therapy were diarrhea, edema (periorbital and leg), fatigue and gastrointestinal bleeding[81]. Overall, the therapy was well tolerated. Blanke *et al*[82] conducted a multicenter randomized phase II trial and they evaluated the efficacy and long-term safety of imatinib (group A 400 *vs* group B 600 mg) in advanced GISTs positive for CD117 antigen. In group A (400 mg, 73 patients), the authors observed GISTs with complete response 0 (0%), partial response 50 (68.5%), stable 10 (13.7%), progressive 11 (15.1%) and unknown 2 (2.7%)[82]. In group B (600 mg, 74 patients), the authors reported GISTS with complete response 2 (2.7%), partial 48 (64.9%), stable 13 (17.6%), progressive 6 (8.1%) and unknown 5 (6.8%)[82]. Overall, imatinib was well tolerated[82]. In the subsequent phase Ⅲ trial, Blanke *et al*[83] evaluated PFS or OS with standard imatinib dose (400 mg) *vs* higher dose (400 mg twice daily) in patients with incurable GISTs. After a median follow up of 4.5 years, median PFS for standard *vs* high dose imatinib was 18 mo *vs* 20 mo, median OS for standard *vs* high dose imatinib was 55 mo *vs* 51 mo respectively[83]. Treatment response in standard *vs* high dose imatinib were divided in to complete response (5% *vs* 3%), partial (40% *vs* 42%), stable (25% *vs* 22%), progressive disease (12% *vs* 10%) and inadequate assessment (10% *vs* 15%) respectively[83]. This study concluded that 400 mg twice daily imatinib was more toxic than 400 mg dose in treatment of incurable GISTs[83]. Debiec-Rychter *et al*[84] evaluated the efficacy of standard dose imatinib (400 mg) *vs* higher dose (400 mg two times daily) in advanced GIST based on mutational status (KIT or PDGFRA). There was a 61% relative risk reduction of PFS in GISTs expressing exon 9 mutation treated with high dose imatinib[84]. Overall, this study concluded that tumor genotype determines PFS and OS in advanced GISTs and also GISTs with KIT exon 9 benefited from 400 mg two times daily imatinib[84].

Heinrich *et al*[85] showed that presence of KIT exon-11 mutation (71.7%) had better treatment outcome with imatinib when compared to KIT exon-9 (44.4%) and wild-type mutation (44.6%) in advanced GISTs.

The authors also showed that there was an improved response rate (complete/partial response) in patients with KIT exon-9 mutation treated with imatinib 800 mg *vs* 400 mg (67% *vs* 17%, $P = 0.02$)[85]. GIST meta-analysis group (MetaGIST) evaluated PFS and OS with imatinib (400 mg *vs* 800 mg) in advanced GISTs[86]. The results showed that there was a small but significant PFS ($P = 0.04$) advantage in high dose (400 mg twice daily) group and no difference in OS between both (400 and 800 mg) groups[86].

SUNITINIB AFTER TREATMENT FAILURE WITH IMATINIB IN ADVANCED GIST

Demetri *et al*[87] evaluated patients treated with sunitinib in advanced GISTS who were intolerant or resistant to previous imatinib treatment. They concluded that median TTP with sunitinib *vs* placebo was 27.3 wk *vs* 6.4 wk respectively[87]. Overall, sunitinib was well tolerated and side effects like nausea, fatigue, skin discoloration and diarrhea were common[87].

REGORAFENIB AFTER TREATMENT FAILURE WITH IMATINIB AND SUNITINIB IN ADVANCED GIST

Demetri *et al*[88] evaluated the efficacy and safety of regorafenib after failure of treatment with imatinib and sunitinib. Results showed that the median PFS in regorafenib *vs* placebo group were 4.8 mo *vs* 0.9 mo respectively[88]. There was no statistical significance in terms of OS between regorafenib and placebo group[88]. Drug related adverse events occurred in 130/132 (98.5%) in regorafenib group and 45/66 (68.2%) in placebo group[88]. The most common adverse effects of regorafenib include hypertension (31/132, 23.5%), hand foot skin reaction (26/132, 19.7%) and diarrhea (7/132, 5.3%)[88]. Overall, this study concluded that regorafenib significantly improved PFS in patients with advanced GISTs who failed treatment with imatinib and sunitinib[88].

FOLLOW-UP AFTER TREATMENT

The goal of follow-up after surgery is early detection and treatment of relapse. CT abdomen and pelvis is used for follow-up. Metastasis of GISTs outside the abdomen is infrequent. MRI or PET-CT can be used as an alternative for follow-up. Annual CT abdomen and pelvis for 5 years is recommended for low risk GISTs after surgery[89]. During adjuvant treatment with imatinib for high risk GISTs, CT abdomen and pelvis is recommended every 6 mo[89]. After adjuvant therapy is stopped, CT is repeated every 3-4 mo for first 2 years and there after every 6-12 mo for 10 years[89].

CONCLUSION

With increasing availability of EUS and improved knowledge of the pathogenesis of GISTs, accurate identification and differentiation of GISTs from other submucosal tumors are achieved. Although surgery is preferred, newer endoscopic techniques can be attempted by experienced endoscopists with the assistance of surgeons in suitable candidates. Neoadjuvant imatinib therapy is recommended for primary GISTs considered unresectable or resectable with high morbidity to reduce the tumor size before surgery and minimize morbidity. Adjuvant therapy with imatinib in intermediate and high risk GISTs improves OS and RFS. Sunitinib and regorafenib can be used in advanced GISTs after treatment failure with imatinib. Multidisciplinary approach involving endoscopist, pathologist, radiologist, medical oncologist and surgeon is required for optimal management of GIST.

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MINIREVIEWS

Immunotherapies in sarcoma: Updates and future perspectives

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Abstract

Sarcomas are malignant tumors that are characterized by a wide diversity of subtypes with various cytogenetic profiles. Despite major treatment breakthroughs, standard treatment modalities combining chemotherapy, radiotherapy, and surgery failed to improve overall survival. Therefore, high expectations are foreseen with immunotherapy upon its maturation and better understanding of its mechanism of action. This paper presents a targeted review of the published data and ongoing clinical trials in immunotherapies of sarcomas, mainly adoptive cell therapies, cancer vaccines and immune checkpoint inhibitors.

Key words: Adoptive cell therapy; Cancer vaccines; Immunotherapy; Immune checkpoint inhibitors; Sarcoma

Core tip: This paper is a review that outlines the most recent updates on the immunotherapy treatment of sarcomas. After a brief review of the concept of immunotherapies and the different treatment modalities, we discuss the available data, the limitations and future perspectives of each treatment option.

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INTRODUCTION

Sarcomas are malignant tumors that derive from embryonic mesodermic tissues including fat, muscles, bones, nerves and blood vessels[1]. Epidemiologic studies report its predominance in the pediatric populations and its rare occurrence in adults[2]. Sarcomas are

characterized by a wide diversity of subtypes with various cytogenetic profiles conferring treatment resistances. These findings combined with an advanced stage at diagnosis substantially increase the years of life lost[3]. The standard treatment modalities combining chemotherapy, radiotherapy, and surgery have failed to improve overall survival (OS)[4]. Despite the major breakthroughs in the treatment armamentarium, the recent data reports a relative 5-year survival rate limited to 66% for bone and soft tissue sarcomas, 53.9% for osteosarcomas, 75.2% for chondrosarcomas, and 50.6% for Ewing's sarcomas[5].

Interestingly, Coley described in 1891 a complete regression of sarcomas secondary to severe episodes of erysipelas but failed to regenerate these results in other patients[6]. The Food and Drug Administration thereafter banned the use of toxin therapy without a new drug-approval process. Fortunately, Coley's paper has encouraged scientists to analyze the role of the immune system in carcinogenesis[7].

After more than a century since Coley's research efforts that marked the history of immunotherapy, we present a review on this elegant treatment modality in the management of sarcomas including adoptive cell therapies (ACT), monoclonal antibodies, vaccines, and immune checkpoint inhibitors (ICI).

APPROVED THERAPIES IN SARCOMAS FROM CHEMOTHERAPY TO TARGETED THERAPIES

Specialized centers in the management of sarcomas have demonstrated a better OS and low recurrence rate[8]. Yet, all patients are managed uniformly according to their prognosis dictated by the stage of the disease, which is determined by the grade, depth and size of the tumor[9]. For patients with localized disease, a complete resection with wide 2-3 cm margins followed by adjuvant radiation therapy is the mainstay treatment for a curative approach. However, survival is not only determined by local control since most patients die from systemic disease. The choice of the chemotherapy regimen depends on the tumor chemosensitivity which varies with the tumor subtype and grade, the patient's performance status, and the timing of metastatic disease[10]. Unfortunately, the benefits of adjuvant chemotherapy are limited to rhabdomyosarcomas, osteosacromas and Ewing's sarcomas. Moreover, Trabectidine is showing promising results encountered in the adjuvant and neoadjuvant settings of patients with myxoid liposarcomas[11]. The role of adjuvant and neoadjuvant chemotherapy in the management of soft tissue sarcomas is yet to be clearly established. The actual recommendations by NCCN and ESMO are to address this issue on a case by case basis according to the patient's performance status, comorbid factors, disease location, tumor size, and histologic subtype. In case of advanced and recurrent sarcomas, induction regimens include Cyclophosphamide and Ifosphamide, Vincristine, Doxorubicin, Dactinomycin, and Etoposide[12]. For patients with unresectable or metastatic disease, the management plan is limited to a palliative approach with Trabectedin or Ifosfamide and Doxorubicin based chemotherapy[13,14].

The rationale of using targeted therapies in sarcomas goes back to 1984 when sarcomagenesis was correlated to recurrent translocations[15]. Genetic profiling thus defined two groups of sarcomas. The first group is characterized by a simple karyotype associated with specific tumor genetic alterations that include chromosomal translocations, oncogenetic mutations, and recurrent gene amplifications. The second group is characterized by a complex karyotype associated with nonspecific and nonrecurring genetic alterations[16]. Subsequent to these advances, Pazopanib, a multitargeted tyrosine kinase inhibitor against VEGFR1-3, PDFGRA-B, and KIT was approved for pretreated metastatic nonlipomatous sarcomas based on the phase Ⅲ PALETTE study[17]. Clinical and preclinical mechanistic studies are being conducted to validate a possible therapeutic role of the various targeted therapies available. Among these novel targeted therapies, we report the trials of Cediranib and Sunitinib in alveolar soft part sarcoma, Tivantinib and Cabozantinib in clear cell sarcoma, Imatinib in dermatofibrosarcoma protuberans, Cabozantinib in endometrial stromal tumors, and Everolimus in perivascular epitheloid cell tumor[18].

ADVANCES IN IMMUNO-ONCOLOGY

In fact, the previous cancer treatment approaches addressed distinctive and complementary hallmarks of carcinogenesis that included sustained proliferative signaling, evasion of growth suppressors, resistance of cell death, enabling of replicative immortality, induction of angiogenesis and activation of invasions and metastasis[19]. The well-known conventional cytotoxic drugs and targeted therapies have reached a plateau in effect that required a re-assessment of the six hallmarks of carcinogenesis. Recent conceptual progress has added two new hallmarks, namely reprogramming of energy metabolism and signaling interactions of the tumor microenvironment[20].

The later resides in the concept of the cancer-immunity cycle and is actually a turning point in the history of cancer therapy[21]. This cycle is the result of a counterbalance between immune-stimulatory and inhibitory factors. It occurs physiologically and starts with the release of cancer cell antigens and ends with the apoptosis of cancer cells *via* the activated effectors of the immune system[22]. Subsequently, cancer immunoediting may proceed with any of the three following phases[23]. The elimination phase describes an activation of the innate and adaptive immune effectors in response to cytokine secretion. The equilibrium phase occurs in the setting of a balance between tumor immune destruction and proliferation. The immunologic phase takes place when the tumor cells are capable of evading the immune system[23].

Recent advances recommend addressing only one step of the immune cycle to avoid potential unwanted

activation of autoimmunity mechanism and normal cells damage. Therefore, immunotherapy aims at initiating or maintaining the cancer-immunity cycle by acting on its rate limiting step. Consequently, ICI often address the immunostar function of the tumor microenvironment[24]. The PD-1/PD-L1 axis is a potential therapeutic target in view of the confirmed expression of PD-L1 in various sarcomas[25]. Inhibition of this axis enables the immune system to quickly adapt to cancer resistances thus allowing durable responses with ICI[26].

IMMUNOTHERAPEUTIC MODALITIES EVALUATED IN SARCOMAS

Sarcomas mainly occur either secondary to the activation of oncogenes *via* translocations and inversions, or secondary to the natural expression of germ cell peptides[27,28]. The issuing peptides generate an immune cascade directed against the aberrant cells[29]. Consequently, multiple rationales to immunotherapy including ACT, therapeutic vaccines, and ICI have been assessed in the treatment of sarcomas (Table 1).

Adoptive cell therapy in sarcomas

Adoptive cell therapy is a new therapeutic strategy based on the modulation, manipulation and selection of autologous T-cells *in vitro* to overcome the tolerance of the immune system to the tumor cells. Those T-cells may be harvested from tumor infiltrating lymphocytes (TIL) and re-transfused into the same patient after ensuring their expansion. Lymphocyte T-cells may also be harvested from peripheral blood, and those that recognize tumor antigens are selectively expanded. Alternatively, lymphocyte T-cells may be genetically engineered either by modifying a T-cell receptor for cancer antigen (transgenic TCR) or by adding a chimeric antigen receptor (CAR) that recognizes a specific cancer antigen[30,31]. Apart from T-cells, NK ACT has also been proven efficacious with several advantages over the classical T-cell ACT in the absence of MHC/HLA restriction, namely their NKG2D-dependent cytotoxicity against autologous tumor cells[32,33].

To our knowledge, the use of TIL has never been reported in the treatment of sarcomas whilst the use of NK ACT has been limited to case reports[33]. On the other hand, tumor antigens such as GD2 (93% of sarcomas) and NY-ESO-1 (80% to 100% of different subtype of sarcomas) were found to represent interesting targets for adoptive cells therapies. Moreover, other cancer testis antigens such as LAGE, MAGE-A3 and PRAME were frequently expressed in sarcomas and would be potential immunotherapeutic targets. In this setting, a phase I study evaluated the ability of adoptively transferred autologous T-cells transduced with a T-cell receptor (TCR) directed against NY-ESO-1 to mediate tumor regression in patients with metastatic synovial cell sarcoma expressing NY-ESO-1. The results showed an objective clinical response in 4 out of 6 patients[31].

Two ongoing trials are evaluating genetically engineered NY-ESO-1 T-cells for children and adults in metastatic synovial sarcoma (NCT01343043). Another phase I trial is testing the role of CAR T-cell therapy targeting the GD2 protein in children and young adults with sarcomas and rhabdomyosarcomas (NCT00743496).

Therapeutic vaccines in sarcomas

The therapeutic effects of cancer vaccines rely on the activation of dendritic cells upon the presence of an immunogenic predetermined antigen. However, most of the initial studies of vaccines in sarcomas did not determine specific antigens and used inefficaciously the entirety of the tumor cells[34,35]. Later studies used SYT-SSX, a fusion derived peptide present in 90% of synovial sarcoma, and also failed to demonstrate an objective response[36-38]. Takahashi *et al*[39] personalized the peptide vaccination patients with refractory sarcoma and administered multiple tumor antigens chosen according to preexisting peptide-specific IgG titers. The median OS was 9.6 mo with disease stabilization occurring in 30% of patients but no objective responses were seen. Another vaccination modality used *in situ* vaccination through combining preoperative gamma radiation (50 Gy) with intratumoral dendritic cells injection. The studied population was limited to high risk, localized, and resected extremity soft tissue sarcoma and resulted in 71% progression free survival at one year[40].

Major efforts in this field are being conducted namely in children with Ewing sarcomas. Recent data demonstrated a 75% OS at one year with FANG immunotherapy in adolescent patients with Ewing's sarcoma. The treatment was well tolerated with a favorable OS[41]. A seemingly interesting phase Ⅰ trial designed for the treatment of pediatric patients with relapsed high-risk Ewing sarcoma, osteogenic sarcoma, rhabdomyosarcoma, synovial sarcoma, and neuroblastoma is using a combination of Decitabine demethylating agent and a cancer vaccine composed of dendritic cells pulsed with overlapping peptides of NY-ESO-1, MAGE-A1, and MAGE-A3 (NCT01241162). Another dendritic cell vaccine is also being assessed in combination with Gemcitabine in a phase Ⅰ trial for adults and children with soft tissue and bone sarcomas (NCT01803152).

Immune checkpoint inhibitors in sarcomas

The concept of ICI relies on deactivating the suppressed activity of the immune system. ICI remove the brakes (PD-1 and CTLA4) thus enhancing the immune function of already sensitized T-cells. Effectively, PD-1 and CTLA4 inhibitors are showing interesting results with acceptable response rates in different cancers, including those considered for a long time as non-immunogenic[42]. Unlike CTLA4 inhibitors, the response to PD1 and PDL-1 inhibitors has been correlated with the expression of PD-1 and PDL-1 on tumor cells and to the mutational load of the tumors[42]. Moreover, PD-1 and PDL-1 expression seems to vary between sarcoma subtypes, a finding that may direct immunotherapy management in patients with sarcomas[43].

Table 1 Summary of the phase Ⅰ/Ⅱ trials of immunotherapies in sarcoma

Treatment modality	Ref.	Agent	Phase/Patients	Indication	RR	Survival
Adoptive cell therapy	Robbins *et al*[31], 2011	Adoptively transferred autologous T cells transduced with a T-cell receptor directed against NY-ESO-1	Ⅰ/6	Metastatic synovial cell sarcoma expressing NY-ESO-1	RR: 4/6	N/A
Vaccines	Mahvi *et al*[34], 2002	GM-CSF treated tumor cells	Ⅰ/16	Melanoma and sarcomas	RR: 1/16	N/A
	Dillman *et al*[35], 2004	Autologous tumor cell line-derived vaccines	Ⅰ, Ⅱ/23	Recurrent or metastatic sarcoma	No objective response assessed	10 patients lived more than 1 year
	Kawaguchi *et al*[36], 2005	Vaccination By SYT-SSX junction peptide	Ⅰ/6	Disseminated synovial sarcoma	RR: 0/6	N/A
	Kawaguchi *et al*[38], 2012	SYT-SSX breakpoint peptide vaccines	Ⅰ, Ⅱ/21	Metastatic synovial sarcoma	RR: 1/21 SD: 6/21	N/A
	Takahashi *et al*[39], 2013	Personalized peptide vaccination	Ⅱ/20	Refractory bone and soft tissue sarcoma	SD in all patients	Median OS: 9.6 mo
	Finkelstein *et al*[40], 2012	Combination of external beam radiotherapy with intratumoral injection of dendritic cells	Ⅰ, Ⅱ/17	Neoadjuvant treatment in high-risk soft tissue sarcoma	RR: 9/17	One-year PFS: 70.6%
	Ghisoli *et al*[41], 2015	FANG autologous immunotherapy	Ⅰ/12	Advanced and metastatic Ewing's sarcoma	RR: 1/12	One-year OS: 75%
Checkpoint inhibitors	Makki *et al*[44], 2013	Ipilimumab	Ⅱ/6	Advanced synovial sarcoma	RR: 0/6 (closed prematurely)	Median OS: 8.75 mo

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; N/A: Not available; OS: Overall survival; PFS: Progression free survival; RR: Response rate.

Unfortunately, the efficacy of ICI in sarcomas has been evaluated in only one study so far. It is a phase Ⅱ study that administered Ipilimumab (3 mg/kg intravenously every 3 wk for 3 cycles), a CTLA-4 inhibitor, to six patients with synovial sarcoma. The median OS was 8.75 mo ranging between 0.8 and 19.7 mo. The study was closed prematurely when none of the patients had an objective tumor response. All patients expressed NY-ESO-1 but its titers did not change after treatment administration[44]. PD-1 and PDL-1 inhibitors present a different mechanism of action compared to anti-CTLA4 agents and consequently may present better response rates[43]. Many ongoing phase Ⅰ trials are assessing the role of anti-PD1 agents in sarcomas as single agent or in combination with Ipilimumab and Dasatinib (NCT0 1643278).

PERSPECTIVE

The proof of the immunotherapy concept in sarcomas has been undoubtedly validated with the benefits encountered upon the use of liposomal muramyl-tripeptide-phosphatidyl-ethanolamine, an immunoactivator agent derived from BCG. However, its role remains controversial in view of the discordant results between the preliminary data and final results in both the adjuvant and metastatic setting. Even though the actual trend is moving towards immunotherapy as an essential tool in the treatment of cancer, the recent ASCO 2016 meeting was unfortunately disappointing in this regard. Five studies have been presented, of which one trial of chemotherapy (Busulphan and Melphalan), three trials of tyrosine kinase inhibitors, monotherapy (Anlotinib and Regorafenib) or in combination with chemotherapy (Gemcitabine plus Pazopanib), and one study reporting the evident detrimental impact of disease progression and altered quality of life on the long-term care and survival of patients with sarcomas. The ongoing trials including the promising results of immunotherapies are awaited. The available results reported a failure of Pembrolizumab in multiple soft tissue sarcomas (NCT02301039) and Nivolumab in metastatic uterine leiomyosarcoma (NCT0 2428192) despite the promising findings encountered with Nivolumab in retrospective experiences[45]. In fact, the biological preclinical rationale is not fully elucidated in view of the absence of any correlation between PD-L1 expression and OS[46]. Thus, the actual state of knowledge does not predict the patient profile that might benefit from immunotherapy.

CONCLUSION

The cornerstone treatment for sarcomas consists of complete surgical resection, chemotherapy, and radiotherapy. Unfortunately, these treatment options fall short from achieving an optimal clinical outcome. Immunotherapy is therefore expected to further improve the survival of patients with sarcomas. Until recently, the field of immunotherapy has not yet matured enough to present robust effects. The better understanding of onco-immunotherapy principles is essential to adjust the design of clinical trials and the selection of inclusion criteria. The published data shows that ACT is yet to be more elucidated and evaluated, vaccine therapy requires tailoring and personalization, and ICI, preferably PD-1 and PDL-1 inhibitors, necessitate better patient selection. Such results

would allow more understanding of the antitumor immunity mechanisms and improvement of the treatment arsenal against sarcomas.

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ORIGINAL ARTICLE

Retrospective Study

Bethesda System for Reporting Thyroid Cytopathology: A three-year study at a tertiary care referral center in Saudi Arabia

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Abstract

AIM

To stratify the malignancy risks in thyroid nodules in a tertiary care referral center using the Bethesda system.

METHODS

From January, 2012 to December, 2014, a retrospective analysis was performed among 1188 patients (15-90 years) who had 1433 thyroid nodules and fine-needle aspiration at Prince Sultan Military Medical City, Saudi Arabia. All thyroid cyto-pathological slides and ultra sound reports were reviewed and classified according to the Bethesda System for Reporting Thyroid Cytopathology. Age, gender, cytological features and histological types of the thyroid cancer were collected from patients' medical chart and cytopathology reports.

RESULTS

There were 124 total cases of malignancy on resection, giving an overall surgical yield malignancy of 33.6%.

Majority of the thyroid cancer nodules (*n* = 57, 46%) in Bethesda VI category followed by Bethesda IV (*n* = 25, 20.2%). Almost 40% of the cancer nodules in 31-45 age group in both sex. Papillary thyroid carcinoma (PTC) was the most common form of thyroid cancer among the study population (111, 89.6%) followed by 8.9% of follicular thyroid carcinoma (FTC), 0.8% of medullary carcinoma and 0.8% of anaplastic carcinoma. Among the Bethesda IV category 68% thyroid nodules were PTC and 32% FTC.

CONCLUSION

The malignancy values reported in our research were constant and comparable with the results of other published data with respect to the risk of malignancy. Patients with follicular neoplasm/suspicious for follicular neoplasm and suspicious of malignancy categories, total thyroidectomy is indicted because of the substantial risk of malignancy.

Key words: Bethesda; Total thyroidectomy; Thyroid nodules; Risk of malignancy; Fine needle aspiration

Core tip: The purpose of this study was to stratify the malignancy risks in thyroid nodules in a tertiary care referral center using the Bethesda system. The study found that there were 124 total cases of malignancy on resection, giving an overall surgical yield malignancy of 33.6%. Majority of the thyroid cancer nodules in Bethesda VI category followed by Bethesda IV. Almost 40% of the cancer nodules in 31-45 age group in both sex. Papillary Thyroid Carcinoma was the most common form of thyroid cancer among the study population followed by follicular thyroid carcinoma, medullary carcinoma and anaplastic carcinoma.

Al Dawish MA, Robert AA, Muna A, Eyad A, Al Ghamdi A, Al Hajeri K, Thabet MA, Braham R. Bethesda System for Reporting Thyroid Cytopathology: A three-year study at a tertiary care referral center in Saudi Arabia. *World J Clin Oncol* 2017; 8(2): 151-157 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i2/151.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i2.151

INTRODUCTION

According to epidemiological and clinical studies thyroid nodules are commonly encountered in clinical exams, palpable in 5% of the population on thyroid examination and detectable in nearly 60% of those subjected to thyroid ultrasound. While the majority of the nodules are benign (non-cancerous), they are normally the first indicators of thyroid cancer; therefore, further investigations are required to identify the cancerous nodule[1,2].

The last decades have revealed a constant and remarkable rise in the occurrence of thyroid cancer across the world, including Saudi Arabia[3-5]. The Saudi Cancer Registry (SCR) report has registered 890 thyroid cancer cases, in nearly 8.1% of all the newly diagnosed cases in 2012. However, studies revealed variations in the incidence of thyroid cancer globally. Thyroid cancer is the 5th most common cancer among females in the United States, whereas in Saudi Arabia it is the 2nd commonest identified cancer in females, and 8th among males[6]. However, compared with the developed countries, research regarding the malignancy risks in thyroid nodules is still insufficient due to lack of appropriate studies being conducted in these specified areas.

One of the most widely used diagnostic tools is fine-needle aspiration (FNA) cytology with ultrasound imaging to determine the necessity for the surgical excision of a thyroid nodule. Today, molecular genetic biomarker analyses are employed to increase the diagnostic accuracy of the FNA biopsies, and can at times drastically change clinical decision procedures as they become more commonly available and better assessed. FNA cytology (FNAC) continues to remain the initial investigation mode for malignancy in patients with thyroid nodules and the selection of patients for thyroid surgery[7]. This minimally invasive and useful method is highly effective in identifying a large percentage of thyroid nodules as benign and eliminating unnecessary surgery for patients with benign disease[8]. However, because a standardized reporting system is still unavailable, pathologists have been employing varying terminologies and diagnostic criteria, thus causing misunderstanding among the referring clinicians while interpreting cytopathology reports, resulting in non-definitive clinical management[9-11]. In 2007, the National Cancer Institute (NCI) established guidelines employing a standardized nomenclature to interpret thyroid FNAs called the Bethesda System for Reporting Thyroid Cytopathology (BSRTC) which is now accepted as the proposed diagnostic categories for thyroid cancer[12]. This study attempts to stratify the malignancy risks in thyroid nodules in a tertiary care referral center in Saudi Arabia utilizing the Bethesda system.

MATERIALS AND METHODS

Study design and setting

From January, 2012 to December, 2014 (36 mo), a retrospective analysis was performed among 1188 patients (15-90 years old) who had 1433 thyroid nodules and FNA at Prince Sultan Military Medical City (PSMMC), a 1200 bedded tertiary care center, Riyadh, Saudi Arabia. The PSMMC caters to the patients referred from different regions of Saudi Arabia and considered a worthy representative of Saudi Arabia in general. The study protocol was approved by the Research and Ethics Committee of PSMMC, Riyadh, Saudi Arabia.

Data collection

All thyroid cytopathological slides and ultra sound

Table 1 The Bethesda system

Diagnostic category	Cytological diagnosis	Risk of malignancy, %	Usual management
Ⅰ	Nondiagnostic or unsatisfactory	1-4	Repeat FNA with ultrasound guidance
Ⅱ	Benign	0-3	Clinical follow-up
Ⅲ	AUS/FLUS	5-15	Repeat FNA
Ⅳ	FNS/SFN	15-30	Surgical lobectomy
Ⅴ	Suspicious for malignancy	60-75	Near-total thyroidectomy or surgical
Ⅵ	Malignant	97-99	Near-total thyroidectomy

FNA: Fine-needle aspiration; AUS/FLUS: Atypia of undetermined significance or follicular lesion of undetermined significance; FNS/SFN: Follicular neoplasm or suspicious for follicular neoplasm.

reports were reviewed and classified according to the BSRTC system. Age, gender, cytological features and histological types of the study population were collected from patients' medical chart and cyto-pathology reports.

Bethesda system

Currently, the Bethesda system of reporting thyroid cytology (TBSRTC) is used for reporting FNAC specimens of thyroid. According to Cibas[13], this system was innovated in 2007 and consists of six categories: (1) Unsatisfactory (UNS) or nondiagnostic (ND); (2) Benign and nonneoplastic; (3) Atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS); (4) Follicular neoplasm or suspicious for follicular neoplasm (FNS/SFN); (5) Suspicious for, but not diagnostic of, malignancy; and (6) Malignant (Table 1).

All FNAs were performed by one of five interventional radiologists under ultrasound (US) guidance, performing 3-5 passes by using 25 gauge needles. On-site FNAs stained with the Diff-Quik stain and adequacy assessment was performed for all samples. All slides interpreted by among of five accredited cyto-pathologists.

Histological diagnoses

The histological diagnoses of thyroid nodules were classified into two types: Benign and nonneoplastic and malignant. For papillary thyroid carcinoma (PTC), subtype variants were documented such as the follicular variant, classical variant, conventional variant and tall cell variant. Also were follicular thyroid carcinoma (FTC) subdivided to minimally invasive follicular thyroid carcinoma (MIFTC) and Widely Invasive follicular thyroid carcinoma (WIFTC).

Statistical analysis

All statistical calculations were performed using IBM SPSS Statistics (IBM SPSS Statistics for Windows, Version 22, SPSS Inc. an IBM Company) program and Microsoft Excel 2010 (Microsoft Corporation, Seattle, WA, United States). The descriptive analysis of the epidemiological data presented as frequencies, percentages and mean ± standard deviation (SD). χ^2 test was performed to find out the variables associated with cancer among the surgical patients.

RESULTS

A total of 1188 patients (range 15-90 years) included in this study. The mean age of the study population was 46.3 ± 15.1 (SD), median 45 years, and mode 49 years. Of the 1188 (212 male; 976 female) patients, 245 patients had two thyroid nodules, which resulted in a total of 1433 FNA cases (nodules). Among the study population, a total of 311 patients underwent surgery and 877 patients did not undergo surgery. Of the 311 patients who underwent surgery, 58 patients had two thyroid nodules, which resulted in a total of 369 cases (245 benign and 124 malignant) (Figure 1). Among patients who underwent surgery, no statistically significant differences were observed on the presence of cancer among both gender (P = 0.463), and different age groups (P = 0.928).

As shown in Table 2, the distribution of all cases in the six Bethesda diagnostic categories were as follows: 46 cases (3.2%) of category Ⅰ, 1080 cases (75.3%) of category Ⅱ, 131 cases (9.1%) of category Ⅲ, 71 cases (5%) of category Ⅳ, 32 cases (2.2%) of category Ⅴ and 73 cases (5.1%) of category Ⅵ.

The distributions of follow-up diagnoses for each initial Bethesda diagnostic classification are shown in Table 3. There were 124 total cases of malignancy on resection, giving an overall surgical yield of malignancy of 33.6%. Eight of (2.2%) 369 thyroid nodules were diagnosed as ND, 181 (49.1%) diagnosed as benign, 42 (11.4%) diagnosed as AUS/FLUS, 53 (14.4%) as FNS/SFN. Category Ⅴ (SM) diagnoses (26 cases) reminded benign in 8 cases, but histologically confirmed as carcinoma in 18 case (69.2%). Finally, category Ⅵ diagnoses (59 cases) reminded benign in 2 cases, but histologically confirmed as carcinoma in 57 cases (96.7%).

Table 4 shows the comparison rates of malignancy on surgical resection for FNA diagnostic categories and malignancy risk of the present findings and previously published data. Table 5 shows the age and sex distribution of thyroid cancer. Majority of the thyroid cancer nodules (n = 57, 46%) in Bethesda Ⅵ category followed by Bethesda Ⅳ (n = 25, 20.2%) and Bethesda Ⅴ (n = 18, 14.5%). Among the Bethesda Ⅳ category 17 (68%) were PTC and 8 (32%) were follicular carcinoma. Almost 40% of the cancer nodules in 31-45 age groups in both sex.

Type and variants of thyroid cancer among histopathological diagnosis are shown in Table 6. Papillary carcinoma was the most common form of thyroid cancer among the study population (111, 89.6%). Among PTC (n = 111), four histologic variants exist, with classic variant PTC accounting for 51.4% of PTC followed by follicular-

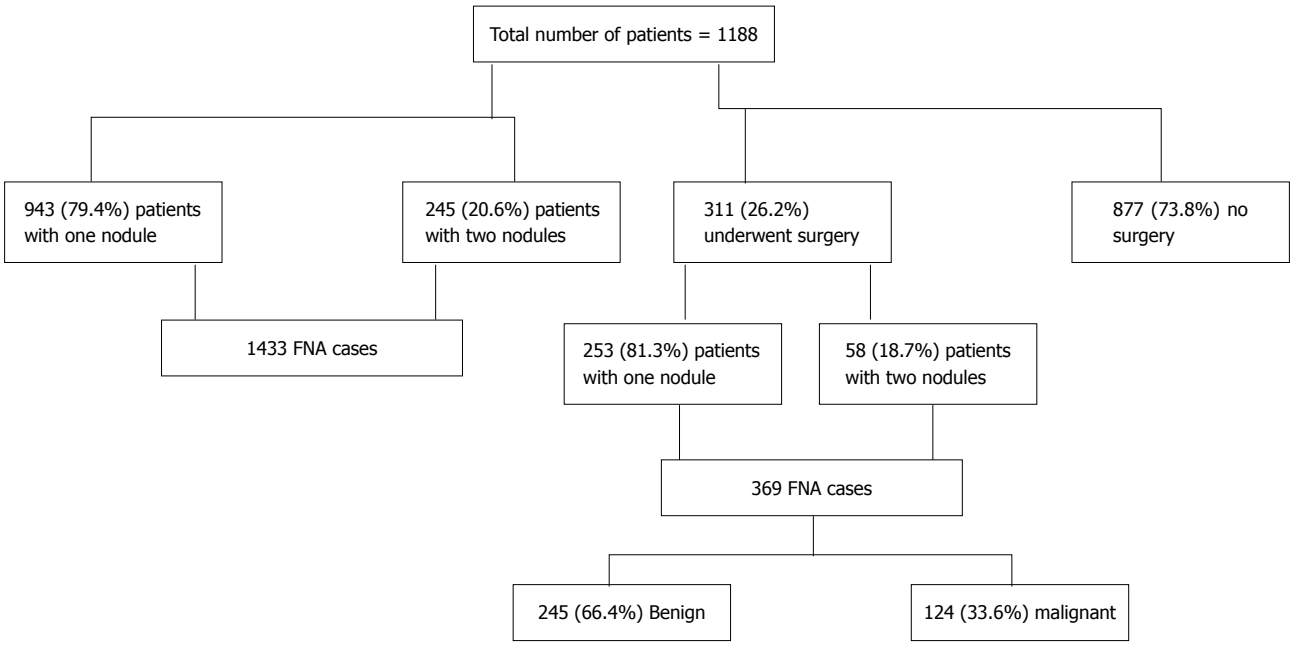

Figure 1 Flowchart of thyroid nodules description among 1188 patients and the risk of malignancy among 311 surgically excised nodules during January, 2012 to December, 2014. FNA: Fine needle aspiration.

Table 2 Age and sex distribution of thyroid lesion (based on fine-needle aspiration cytology according to Bethesda system)

Age (yr)	Total number of patients	Gender	All FNAs (n = 1433) n, %						
		F/M	Bethesda Ⅰ	Bethesda Ⅱ	Bethesda Ⅲ	Bethesda Ⅳ	Bethesda Ⅴ	Bethesda Ⅵ	Total
15-30	176 (14.8)	159/17	9 (4.5)	149 (74.9)	17 (8.5)	12 (6)	4 (2)	8 (4)	199
31-45	420 (35.4)	362/58	12 (2.4)	375 (74.7)	41 (8.2)	28 (5.6)	14 (2.8)	32 (6.4)	502
46-60	374 (31.5)	301/73	15 (3.3)	347 (75.1)	40 (8.8)	22 (4.8)	9 (2)	23 (5)	456
61-75	175 (14.7)	126/49	10 (4.5)	162 (72.3)	33 (14.7)	7 (3.1)	4 (1.8)	8 (3.6)	224
> 75	43 (3.6)	28/15	0	47 (90.4)	0	2 (3.8)	1 (1.9)	2 (3.8)	52
Total	1188	976/212	46 (3.2)	1080 (75.3)	131 (9.1)	71 (5)	32 (2.2)	73 (5.1)	1433

FNA: Fine-needle aspiration; F: Female; M: Male.

Table 3 Cyto-Histopathological correlation of thyroid lesion

Cytopathology	Histopathological diagnosis		Total
	Benign	Malignant, n (%)	
Bethesda Ⅰ	6	2 (25)	8
Bethesda Ⅱ	165	16 (8.9)	181
Bethesda Ⅲ	36	6 (14.3)	42
Bethesda Ⅳ	28	25 (47.2)	53
Bethesda Ⅴ	8	18 (69.3)	26
Bethesda Ⅵ	2	57 (96.7)	59
Total	245	124 (33.6)	369

variant PTC (30.6%). Furthermore, 8.9% of malignancies were FTC (including 0.8% of the highest risk widely invasive phenotype), 0.8% of medullary thyroid carcinoma (MTC) and 0.8% of anaplastic thyroid carcinoma (ATC). Among the Bethesda Ⅳ category 17 (68%) thyroid nodules were PTC and 8 (32%) were FTC.

DISCUSSION

Over the last few decades thyroid cancer has been on the rise considerably, globally, while mortality has steadily dropped, including in Saudi Arabia[14]. This reduction in the mortality resulting from thyroid cancer reflects the variations in the exposure to risk factors and alters the diagnosis and treatment of the disease, while the rise in the incidence is probably due to the improvement in the identification of this neoplasm[14]. However, in comparison with the developed countries, research on the incidence, prevalence and type of thyroid cancer in Saudi Arabia is still inadequate due to the lack of suitable studies being done on this specific aspect. Therefore, the objective of the current study is to stratify the risk of malignancy in the thyroid nodules based on the Bethesda system, which enhances the interpretation of the FNAC reports and enables a more accurate study and diagnosis of such thyroid nodules[13,15]. In this study, the distribution of age and gender among the patients is almost similar to those recorded in identical studies[1,2,16]. Besides, the female/male ratio reported in this study for thyroid cancer (4.7:1) concurs with the concept that thyroid cancer occurs more commonly among women. In the present study we found that the overall

Table 4 Comparison rates of malignancy (%) on surgical resection for fine-needle aspiration diagnostic categories and malignancy risk of recent studies

	Published year	Comparison of diagnostic categories					
		Ⅰ (ND)	Ⅱ (Benign)	Ⅲ (AUS/FLUS)	Ⅳ (FN/SFN)	Ⅴ (SM)	Ⅵ (malignant)
Recent studies							
Park *et al*[22]	2014	13.3	40.6	9.1	0.4	19.3	17.6
Mondal *et al*[10]	2013	1.2	87.5	1	4.2	1.4	4.7
Mufti *et al*[29]	2012	11.6	77.6	0.8	4	2.4	3.6
Wu *et al*[30]	2012	20.1	39	27.2	8.4	2.6	2.7
Bongiovanni *et al*[31]	2012	2	54.7	6.3	25.3	6.3	5.4
Present study		3.2	75.3	9.1	5	2.2	5.1
Comparison of malignancy risk							
Haugen *et al*[32] (meta-analysis)	2016	9-32	1-10	6-48	14-34	53-97	94-100
Pantola *et al*[33] 2016	2016	0	0	8.3	10	100	100
Park *et al*[22]	2014	35.3	5.6	69	50	38.7	98.9
Mondal *et al*[10]	2013	0	4.5	20	30.6	75	97.8
Mufti *et al*[29]	2012	20	3.1	50	20	80	100
Wu *et al*[30]	2012	12	8	27	33	68	100
Present study		25	8.9	14.3	47.2	69.3	96.7

ND: Nondiagnostic; AUS/FLUS: Atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN: Follicular neoplasm/suspicious for follicular neoplasm; SM: Suspicious for malignancy.

Table 5 Age and sex distribution of thyroid cancer

Age (yr)	Total number of nodules	Gender	All FNAs (n = 124) n, %					
		F/M	Bethesda Ⅰ	Bethesda Ⅱ	Bethesda Ⅲ	Bethesda Ⅳ	Bethesda Ⅴ	Bethesda Ⅵ
15-30	18 (14.5)	3/15	0	3	1	3	4	7
31-45	49 (39.5)	39/10	1	5	2	9	7	25
46-60	43 (34.7)	35/8	1	7	3	9	5	18
61-75	12 (9.7)	8/4	0	1	0	3	2	6
> 75	2 (1.6)	2/0	0	0	0	1	0	1
Total	124	87/37	2 (1.6)	16 (12.9)	6 (4.8)	25 (20.2)	18 (14.5)	57 (46)

FNA: Fine-needle aspiration; F: Female; M: Male.

Table 6 Type and variants of thyroid cancer among histopathological diagnosis

Type of cancer	Total = 124 (n, %)	BETHESDA (n, %)					
		Ⅰ	Ⅱ	Ⅲ	Ⅳ (n = 25)	Ⅴ	Ⅵ
PTC							
Classic variant	57	1	5	1	3	8	39
Follicular variant	34	1	8	2	11	6	6
Conventional	19	0	2	2	3	3	9
Tall-cell variant	1	0	0	0	0	0	1
Total PTC	111 (89.6)	2	15	5	17 (68)	17	55
FTC							
MIFTC	10	0	1	1	7	1	0
WIFTC	1	0	0	0	1	0	0
Total FTC	11 (8.9)	0	1	1	8 (32)	1	0
MTC	1 (0.8)	0	0	0	0	0	1
ATC	1 (0.8)	0	0	0	0	0	1

PTC: Papillary thyroid carcinoma; MTC: Medullary thyroid carcinoma; ATC: Anaplastic thyroid carcinoma; FTC: Follicular thyroid carcinoma; MIFTC: Minimally invasive follicular thyroid carcinoma; WIFTC: Widely invasive follicular thyroid carcinoma.

malignant rate was 33.6% which exactly matches the percentage (33.8%) of 25445 thyroid FNAs used in the meta-analysis done by Bongiovanni *et al*[17], as well as Jo *et al*[18] who reported 30.9%. However, this high malignancy rate is not unusual if it is understood that the FNAC is consistently being performed today for most patients with thyroid nodules. This has resulted in a drop in the number of unwarranted surgeries and thereby to an increase in the percentage for reported malignancies[1]. It is noteworthy that the number of FNA

cases in this study steadily rose from 2012 (n = 357) to 2014 (n = 449). From various studies it was evident that the percentage of cases that were subjected to surgery differed widely among different institutions, reporting a range from 11.8%[19] to 45.1%[20] with an average rate of 25%[17]; the current study identified 26.2% of the study population who had surgical outcome.

Each Bethesda category showed a malignancy rate ranging from 1%-10% ("benign category) to 94%-100% ("malignant" category). This comprehensive range highlights the ability of the Bethesda system to differentiate and determine the likelihood of malignancy. The results recorded in our research concurred closely with the results reported in the American Thyroid Association Management Guidelines and other studies: 25% *vs* 9%-32% ("non-diagnostic or unsatisfactory" category), 9.3% *vs* 1%-10% ("benign and non-neoplastic" category), 14.3% *vs* 6%-48% (AUS/FLUS), 69.2% *vs* 53%-97% ("suspicious for malignancy" category), and 96.7% *vs* 94%-100% ("malignant" category)[13,17]. Among Bethesda, category Ⅳ found 47.2% malignancy risk, a value higher than the meta-analysis results of 14%-34% (FNS/SFN), published recently by Bongiovanni *et al*[17]. However, many studies revealed the greatest variation in the risk of malignancy class Ⅳ, some of which are higher (malignancy rate 50%-67%) than the present values[21-23].

The current study reported PTC (89.6%) as the commonest type of thyroid cancer in the population under study. Studies also reported that overall PTC as the commonest kind of thyroid cancer represents 80% of all the thyroid malignancies and more than 90% of the differentiated thyroid cancers[13,24,25]. A spurt in the occurrence of PTC over the past decades has triggered greater interest in this disease. This is one of the fastest growing kinds of cancer recording over 20000 new cases annually. Although individuals are susceptible to papillary carcinoma irrespective of age, most patients will show the disease prior to 45 years of age[26], a fact corroborated by the current findings (42% PTC between 31-45 years of age). Unfortunately, FTC is not being diagnosed as often, although there is an increasing incidence of well-differentiated thyroid carcinomas everywhere else[27,28], concurring with the results of the current study.

There are a two limitations to this study, mainly the retrospective design and performance in a single center. As the PSMMC is a tertiary center for thyroid lesions, the data of this study may not precisely reflect the general population. More research is warranted to overcome the limitations of the study.

In conclusion, 33.6% of the cases overall among the surgically excised nodules, showed malignancy. The malignancy values reported in our research were constant and comparable with the results of other data with respect to the risk of malignancy. For the FN/SF patients and those with suspicions of malignancy, total thyroidectomy is indicated because of the substantial risk of malignancy. It is clear, that reviewing the thyroid FNAs with the Bethesda system allowed a more precise cytological diagnosis. However, the impact of Bethesda application may vary among different institutions. Clinicians are advised to be aware of the malignancy rate in the Bethesda categories in their respective institutions to improve the investigation and decision regarding patients with thyroid nodules.

COMMENTS

Background

The National Cancer Institute, United States, established guidelines employing a standardized nomenclature to interpret thyroid fine-needle aspirations (FNAs) called the Bethesda System for Reporting Thyroid Cytopathology (BSRTC) which is now accepted as the proposed diagnostic categories for thyroid cancer.

Research frontiers

Compared with the developed countries, research regarding the malignancy risks in thyroid nodules is still inadequate due to lack of appropriate studies being conducted in these specified areas in Saudi Arabia. Hence, this present study attempts to stratify the malignancy risks in thyroid nodules in a tertiary care referral center in Saudi Arabia utilizing the Bethesda system.

Innovations and breakthroughs

The study found that there were 124 total cases of malignancy on resection, giving an overall surgical yield malignancy of 33.6%. Majority of the thyroid cancer nodules in Bethesda Ⅵ category followed by Bethesda Ⅳ. Almost 40% of the cancer nodules in 31-45 age group in both sex. Papillary thyroid carcinoma was the most common form of thyroid cancer among the study population followed by follicular thyroid carcinoma, medullary carcinoma and anaplastic carcinoma.

Applications

Reviewing the thyroid FNAs with the Bethesda system allowed a more precise cytological diagnosis. However, the impact of Bethesda application may vary among different institutions. Clinicians are advised to be aware of the malignancy rate in the Bethesda categories in their respective institutions to improve the investigation and decision regarding patients with thyroid nodules.

Terminology

PTC: Papillary thyroid carcinoma; FTC: Follicular thyroid carcinoma; SCR: Saudi Cancer Registry; FNA: Fine-needle aspiration; FNAC: Fine-needle aspiration cytology; NCI: National Cancer Institute, United States; BSRTC: Bethesda System for Reporting Thyroid Cytopathology; PSMMC: Prince Sultan Military Medical City; TBSRTC: The Bethesda system of reporting thyroid cytology; UNS: Unsatisfactory; ND: Nondiagnostic; AUS/FLUS: Atypia of undetermined significance or follicular lesion of undetermined significance; US: Ultrasound; MIFTC: Minimally invasive follicular thyroid carcinoma; WIFTC: Widely Invasive follicular thyroid carcinoma; ATC: Anaplastic thyroid carcinoma.

Peer-review

The study shows a very exhaustive analysis of the throughput of thyroid cytopathology over a three-year period. The manuscript contains a detailed exposition of the results, including comprehensive tables and a comparison to other recent studies. In my opinion, this manuscript fulfills all the requirements to be published.

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ORIGINAL ARTICLE

Clinical Trials Study

Study of recombinant human interleukin-12 for treatment of complications after radiotherapy for tumor patients

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Author contributions: Wang YS and Zhao Y contributed to the conception of the study; Guo N and Wang WQ contributed significantly to analysis and manuscript preparation; Guo N, Gao L and Shen HY performed the data analyses and wrote the manuscript; Wang WQ and Gong XJ helped perform the analysis with constructive discussions; Yang LR and Yu WN collected specimens and detected indexes; all the authors contributed to this article.

Institutional review board statement: This study was reviewed and approved by the scientific ethical committee of the Hospital. All operations were performed according to international guidelines concerning the care and treatment of cancer patients.

Informed consent statement: Patients were informed of the purpose of the experiment and agreed to treatment with rhuIL-12. Informed consent was obtained in all cases, and protocols were approved by the scientific ethical committee of the Hospital.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Abstract

AIM

To evaluate the treatment effects of recombinant human interleukin-12 (rhIL-12) on radiotherapy complications, such as severe myelosuppression or pancytopenia, the decline or imbalance of immune function, *etc*.

METHODS

The patients received high-dose and short-course precise radiotherapy, such as Cyber knife and image-guided radiotherapy (IGRT), which can cause myelosuppression or pancytopenia and immune function decline within a short time. One-hundred subjects were enrolled in the study, and 50 were randomized to a treatment group which used rhIL-12 and 50 were randomized to a control group which used symptomatic and supportive therapy after radiotherapy. The 50 subjects in the treatment group were further divided into five subgroups and intervened

with rhIL-12 at a dose of 50, 100, 150, 200 or 250 ng/kg respectively. The dose-effect relationship was observed.

RESULTS

RhIL-12 significantly attenuated the decrease of peripheral blood cells in the treatment group, and immune function was improved after treatment. Due to the different radiation doses, there was a fluctuation within 12 h after treatment but mostly showing an increasing trend. As to the clinical manifestations, 2 patients in the 250 ng/kg subgroup showed low fever after administration, 1 patient in the 200 ng/kg subgroup and 2 patients in the 250 ng/kg subgroup showed mild impairment of liver function during the observation period.

CONCLUSION

RhIL-12 has effective therapeutic and protective effects on complications following radiotherapy, such as the decline of blood cells, myelosuppression and the decline or imbalance of immune function, which indicated good prospects for development and application.

Key words: Recombinant human interleukin-12; Cancer prevention; Radiotherapy complications; Clinical research

Core tip: Recombinant human interleukin-12 (rhIL-12) is a new kind of biological agent secreted by Chinese hamster ovary cells. Study has shown that it has the advantage of promoting recovery of hematopoietic function, regulating the body's immunity and inhibiting angiogenesis growth. At present, the research of rhIL-12 stays in the foundational realm and in animal experimentation. In our study, however, there were 100 patients with large or numerous tumors (more than two) and who received precision radiotherapy (Cyber knife or image-guided radiotherapy). The results showed that rhIL-12 can prevent radiation damage, improve hematopoietic function, regulate immunity, reduce the side effect of radiotherapy and improve the quality of life of patients.

Guo N, Wang WQ, Gong XJ, Gao L, Yang LR, Yu WN, Shen HY, Wan LQ, Jia XF, Wang YS, Zhao Y. Study of recombinant human interleukin-12 for treatment of complications after radiotherapy for tumor patients. *World J Clin Oncol* 2017; 8(2): 158-167 Available from: URL: http://www.wjgnet.com/2218-4333/full/v8/i2/158.htm DOI: http://dx.doi.org/10.5306/wjco.v8.i2.158

INTRODUCTION

Interleukin-12 (IL-12) is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells, and its target point lies in early undifferentiated pluripotent hematopoietic stem cells. The studies of IL-2 trace back to early in 1986. Subsequently, many studies have confirmed that IL-12 can contribute to enhancing immunity. For example, Zhang *et al*[1] found a cytokine which can promote secretion of cytotoxic T cells (CTLs) and lymphatic factor-activated killer cells (LAK) in synergy with IL-2. In 1989, Bellone and Trinchieri[2] found a cytokine called natural killer cell-stimulating factor (NKSF), which can stimulate the production of IFN-γ. Eventually, it became known that the two cytokines were the same substance, now known as IL-12.

Based on subsequent research studies, IL-12 seems to serve as an immunoregulatory anti-cancer agent for oncology patients. However, the adverse events related to IL-12, including fever, chills, decreased peripheral blood cells and organ dysfunction, have limited the clinical application of IL-12[3]. Recombinant human interleukin-12 (rhIL-12) is an immunoregulatory protein produced by gene engineering technology. RhIL-12 has similar biological activity to IL-12. With the advantage of high purity (> 98%), high activity and low therapeutic dose, rhIL-12 became the only agent which could not only restore hematopoietic function but also improve immune function[4].

The basic experimental studies have found that the recovery and reconstitution of hematopoiesis system after radiotherapy is helpful to avoid the rapid increase of single blood cells, which lead to high fever, conjunctival hemorrhage, abnormal immune response, embolism and other detrimental side effects[5]. But a large number of studies are only based on animal experiments. The aim of our study, then, was to explore the interventional effects of rhIL-12 in tumor patients receiving radiotherapy, including the complications after radiotherapy, the curative effects on hematopoietic function and immune function as well as dose-effect relationship, and to provide scientific basis for clinical application.

MATERIALS AND METHODS

Objectives

To observe 100 patients with mid-advanced tumors who were treated with Cyber knife or image-guided radiotherapy (IGRT) in the People's Liberation Army 107th Hospital, from October 2014 to June 2016. Inclusion criteria were: (1) Tumor confirmed by pathology, CT or MRI diagnosis, for which the clinical staging criteria were Ⅲ-Ⅳ according to the World Health Organization (WHO); (2) ECOG score of 1 to 4 points; (3) Postoperative recurrence or lymphocytes invasion and metastasis, and need for radiosurgery; and (4) Provision of written informed consent for research and therapy. Exclusion criteria were: (1) Illness combined with tuberculosis or serious failure of important organs, such as heart, liver, kidney, lung, *etc*.; (2) Presence of benign tumors; (3) History of organ transplantation or allergies; and (4) Pregnant or lactating women or women of childbearing age. The experimental study was approved by the hospital's ethics committee.

In the treatment group, 34 of the subjects (68%) were

males and 16 (32%) were females; the mean age was 58.5-year-old (range: 27-83 years). Fifty subjects (30%) had lung cancers, 12 (24%) had liver cancers, 8 (16%) had head tumors, 1 (2%) had pancreatic cancer and 14 (28%) had other tumors. Solid relapse and metastasis tumor, for which size of the nidus could be assessed, accounted for 98% (n = 49) of the cases. Diffuse invasive metastatic tumor accounted for 2% (n = 1) of the cases, and the tumor diameter ranged from 3 cm to 16 cm. The patients who were classified as recurrent after the surgery or with more than 2 lesions accounted for 96% (n = 48) of the cases. In the control group, 28 of the subjects (56%) were males and 22 (44%) were females; the mean age was 57.4-year-old (range: 25-79 years). Twenty subjects (40%) had lung cancers, 15 (30%) had liver cancers and 15 (30%) had pancreatic cancers.

Main equipment, drugs and reagents

Equipment: Cyber knife, third-generation model produced by a United States' accuracy company; IGRT, Eiekta Synergy model produced by a Swedish medical company; Flow cytometer produced by the United States' BD Biosciences; Enzyme-mark instrument produced by a United States' automation company; Chemiluminescence apparatus and automatic biochemistry analyzer produced by Roche Company; Hematology analyzer produced in Japan.

Drugs and reagents: RhIL-12 (for injection) produced by Qingdao Litai Kang Pharmaceutical Co. Ltd. Antibodies used in the study were purchased from BD Biosciences, including anti-human-IgG-FITC, anti-human-CD45-FITC, anti-human-CD56-PE and anti-human-CD3-PerCP-CD4-FITC-CD8-PE. Hemolysin was produced by an American research and development company.

Methods: After being admitted to hospital, all patients' data were recorded for the three routine(liver and kidney function, heart function, bleeding and clotting time) and the imaging examination (such as electrolytes, color Doppler, CT and IMT), as well as adverse reactions, *etc*. All patients signed "consent form of precise radiotherapy", "consent form of experimental study" and "agreement about the clinical application of rhIL-12 for prevention and treatment of malignant tumor radiotherapy complications".

According to the research program, the patients were divided into 50, 100, 150, 200 and 250 ng/kg different-dose subgroups, and injected with rhIL-12 subcutaneously. Peripheral blood samples (for red blood cell (RBC), white blood cell (WBC) and platelet (PLT) assessment) and the immunophenotypes (CD4/8, CD45 and CD56) were collected before dosing (0 d) and at 12 h, 3 d, 7 d, 10 d, 14 d, 21 d, 28 d after dosing respectively. The effects on hematopoietic function and immune function were observed, as well as the dose-effect relationship. The control group used symptomatic supportive treatment.

Efficacy evaluation criteria

Test evaluation: The patients who accepted high-dose and short-course of accurate radiotherapy, such as cyber knife and IGRT, could experience induction of decrease of peripheral blood cells and decline or imbalance of immune function. In this study, rhIL-12 was given to explore the interventional effects on radiation oncology surgery patients, including effects on hematopoietic function and immune function as well as dose-effect relationship and to provide scientific basis for drug development and clinical application.

WHO objective evaluation criteria: Complete remission (CR) indicated all symptoms and signs disappearing for 4 wk; partial remission (PR) indicated the tumor size was estimated to have reduced by more than 50% for at least 4 wk. No change (NC) indicated the patient's condition had no obvious change for at least 4 wk, the tumor size has increased less than 25% and decreased less than 50%. PD (worsen) indicated new lesions having appeared or lesions had increased by more than 25%.

Zubrod-ECOG-WHO score: 0 score stood for normal activities; 1 score stood for mild symptoms and almost normal activities; 2 score stood for the time staying in bed as less than 50% of the daytime; 3 score stood for the time staying in bed as more than 50% of the daytime; 4 score stood for completely bedridden; 5 score stood for death. Total efficiency = (CR + PR)/total number of cases × 100%.

Statistical analysis

SPSS16.0 software was used for statistical analysis. Continuous variables are expressed as a mean and standard deviation; the mean differences between the groups were compared by independent t-test and ANOVA, and χ^2 test was used to compare classified variables. Two values of data used two distribution tests. $P < 0.05$ indicated that the difference was statistically significant. Charts and tables were made by Prism GraphPad 4 software.

RESULTS

Analysis of research subjects' number

All of the 100 patients completed the study.

Results of whole blood test

Treatment group: There was a transient decline of WBC and PLT within 12 h after treatment and by 3 d the lowest level was reached; the recovery rate decreased after 7 d, and the trend became stable after 21 d until the end of observation. This trend was relatively significant for WBC.

Control group: The whole blood cells declined on 3 d after radiotherapy, decreased significantly after 7 d, and reached the lowest point on 14 d. The degree of decrease was related to the radiation dose and tumor size. The difference between the treatment group and the control

Table 1 Results of whole blood test for the treatment group and the control group (mean ± SD, *n* = 10)

Group	Indicator	0 d	12 h	3 d	7 d	14 d	21 d	28 d
Control	WBC (× 10^9/L)	7.66 ± 0.82	5.5 ± 0.67	4.2 ± 0.39	4.3 ± 0.48	4.21 ± 0.62	4.69 ± 0.38	4.89 ± 0.63
	RBC (× 10^{12}/L)	3.92 ± 0.31	4.26 ± 0.43	3.9 ± 0.41	3.93 ± 0.22	4.38 ± 0.36	3.86 ± 0.34	3.89 ± 0.28
	PLT (× 10^9/L)	358 ± 0.43	339 ± 31.45	232 ± 20.43	258 ± 19.2	275 ± 0.31	296 ± 0.29	321 ± 0.26
50 ng/kg	WBC (× 10^9/L)	6.31 ± 0.59	3.6 ± 0.35	4.27 ± 0.46	4.85 ± 0.35	4.52 ± 0.42	4.72 ± 0.39	5.18 ± 0.52
	RBC (× 10^{12}/L)	5.43 ± 0.54	4.92 ± 0.53	4.16 ± 0.38	4.59 ± 0.82	4.73 ± 0.32	5.26 ± 0.37	5.63 ± 0.41
	PLT (× 10^9/L)	231 ± 20.81	185 ± 18.24	195 ± 18.97	205 ± 18	226 ± 18.2	246 ± 17.5	229 ± 19.4
100 ng/kg	WBC	8.4 ± 0.45	7.7 ± 7.89	5.3 ± 10.64	6.6 ± 0.98	6.1 ± 0.64	5.73 ± 0.47	5.9 ± 0.21
	RBC (× 10^{12}/L)	6.3 ± 0.72	3.37 ± 0.43	3.2 ± 0.34	4.2 ± 0.37	4.78 ± 0.46	4.5 ± 0.42	4.6 ± 0.34
	PLT (× 10^9/L)	231 ± 27.2	185 ± 10.7	178 ± 12.6	195 ± 12.8	166 ± 10.9	182 ± 12.5	205 ± 15.3
150 ng/kg	WBC (× 10^9/L)	6.6 ± 0.73	5.4 ± 0.76	3.8 ± 0.35	5.2 ± 0.37	5.7 ± 0.42	6.2 ± 0.54	6.3 ± 0.54
	RBC (× 10^{12}/L)	3.4 ± 0.36	3.5 ± 0.37	3.2 ± 0.29	3.3 ± 0.28	3.6 ± 0.24	3.6 ± 0.26	3.44 ± 0.21
	PLT (× 10^9/L)	367 ± 35.75	352 ± 32.45	306 ± 30.12	316 ± 16	357 ± 17	348 ± 26	317 ± 16
200 ng/kg	WBC (× 10^9/L)	5.3 ± 0.8	4.2 ± 0.3	3.2 ± 0.1	3.5 ± 0.32	3.6 ± 0.27	4.3 ± 0.31	5.4 ± 0.43
	RBC (× 10^{12}/L)	4.6 ± 0.51	4.2 ± 0.41	4.1 ± 0.45	4.2 ± 0.3	4 ± 0.2	4.12 ± 0.34	4.5 ± 0.42
	PLT (× 10^9/L)	278 ± 36	183 ± 19	149 ± 14	208 ± 22	259 ± 24	267 ± 25	271 ± 21
250 ng/kg	WBC (× 10^9/L)	3.6 ±	3 ± 0.37	2.7 ± 0.24	3.4 ± 0.4	4.2 ± 0.3	4.5 ± 0.4	4.2 ± 0.4
	RBC (× 10^{12}/L)	3.6 ± 0.3	3.3 ± 0.2	3.1 ± 0.2	3.2 ± 0.3	3.4 ± 0.3	3.7 ± 0.2	4.1 ± 0.3
	PLT (× 10^9/L)	364 ± 35	235 ± 21	240 ± 20	276 ± 22	314 ± 19	342 ± 21	312 ± 20

WBC: White blood cell; RBC: Red blood cell; PLT: Platelet.

Table 2 Results of immunologic detection for the treatment group and the control group (mean ± SD, *n* = 10)

Group	Indicator	0 d	12 h	3 d	7 d	14 d	21 d	28 d
Control	CD4/8	20.4 ± 2.6	18 ± 1.2	17.5 ± 1.2	17.8 ± 1.3	18.4 ± 1.2	17.3 ± 1.4	16.5 ± 0.4
	CD45	75.2 ± 7.5	68.1 ± 5.2	65.4 ± 4.2	83.3 ± 5.2	79.3 ± 3.7	62.4 ± 5.1	60.3 ± 3.6
	CD56	12.3 ± 1.2	8.7 ± 0.6	7.6 ± 0.5	11.4 ± 1.3	10.4 ± 0.9	8.2 ± 0.6	6.7 ± 0.5
50 ng/kg	CD4/8	28.2 ± 1.8	22.4 ± 1.6	19.8 ± 1.6	19.6 ± 1.6	21.4 ± 1.8	18.8 ± 1.2	16.9 ± 1.4
	CD45	81.9 ± 2.4	78.8 ± 5.1	75.2 ± 3.6	82.9 ± 3.8	79.8 ± 5.6	62.4 ± 4.6	51.8 ± 4.3
	CD56	27.9 ± 2.2	21.9 ± 1.8	19.2 ± 1.2	22.1 ± 1.8	19.7 ± 2.1	21.6 ± 1.3	22.4 ± 0.9
100 ng/kg	CD4/8	15.4 ± 1.5	12.7 ± 1.2	11.5 ± 1.1	10.6 ± 1.2	10.1 ± 0.3	9.2 ± 0.4	8.7 ± 0.2
	CD45	84.3 ± 2.6	66.1 ± 3.8	68.7 ± 3.5	67.9 ± 4.4	57.6 ± 4.6	63.1 ± 3.6	70.2 ± 3.1
	CD56	14.8 ± 1.8	8.9 ± 0.6	10.8 ± 0.9	12.3 ± 1.5	10.4 ± 4.6	12.9 ± 0.3	11.6 ± 0.4
150 ng/kg	CD4/8	16.5 ± 1.2	12.8 ± 1.2	11.6 ± 0.9	13.1 ± 4.6	12.6 ± 1.5	10.8 ± 0.9	7.6 ± 0.5
	CD45	74.2 ± 3.6	63.2 ± 3.2	66.1 ± 4.8	82.7 ± 5.2	68.1 ± 4.6	75.2 ± 4.2	65.1 ± 4.1
	CD56	6.2 ± 0.3	6.5 ± 0.3	5.9 ± 0.3	7.9 ± 0.6	8.2 ± 7.4	8.8 ± 0.9	6.5 ± 0.1
200 ng/kg	CD4/8	9.6 ± 0.4	8.3 ± 0.61	7.6 ± 0.6	7.2 ± 0.9	8.6 ± 4.6	8.2 ± 1.2	6.1 ± 0.2
	CD45	64.5 ± 4.2	60.7 ± 4.2	65.8 ± 4.4	76.6 ± 5.9	74.3 ± 4.6	73.2 ± 5.2	55.9 ± 5.0
	CD56	3.8 ± 0.2	2.8 ± 0.2	4.4 ± 0.4	4.9 ± 0.9	6.2 ± 0.6	5.9 ± 0.3	4.5 ± 0.1
250 ng/kg	CD4/8	6.4 ± 0.2	5.7 ± 0.2	4.2 ± 0.3	4.7 ± 0.3	3.5 ± 0.3	3.4 ± 0.9	3.1 ± 0.9
	CD45	46.5 ± 3.9	40.2 ± 3.2	57.9 ± 4.1	66.5 ± 3.8	54.9 ± 4.2	50.4 ± 4.9	35.9 ± 3.2
	CD56	4.3 ± 0.2	3.9 ± 0.2	5.4 ± 0.4	6.3 ± 0.5	6.6 ± 0.3	7.2 ± 0.6	6.4 ± 0.8

group was statistically significant (Table 1 and Figure 1).

Results of immunologic detection

The aim was to observe the immune indexes, including CD4/8, CD45 and CD56.

Treatment group: There was a transient decline of CD4/CD8 within 12 h in the 150, 200 and 250 ng/kg subgroups. There was volatility rise between 3-14 d but the level remained below the pre-medication level, and went down after 21 d. There was a transient decline of CD45 and CD56 within 12 h, which rose after 3 d and went down after 21 d. The overall recovery improvement trend was obvious.

Control group: The trend of the immune indexes showed rebound on 3 d and a continuous downward trend after 7 d. There was no significant difference in these immune indexes between the other two groups (50 and 100 ng/kg subgroups) and the control group (Table 2 and Figure 2).

Objective evaluation results

The remission rate in the treatment group (84%) was obviously higher than that in the control group (60%), and the difference was statistically significant ($P < 0.05$). During the observation period, there were no recurrence, metastasis or death, and the survival time of patients was significantly prolonged. There were 2 patients in the 250 ng/kg subgroup that had low fever after administration, 1 in the 200 ng/kg subgroup and 2 in the 250 ng/kg subgroup that had mild impairment of liver function during the observation period. There was no other adverse event

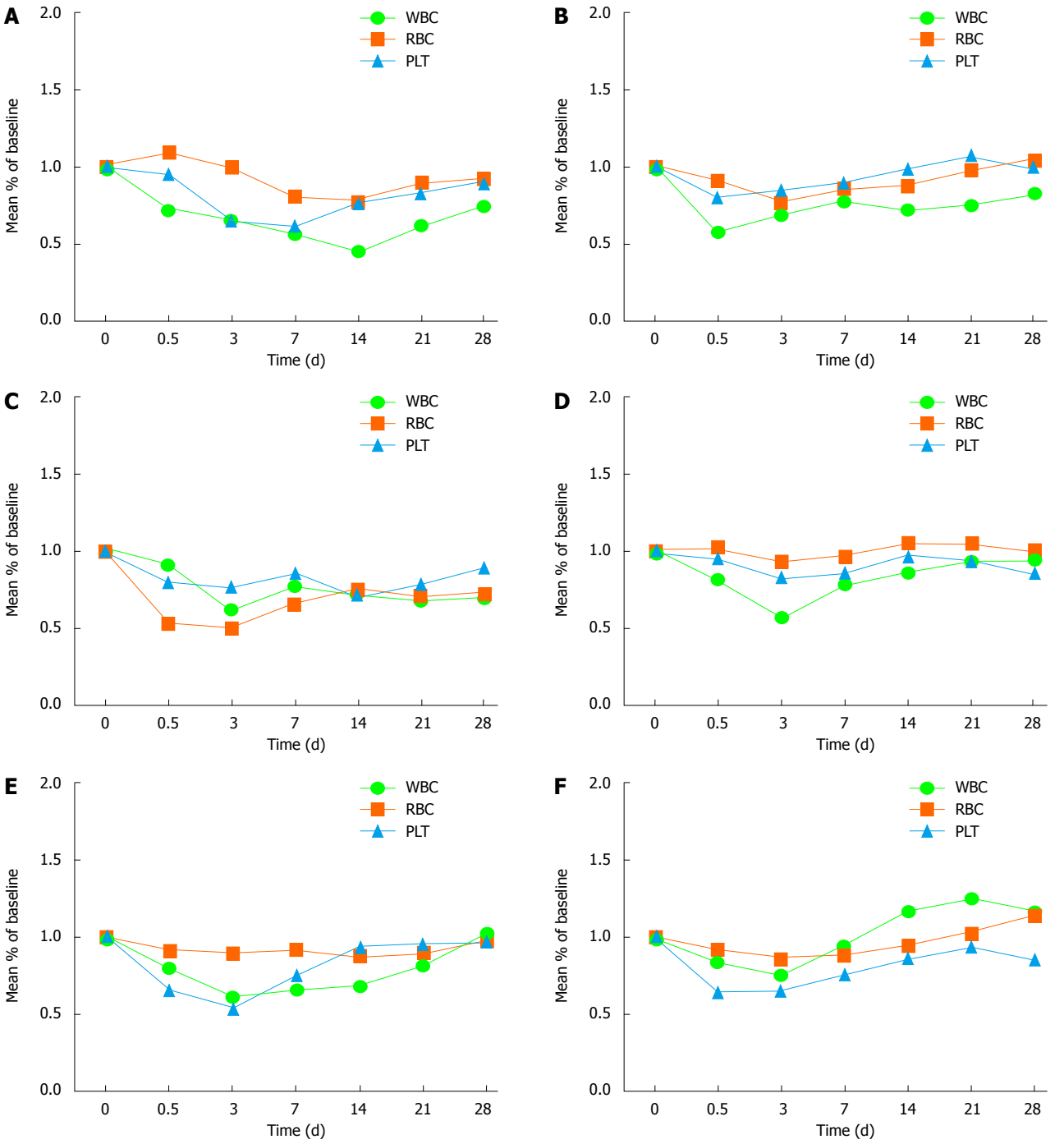

Figure 1 Count changes of the whole blood test. A: Control group; B: 50 ng/kg subgroup; C: 100 ng/kg subgroup; D: 150 ng/kg subgroup; E: 200 ng/kg subgroup; F: 250 ng/kg subgroup. WBC: White blood cell; RBC: Red blood cell; PLT: Platelet.

found (Table 3).

ECOG score results

The aim was to observe the ECOG score for a period of a month after rhIL-12 intervention.

Treatment group: There were 26 patients (52%) with normal activities after treatment, and the difference was statistically significant as compared with the 10 cases (20%) before treatment ($P < 0.05$). The life quality of patients was significantly improved.

Control group: There were 20 patients (40%) with normal activities after treatment, and the difference was not statistically significant as compared with the 12 cases (24%) before treatment ($P > 0.05$) (Table 4).

Imaging evaluation results

There were two CT pictures, including 1 case of pancreatic cancer and 1 case of lung cancer in the treatment group, before and after treatment. The results showed that the original lesion was significantly reduced after treatment and no new lesions appeared (Figures 3 and 4).

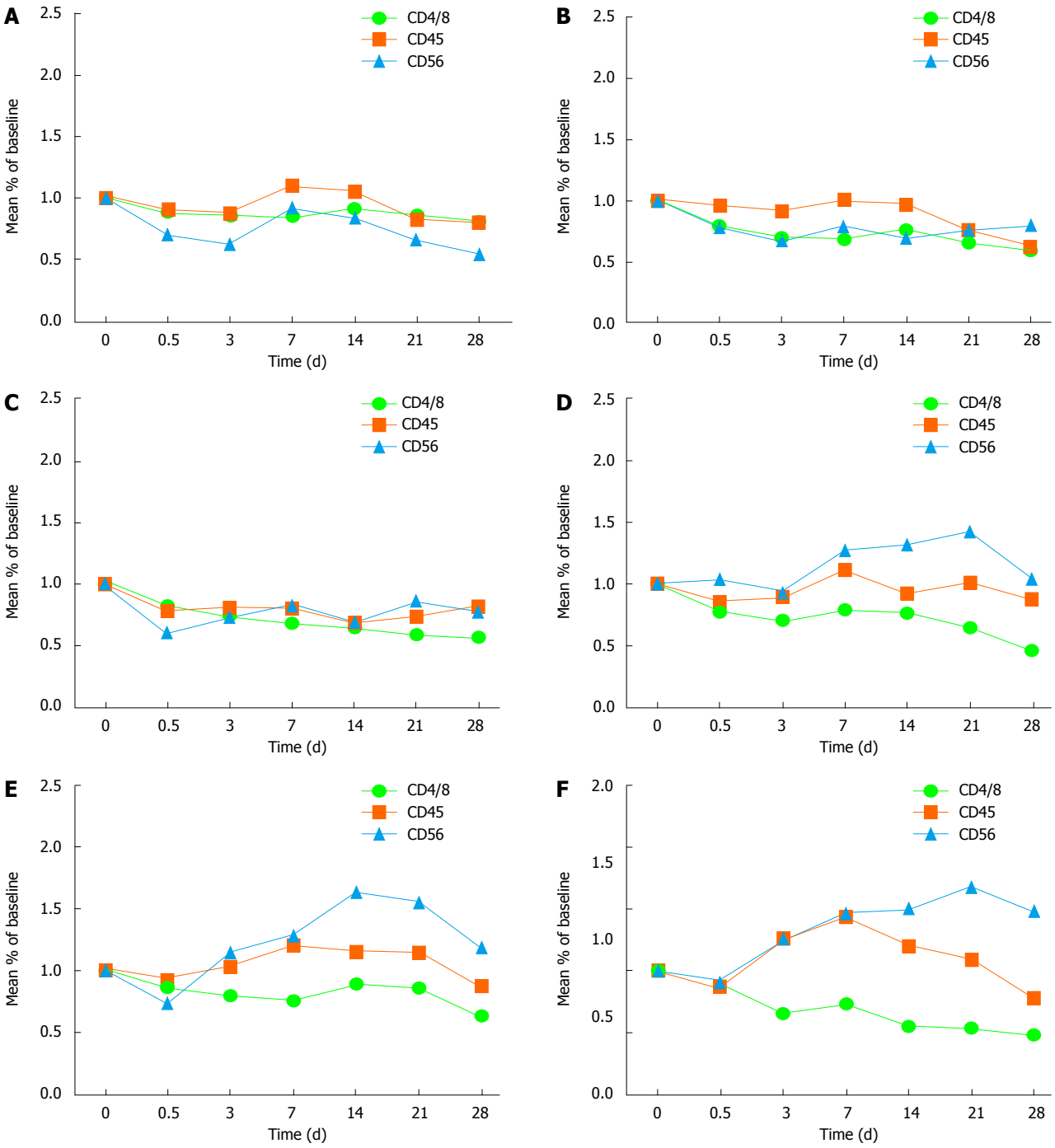

Figure 2 Changes of immune indexes. A: Control group; B: 50 ng/kg subgroup; C: 100 ng/kg subgroup; D: 150 ng/kg subgroup; E: 200 ng/kg subgroup; F: 250 ng/kg subgroup.

DISCUSSION

The incidence of cancer is rising, and cancer has become one of the main causes of death[6]. In recent years, radiotherapy of malignant tumors has developed rapidly, especially for accurate radiotherapy. However, the clinical curative effect for patients who have larger or more numerous tumor lesions are often reduced due to adverse reactions after radiotherapy, such as immune injury and myelosuppression. Under normal circumstances, the immune systems maintain the physiological balance and stabilization of the body.

Immune cells are the first line of the anti-tumor system. Immune regulatory factors or cytokines participate in immune regulation by means of signal transduction[7]. Studies have shown that tumor cells can escape immunosurveillance through a number of special mechanisms. The immune system is critical to the body's surveillance against cancer.

The immune function of about 86% of patients has been shown to be on the decline in the early stage of cancer, and to further decline after treatment, which is

Table 3 Results of objective evaluation for the treatment group and the control group

Group	*n*	CR	%	PR	%	MR	%	PD	%
Treatment	50	12	24[a]	30	60	5[a]	10[a]	3[a]	6[a]
Control	10	5	10	25	50	10	20	10	20

Compared with the control group, [a]$P < 0.05$. CR: Complete remission; PR: Partial remission.

Table 4 Comparison of ECOG scores before and 1 mo after intervention for the treatment and control groups

ECOG scores before treatment	after treatment	
Treatment group (n = 50)		
0	0	11[a]
1	10	15[a]
2	15	19[a]
3	20	5[a]
4	5	0[a]
Control group (n = 50)		
0	1	4
1	11	16
2	17	20
3	15	10
4	6	0

There was significant difference between the two groups, [a]$P < 0.05$.

the main cause of tumor metastasis and recurrence[8]. What's more, the complications including infection and bleeding that are caused by the decrease of peripheral blood cells counts are also common causes of death in patients with cancer.

Therefore, improving immune function and reducing myelosuppression are indispensable auxiliary treatments in the process of tumor radiotherapy. At present, the treatment of cancer has entered into the era of personalized multidisciplinary treatment. The research shows that the combination of radiotherapy and immunotherapy has a unique advantage[9]. In this respect, IL-12 has received more and more attention.

IL-12 is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells. IL-12 mainly functions to mediate cellular immunity, and it can induce the differentiation of T helper cell 1 (Th1), as well as promote the proliferation of NK cells and T cells, further stimulate IFN-γ secretion, and enhance the ability to kill target cells. IL-12 also can promote the formation of interferon-inducible protein-10 (IP-10). IP-10 can prevent the formation of tumor blood vessels, thereby reducing and blocking the nutrition source of tumor cells and inhibiting their growth[10,11].

It has been nearly 30 years since IL-12 was discovered, and a large amount of the related research is still at the stage of basic study and animal study. Many studies have found its abilities to improve immunity capability, adjust the immune function and inhibit the production of tumor blood vessels, but the adverse reactions such as chills and fever, nausea and vomiting, pulmonary edema and allergic reactions limit its clinical application and temper its promotion.

RhIL-12 is a new kind of biological agent secreted by Chinese hamster ovary cells through gene engineering. Its biological activity is similar to that of IL-12. The advantage of rhIL-12, however, is its high purity (> 98%), high activity (≥ 10000 IU/μg), low therapeutic dose and dosage that can be tolerated. Preliminary experimental study[12-14] found that rhIL-12 is currently the only biological agent with the advantage of comprehensive recovery of hematopoietic function, regulation of the body's immunity, inhibition of tumor angiogenesis and inhibition of tumor growth, thereby improving the life quality of patients with cancer[15]. As an immune regulatory factor, it plays an important role in both primary and secondary immunity[16]. Especially for radiotherapy patients who present with larger or more numerous tumor targets (more than two), it has important research value.

In our study, there were 100 patients who had larger or more numerous tumor s (more than two) and who received precision radiotherapy (Cyber knife or IGRT). The following conclusions are drawn. In the treatment group, the whole blood cells showed a transient decline within 12 h after treatment. The reason for this may be that the cell changes into the microcirculation or the bone marrow microenvironment, which may affect the proliferation of hematopoietic stem cells. The whole blood cells reached the lowest level at 3 d. People have always stopped treatment at this time, which represents a misunderstanding of the early research. The recovery rate decreased after 7 d, and the trend became stable after 21 d until the end of observation. This trend is relatively significant for WBC. Compared with the control group, the difference was significant, which indicated that the rhIL-12 was effective.

Observation of the immune indexes, including CD4/8, CD45 and CD56, showed a transient decline of CD4/CD8 within 12 h in the 150, 200 and 250 ng/kg subgroups in the treatment group, with volatile rises between 3-14 d, but remaining below the pre-medication level, and then decreasing after 21 d. There was a transient decline of CD45 and CD56 within 12 h, which rose up after 3 d and went down after 21 d. Compared with the control group, the difference was significant. The improvement tendency was obvious, which suggested that rhIL-12 could promote the immune function of the patients after radiotherapy.

From our observations of the clinical manifestations, 2 patients in the 250 ng/kg subgroup showed low fever after administration, which could be returned to normal after physical cooling. One patient in the 200 ng/kg subgroup and 2 in the 250 ng/kg subgroup showed mild injury of

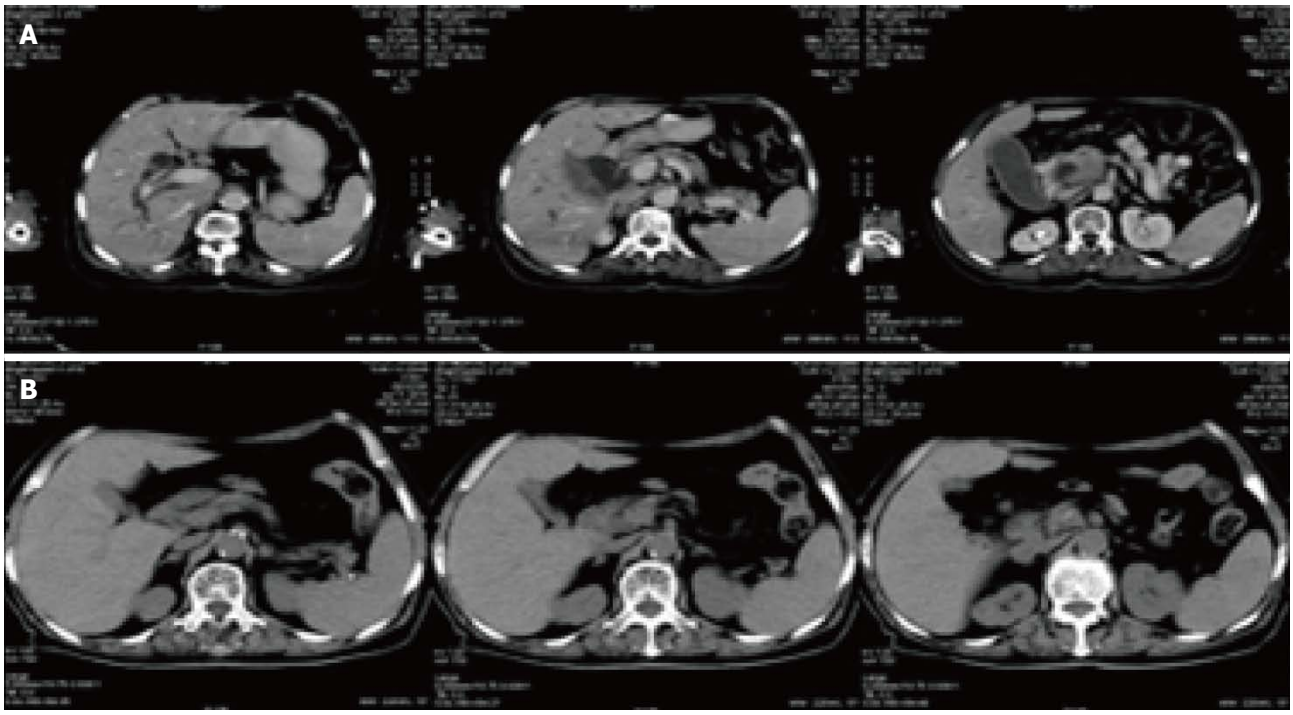

Figure 3 Changes of computed tomography slice before and after treatment in pancreatic cancer patients. A: Pancreatic head tumor mass (4.4 cm × 3.6 cm × 5.3 cm) accompanied by dilation of intrahepatic and extrahepatic bile duct, pancreatic duct and gallbladder before treatment; B: Most of the pancreatic head mass disappeared in 2 mo after B treatment. Low obstruction disappeared.

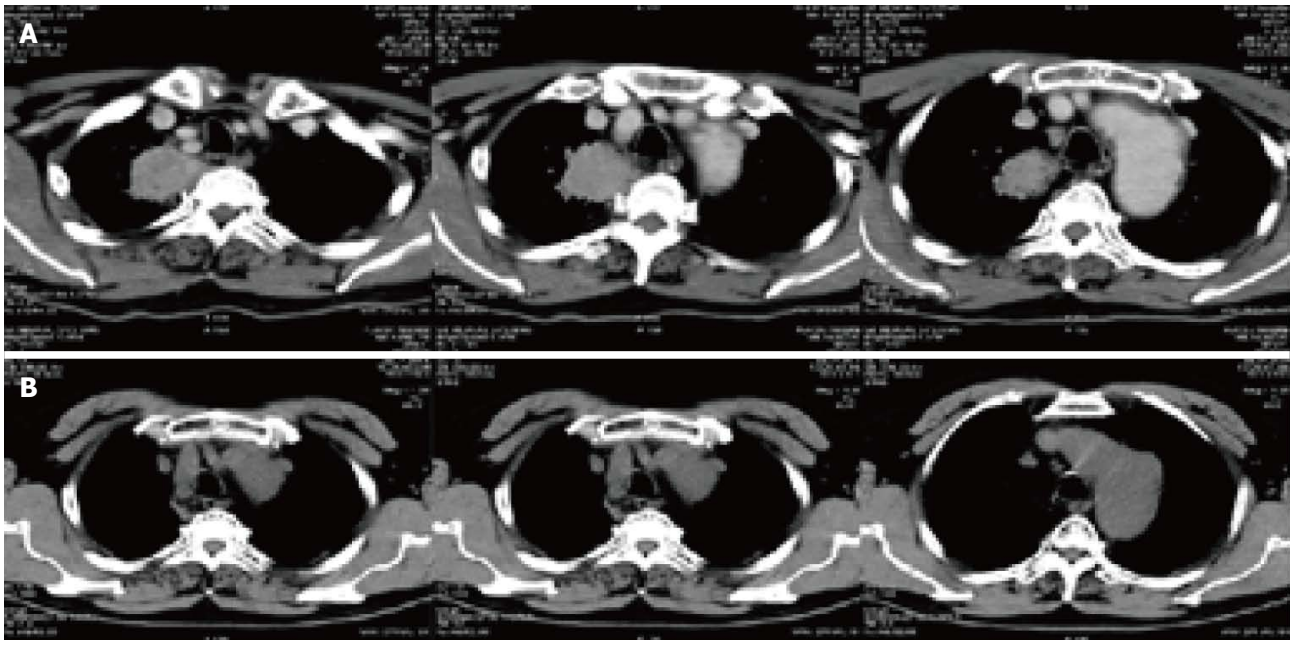

Figure 4 Changes of computed tomography slice before and after treatment in lung cancer patients. A: Right peripheral lung tumor mass (4.5 cm × 4.8 cm × 4.0 cm) located in the right upper lobe of the right lung before treatment; B: The mass disappeared 1 year after treatment.

liver function during the observation period, which could be returned to normal after liver-protecting therapy. The injury related to radiation or rhIL-12 needs to be further studied.

In addition, this study explored the relationship between biological activity and concentration. The result showed that there was no statistical difference among the 50 ng/kg subgroup, the 100 ng/kg subgroup and the control group for immune response. What's more, the adverse reactions were mainly concentrated in the 200 and 250 ng/kg subgroups, which suggests that the suitable clinical dosage concentration in our study is 150 ng/kg. The anti-tumor activity of rhIL-12 has been shown in clinical trials, but its toxicity to some extent

limits the application. Therefore, rhIL-12 still needs to be further researched.

In conclusion, rhIL-12 can prevent radiation damage, improve hematopoietic function, regulate immunity, reduce the side effect of radiotherapy and improve the quality of life of patients. It has good clinical application value and good development prospect as tumor auxiliary treatment.

COMMENTS

Background

Interleukin-12 (IL-12) is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells, and its target point lies in early undifferentiated pluripotent hematopoietic stem cells. However, the adverse events related to IL-12, including fever, chills, decrease of peripheral blood cells and organ dysfunction, have limited its clinical application. Recombinant human interleukin-12 (rhIL-12) is an immunoregulatory protein produced by gene engineering technology. RhIL-12 has similar biological activity to IL-12, but with the advantage of high purity (> 98%), high activity and low therapeutic dose. RhIL-12 has become the only available agent which can not only restore hematopoietic function but also improve immune function. Basic experimental study has found that the recovery of hematopoietic function after radiotherapy is helpful to avoid the rapid increase of single blood cells, which can lead to high fever, conjunctival hemorrhage, abnormal immune response, embolism and other detrimental side effects. But a large number of studies are basic in nature and based on animal experiments. The aim of the study was to explore the interventional effects of rhIL-12 on tumor patients receiving radiotherapy, including the complications after radiotherapy, the curative effects on hematopoietic function and immune function as well as dose-effect relationship, and to provide scientific basis for drug development and clinical application.

Research frontiers

Some studies have shown that rhIL-12 can stimulate various kinds of cytokines through stimulating the bone marrow microenvironment, either directly or indirectly, further promoting long-term hematopoietic reconstitution progenitor cells' differentiation and maturation, and instigate short-term hematopoietic reconstitution progenitor cells. These help to achieve a comprehensive recovery of hematopoietic function. In addition, in the circumstances of no supportive treatments, primate experiments demonstrated that using a low dose of rhIL-12 within 24 h after lethal dose irradiation could significantly improve (4-times) the animal's survival rate. What's more, rhIL-12 could promote the healing of skin wounds after radiation injury. As a radiation injury prevention drug, rhIL-12 is still effective at 24 h to 48 h after radiation. At the same time, a large number of animal experiments have shown that IL-12 can significantly inhibit the growth and metastasis of malignant tumors, prolonging the survival time of tumor-bearing animals. IL-12 can enhance the natural killer (NK) cell and cytotoxic T lymphocyte (CTL) cell response and the ability to induce production of IFN-C, which indicates that it may have anti-tumor activity. IL-12 enhances the binding ability of NK cells and K562 target cells and tumor cell monolayer, and enhances the cytotoxicity of NK cells to tumor cells. Because rhIL-12 and IL-12 have similar biological activities, some studies have shown that low dose of rhIL-12 can inhibit tumor cell growth, and rhIL-12 has synergistic anti-cancer effect on radiotherapy and chemotherapy, which needs further clinical validation.

Innovations and breakthroughs

The study found that low-dose rhIL-12 has the effect of prevention and treatment for the decrease of blood cells after radiotherapy, and could effectively improve the immune function and reduce the complications of radiotherapy.

Applications

RhIL-12 can prevent radiation damage, improve hematopoietic function, regulate immunity, reduce the detrimental side effects of radiotherapy and improve the quality of life of patients.

Terminology

IL-12: Interleukin-12 is an immunoregulatory protein produced by macrophages, B cells, mononuclear cells, keratinocytes and dendritic cells, and its target point lies in early undifferentiated pluripotent hematopoietic stem cells; RhIL-12: Recombinant human interleukin-12 is an immunoregulatory protein produced by gene engineering technology; its biological activity is similar to IL-12.

Peer-review

The authors conducted an interesting clinical study on rhIL-12 for the prevention and treatment of complications after radiotherapy in patients with malignant tumors. The manuscript was well written. The methodology was clear and accurate. The results section was adequate.

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ORIGINAL ARTICLE

Observational Study

Gastric and duodenal polyps in familial adenomatous polyposis patients: Conventional endoscopy *vs* virtual chromoendoscopy (fujinon intelligent color enhancement) in dysplasia evaluation

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Abstract

AIM

To test the fujinon intelligent color enhancement (FICE) in identifying dysplastic or adenomatous polyps in familial adenomatous polyposis (FAP) patients.

METHODS
Seventy-six consecutive FAP patients, already treated by colectomy and members of sixty-five families, were enrolled. A FICE system for the upper gastro-intestinal tract with an electronic endoscope system and a standard duodenoscope (for side-viewing examination) were used by two expert examiners. Endoscopic resection was performed with diathermic loop for polyps ≥ 6 mm and with forceps for polyps < 6 mm. Formalin-fixed biopsy specimens were analyzed by two expert gastrointestinal pathologists blinded to size, location and number of FAP-associated fundic gland polyps.

RESULTS
Sixty-nine (90.8%) patients had gastric polyps (34 only in the corpus-fundus, 7 only in the antrum and 28 in the whole stomach) and 52 (68.4%) in duodenum (7 in the bulb, 35 in second/third duodenal portion, 10 both in the bulb and the second portion of duodenum). In the stomach fundus after FICE evaluation, 10 more polyps were removed from 10 patients for suspicious features of dysplasia or adenomas, but they were classified as cystic fundic gland after histology. In the antrum FICE identified more polyps than traditional endoscopy, showing a better tendency to identify adenomas and displastic areas. In the duodenum FICE added a significant advantage in identifying adenomas in the bulb and identified more polyps in the Ⅱ/Ⅲ portion.

CONCLUSION
FICE significantly increases adenoma detection rate in FAP patients but does not change any Spigelman stage and thus does not modify patient's prognosis and treatment strategies.

Key words: Fujinon intelligent color enhancement; Familial adenomatous polyposis; Spigelman; Endoscopy; Polyp; Adenoma; Stomach; Duodenum

Core tip: Colon endoscopic surveillance and prophylactic colectomy have strongly reduced mortality due to colorectal carcinoma and have improved survival of familial adenomatous polyposis (FAP) patients, leading to the development of surveillance for extra-colonic cancers. Polyps in the duodenum and stomach are frequent findings in FAP. The timing of endoscopic and histology surveillance is currently a great challenge. Spectral estimation by fujinon intelligent color enhancement (FICE) may identify dysplasia and discriminate between adenomatous and non-adenomatous polyps. Interestingly, application of FICE to FAP patients significantly increases the detection of adenomas but does not yet change the prognosis, surveillance program and treatment strategies.

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INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited syndrome characterized by the development of colorectal cancer by the age of 40 years in nearly 100% of individuals[1]. Colon Endoscopic surveillance and prophylactic colectomy have strongly reduced mortality due to colorectal carcinoma and have improved survival of FAP patients with minimal consequences[2,3], leading to the introduction of surveillance strategies for the prevention of other extra-colonic malignancies[4].

The duodenum is the second most common site of polyps development after colon, with a life-time risk of duodenal adenomas that approaches 100% in FAP affected individuals[5,6]. The cumulative risk of duodenal cancer or high grade of dysplasia by the age of 60 years is 4%-10%[6-8].

Endoscopic surveillance and removal of neoplastic tissue is useful in the prevention of duodenal cancer[8]. However, the choice of treatment and the optimal timing of surveillance based on endoscopic and histopathology examination for each patient is currently a great challenge. Currently the surveillance of duodenum is based on the Spigelman classification of duodenal adenomatosis (Table 1); however, this staging system has low predictive values and has never been validated[6,8].

Gastric polyps are also a common finding in patients with FAP: they mostly consist of FAP-associated fundic gland polyps (FGPs) reported to occur at variable rates, up to 88%[9,10], against a strongly smaller rate (0.8%-5.0%)[11,12] in non-FAP subjects who undergo an esophagogastroduodenoscopy (EGD).

FGPs have historically been considered non-neoplastic lesions without malignant potential[13]; however recent studies have questioned this assumption reporting high rates of low and high grade dysplasia (up to 54%)[9,14,15]. In particular, European and Asian registries of FAP patients proved the presence of gastric carcinoma arising from FGPs in FAP patients and an incidence of gastric adenocarcinoma between 0.6% and 4.0%[16-19].

Other common types of gastric polyps are represented by adenomas (gastric foveolar or intestinal type-gastric adenomas and pyloric gland adenomas) which are reported in approximately 10% of gastric polyps in FAP patients[10,20,21] and which can arise in the gastric antrum, in the gastric body-fundus or in the context of FGP[22]. So, identification of dysplastic lesions or adenomatous tissue in these patients is often made difficult by the great number of polyps (up to hundreds) and by the patchy

Table 1 Demographic features

Features	Patients
Total	76
Age (yr)	Mean 40.3 (24-64)
Gender	
Male	41 (53.9%)
Female	35 (46.1%)
Prior surgery	
IRA	10 (13.2%)
IPAA	66 (86.8%)
Chemoprevention	16 (21.1%)
NSAIDS intake	17 (22.4%)
Tobacco exposure	21 (27.6%)
PPI/anti-H2 intake	14 (18.4%)
Family history of GI malignancies	
None	31 (40.8%)
1 member	32 (42.1%)
2 members	10 (13.2%)
3 members	3 (3.9%)
Spigelman duodenal stage	
0	28 (36.8%)
I	7 (9.2%)
II	34 (44.8%)
III	7 (9.2%)
IV	0 (0.0%)

IRA: Total colectomy and ileo-rectal anastomosis; IPAA: Total proctocolectomy and ileopouch-anal anastomosis; NSAIDS: Non steroidal anti-inflammatory drugs; PPI: Proton pump inhibitors.

distribution of dysplasia.

By now, dysplasia finding in this subgroup of subjects is made on the basis of random biopsies[9] which lead to a time consuming, laborious and poorly performing procedure, that can result in a high rate of missed lesions. According to that, it would be useful identifying FGPs at risk of malignant degeneration.

A better characterization of patients, an optimized program of surveillance and a good survival are possible with a selective and complete asportation and with a careful histological evaluation.

It is well known that is possible to predict the histology of a mucosal lesion by observing the crypt orifices (the so called pit pattern) of mucosal glands[23] and the capillary pattern of the mucosa[24]. Several endoscopic imaging techniques have been proposed to enhance the details of these patterns[25]. Among these, chromoendoscopy is a widely applied staining method that uses biocompatible dye agents which accumulate within crypt orifices during endoscopy[26]. Although chromoendoscopy is effective for many applications, it still has some problems, such as difficulty in achieving complete and even coating of the mucosal surface with the dye, the extra cost of the equipment needed for dye spraying and the extra time required to perform the procedure. Moreover, traditional chromoendoscopy isn't able to enhance the capillary pattern, whose evaluation is essential in early diagnoses of malignant lesions[24]. In attempt to resolve these problems, other endoscopic technologies have been developed. Fujinon intelligent color enhancement (FICE™, Fujinon Corp, Saitama, Japan) represents a spectral estimation technique based on arithmetically processing of a white-light image captured by a video endoscope and sent to the spectral-estimation matrix-processing circuit. The image of the white-light endoscopic observation is resolved in each color image of the red, green and blue signal. Next, each resolved image is converted into various presumed wavelength images by a pixel unit. The images of an arbitrary single wavelength are then extracted and reconstructed. Due to its variable setting functions (up to 10) it is possible to select flexibly the most suitable wavelengths required for examination. Preliminary studies suggested that FICE successfully achieved enhancements of real-time observations of mucosal and microvascular patterns[27,28].

The light penetration into the mucosa varies according to the wavelengths: Those in the 400-500 nm range are ideal for analyzing surface structures whereas, because of the absorption properties of hemoglobin, longer wavelengths of about 550 nm are more effective for the visualization of blood vessels.

FICE seems able to discriminate between adenomatous and non-adenomatous polyps and to identify the presence of dysplasia[29-32]. Few studies have been conducted to determine the efficacy of chromoendoscopy, both traditional and virtual, in the evaluation of duodenal and peri-ampullary adenomatous polyps in FAP patients[33-35]. Interestingly, FICE application in the discrimination between neoplastic and non-neoplastic gastric lesions has not been throughly investigated[36-39], and no data are available about FICE in evaluating FGPs dysplasia or application of FICE for the screening of FAP patients.

In FAP cohort, the specific identification of who is at a greater risk of cancer development could be of paramount importance to assure a personalized program of surveillance or a therapeutic intervention.

The primary aim of this study was to assess the capability of FICE in identifying gastric polyps with dysplastic or adenomatous tissue in comparison to traditional endoscopy and in identifying a greater number of duodenal adenomas with advanced histological features.

Secondary aim was to assess the capability of FICE in identifying adenomas not seen on white light evaluation.

MATERIALS AND METHODS

Patients

Seventy-six consecutive FAP patients, already treated by colectomy and members of sixty-five families, were enrolled. Exclusion criteria were: Uncorrectable coagulopathy, inability to give informed consent, age < 18 years, prior gastro-duodenal surgery or a personal history of gastro-duodenal cancer. All patients underwent a surveillance esophagogastroduodenoscopy (EGD) in deep sedation at the Gastroenterology U.O. of the Azienda Ospedaliero Universitaria di Careggi, Firenze, Italy. Written informed consent were obtained before EGD and sedation.

Endoscopy

A FICE system (EG-590ZW; Fujinon Corp, Saitama,

Japan) for the upper gastro-intestinal tract with an electronic endoscope system (EPX-4400; Fujinon Corp, Saitama, Japan) was used for this study. In this system, ten channels with different predefined combinations of absorption wavelengths are available. We used channel 5, corresponding to R 500 nm, G 480 nm, B 420 nm, on the basis of previous studies.

A standard duodenoscope was used for side-viewing examination. Because this model of duodenoscope does not support FICE system, ampullary and periampullary evaluations were not included in the analysis. All of the endoscopic procedures were performed by two experts examiners.

"A" operator performed the exam on white light, while "B" operator used only FICE system for gastroduodenal visualization. Each EGD was divided into three phases.

During the first phase, "A" operator observed stomach and duodenum by white light recording photographic images of suspected polyps and pointing them. We intended suspected polyps on white light those larger than 1 cm and those with irregular shape or surface features.

During the second phase, "B" operator performed the exam using FICE and, like "A" operator, recorded photographic images of suspected polyps on the basis of Kudo classification[23] and capillary pattern, and pointed them.

Kudo classification classifies mucosal crypt patterns into five types, with type Ⅰ and Ⅱ predicting non-adenomatous lesions and type Ⅲ-Ⅴ predicting adenomatous lesions.

Hyperplastic polyps were suspected when the surface showed pale color with only minute thin superficial (couperose-like) vessels and round or asteroid pattern (type Ⅰ and Ⅱ). Adenomas were suspected in the presence of increased vascular density (darkening of the mucosal pattern or a fine meshwork of brownish/bluish vessels) and a typical tubular or gyrus-like pattern (type Ⅲ-Ⅳ). Finally, type V have a non structural pattern which identifies high dysplastic or yet carcinomatous lesions.

During this phase we intended suspected those lesions with a pit pattern type Ⅲ-Ⅴ and those with an increased capillary density.

During the third phase, after the two endoscopists' cross-evaluation, lesions which seemed suspected only by FICE, only by white light or by both methods were resected or biopsied according to Kudo class.

Endoscopic resection was performed with diatermic loop for polyps ⩾ 6 mm and with forceps for polyps < 6 mm. The size was estimated using on open biopsy forceps (8 mm) for comparison and recorded as smaller than 6 mm, 6 to 10 mm, 11 to 20 mm and greater than 20 mm.

The total number of FGPs was documented as below: 0 to 2 polyps, 3 to 20, 21 to 30 and more than 30 polyps. On the basis of location we identified: Fundus-corpus, antrum, duodenal bulb, Ⅱ°/Ⅲ° duodenal portion.

For fundic polyps seen on white light, the number of FGPs from which a biopsy specimen was taken was based on the total number of FGPs present: 3 biopsies if 3-20 polyps, 5 biopsies if 21-30 polyps, 7 biopsies if > 30 polyps[9].

On FICE, only suspected polyps (Kudo Ⅲ-Ⅴ, high capillary density) were removed.

For antral and bulbar polyps, all of them were removed or biopsied both on withe light than on FICE.

In the second and third duodenal portion, on white light only suspected polyps were resected or biopsied, while on FICE were biopsied those with Kudo Ⅴ and those with Kudo Ⅳ and high capillary density.

Macroscopic classification of lesions followed the Paris classification[40] as polyp, superficially flat or depressed lesion, and lateral spreading tumor.

Histology

All biopsy specimens, fixed in 10% neutral buffered formalin, were analyzed by two expert gastrointestinal pathologists blinded to size, location and number of FGPs.

In the case of multiple lesions in the same patient, each lesion was identified and placed in different flasks. Lesions were histological classified in adenomatous, hyperplastic or inflammatory polyps, fundic gland polyps, and metaplastic areas.

Adenomatous polyps were classified according to OMS classification: Tubular if holding more than 75% of tubular glands, villus if holding more than 75% of villus glands, tubulo-villus if not prevailing none of the two patterns.

Dysplasia was classified according to Vienna criteria[41] in low grade if holding nuclear enlargement, stratification and hyperchromasia with overall preservation of nuclear polarity; high grade as above but with nuclear polarity loss and glandular crowding; indefinite for dysplasia if present mild nuclear enlargment and insufficient hypercromasia to be classified as dysplasia or if present a significant obscuring background inflammation.

The stage of duodenal polyposis was graded according to Spigelman classification modified sec. Saurin[42], which take into account duodenal polyp number, size, histological type and grade of dysplasia. It was noted before and after FICE evaluation.

Statistical analysis

The diagnostic performances (sensitivities, specificities, positive and negative predictive values) of FICE and WL were determined by comparing the endoscopic diagnoses with the histo-pathological findings. To identify associations of demographic, clinical and endoscopic features with the presence of FGP dysplasia or with adenomas, the Fisher exact test was used to study univariable associations of categoric demographic and endoscopic factors with the presence of dysplasia or adenomatous tissue. The Student t test was used for continuous factors. A *P* value "two tailed" < 0.05 was considered statistical significant. The strength of association was calculated by odds ratio (OR). The statistical methods of this study were reviewed by S. Milani, University of Florence.

Table 2 Stomach and duodenum polyps

	Patients		Patients
Fundus	34 (49.3%)	Bulb	7 (13.5%)
Antrum	7 (10.1%)	Ii°/iii° portion	35 (67.3%)
Fundus + antrum	28 (40.6%)	Bulb+ii°/iii°	10 (19.2%)
Total stomach	69 (100%)	Total duodenum	52 (100%)

Table 3 Features of fundic polyps identified by white light endoscopy

	P1-P3	P4-P10	P11-P24	P25-P55	P56-P397
Kudo	I	II	II	II	I
Size (mm)	5	5	6-10	5	5
Paris CL	Is	Is	Is	Is	Is
Histology	HYP	IN	FGP	FGP	FGP

IN: Inflammatory; FGP: Fundic gland polyp; IP: Hyperplastic.

RESULTS

Seventy-six consecutive FAP patients (41 male and 35 female; mean age 40.3 years old, range 24-64) underwent EGD. Among all patients, 69 (90.8%) had gastric polyps (34 only in the corpus-fundus, 7 only in the antrum and 28 in the whole stomach) and 52 (68.4%) in duodenum (7 in the bulb, 35 in second/third duodenal portion, 10 both in the bulb and the second portion of duodenum) (Table 2).

Identification of polyps in the stomach

Fundus: 62 patients had a widespread fundic polyposis (81.6%); 52 of them had more than 30 fundic polyps (68.5%), 3 between 21 and 30 (3.9%) and 7 between 5 and 20 (9.2%).

On white light visualization, 397 polyps in 62 patients (6.4 polyps per patient) were removed. Three were hyperplastic polyps, 7 inflammatory while the rest were cystic fundic gland polyps. No polyp harboured dysplasia nor adenomatous foci (specificity 100%, sensitivity NV, positive predictive value NV, negative predictive value 100%, 95%CI) (Table 3).

After FICE evaluation, 10 polyps were removed from 10 patients on the basis of suspicious features of dysplasia or adenomatous tissue. All of them were cystic fundic gland polyps, none of them harboring dysplasia or adenomatous foci (specificity 97%; sensitivity NV; positive predictive value 0%; negative predictive value 100%) (Table 4).

Thirty-eight patients with fundic polyposis had also duodenal polyposis (61.2%), while among the 14 patients without fundic polyps, 10 had polyps in the duodenum (71.4%). Thus the presence of fundic polyps doesn't correlate with a higher risk to develop duodenal polyps (P = 0.55; OR = 0.6).

Antrum: A total of 56 polyps were identified and removed in the antrum of 35 patients (average 1.6 polyps per patient). Twenty-four polyps were identified in 35 patients by white light endoscopy (0.7 polyps per patient); 21 of them were tubular adenomas with low grade dysplasia while 3 were inflammatory polyps (specificity 88.0%; sensitivity 67.7%; positive predictive value 87.5%; negative predictive value 68.7%) (Table 5).

Beside polyps seen with conventional endoscopy, FICE was further able to identify 32 polyps in 28 patients. They were 7 tubular adenomas with low grade dysplasia, 14 inflammatory polyps, 3 areas with low grade dysplasia in the context of flogistic mucosa, 8 metaplasic areas (specificity 12.0%; sensitivity 100%; positive predictive value 58.5%; negative predictive value 100%) (Table 6).

FICE identified a higher number of polyps than traditional endoscopy (56 *vs* 24; $P < 0.0001$), showing a better, but not statistically significant, tendency to identify adenomas and displastic areas (31 *vs* 21; $P = 0.0857$). All but 4 polyps missed out by white light, were flat.

Eighteen of patients with antral lesions (51.4%) had polyps also in the duodenum. There is no relationship between presence of dysplasia in antral stomach and Spigelman advanced stages (P = 1; OR = NV).

Table 4 Features of fundic polyps identified by fujinon intelligent color enhancement

	P1-P6	P7-P10
Kudo	IIIS	IIIL
Size (mm)	5	5
Paris CL	Is	Is
Histology	FGP	FGP

FGP: Fundic gland polyp.

Table 5 Features of antral polyps identified by white light endoscopy

	P1-P3	P4	P5-P10	P11-P17	P18-P24
Kudo	II	IIIS	IIIS	IIIS	IV
Size (mm)	6-10	6-10	5	5	5
Paris CL	IIa	IIa + IIc	Is	Is	Is
Histology	IN	TA, LGD	TA, LGD	TA, LGD	TA, LGD
Spigelman	0	0	I°	0	0

IN: Inflammatory; TA: Tubular adenoma; LGD: Low grade dysplasia.

Identification of duodenal polyps

Bulb: 21 polyps were seen in 17 patients (1.2 polyps per patient). All of them were endoscopically removed. White light endoscopy identified 14 polyps in 12 patients; 8 polyps were inflammatory, while 6 were tubular adenomas with low grade dysplasia (specificity 0%; sensitivity 46.2%; positive predictive value 42.9%; negative predictive value 0%) (Table 7).

During FICE evaluation, beside polyps seen with conventional endoscopy, 7 more polyps in 7 patients, five

Table 6 Features of antral polyps identified by fujinon intelligent color enhancement

	P25	P26	P27	P28-P30	P31	P32-P33	P34-P35	P36-P38	P39-P44	P45-P49	P50-P53	P54-P56
Kudo	V	V	IIIS	IIIL	IIIL	IV	IV	IIIL	IIIL	IIIS	IIIS	V
Size (mm)	5	5	5	6-10	5	5	6-10	5	5	5	6-10	5
Paris CL	IIb	IIa	IIb	IIb	IIa	IIb	IIa	IIa	IIb	IIb	Is	IIb
Histology	IN LGD	IN LGD	IN LGD	MET	MET	MET	MET	IN	IN	IN	TA LGD	TA LGD
Spigelman	III°	II°	I°	II°	II°	0	II°	0	0	0	I°	II°

IN: Inflammatory; TA: Tubular adenoma; LGD: Low grade dysplasia; MET: Metaplasia.

Table 7 Features of bulbal polyps identified by white light endoscopy

	P1-P5	P6-P8	P9	P10-P12	P13	P14
Kudo	II	IIIS	IIIS	IIIL	IV	IV
Size (mm)	5	6-10	6-10	5	6-10	6-10
Paris CL	Is	Is	Is	IIa	Is	Is
Histology	IN	TA LGD	TA LGD	IN	TA LGD	TA LGD
Spigelman	0	II°	I°	0	III°	II°

TA: Tubular adenoma; LGD: Low grade dysplasia.

Table 8 Features of bulbal polyps identified by fujinon intelligent color enhancement

	P15	P16-P17	P18	P19	P20-P21
Kudo	IIIS	IV	IIIS	IIIL	IIIS
Size (mm)	5	6-10	6-10	5	5
Paris CL	IIa	IIb	IIa	IIb	IIb
Histology	TA LGD	TA LGD	TA LGD	TA LGD	TA LGD
Spigelman	I°	II°	II°	I°	I°

TA: Tubular adenoma; LGD: Low grade dysplasia.

of them new, were discovered. All of them were tubular adenomas with low grade dysplasia (specificity 62.5%; sensitivity 100%; positive predictive value 81.3%; negative predictive value 100%) (Table 8).

FICE was able to see further 7 polyps than traditional endoscopy, and it was able to identify a quite significant higher number of polyp in the duodenal bulb (21 *vs* 14; $P = 0.069$). FICE added a statistical significant advantage in identifying adenomas (13 *vs* 6; $P = 0.03$). All FICE identified polyps were flat lesions. All patients with bulbal polyps had also lesions in the gastric fundus and no adenoma in the antrum.

All patients with bulbal adenomas had polyps in the second/third portion of duodenum, while patients with inflammatory polyps had a Spigelman's stage 0.

II°/III° duodenal portion: Totally, 391 polyps in 45 patients (8.7 polyps per patient) were identified. Of them, 105 were removed or biopsied (26.5%). Conventional endoscopy identify 324 polyps in 45 patients (7.2 polyps per patient). Of them, 94 were removed or biopsied (2.1 polyps per patient) and they resulted: 80 tubular adenomas with low grade dysplasia, 10 inflammatory polyps and 4 tubulo-villous adenomas with low grade dysplasia. No case of high grade dysplasia (3 suspected). (Table 9). FICE identified further 67 polyps in 35 patients and 11 were removed or biopsied in 11 subjects. All of them were tubular adenomas with low grade dysplasia. No case of high grade dysplasia (Table 10). FICE was able to identify a higher number of polyps than traditional endoscopy (8.7 *vs* 7.2; $P < 0.001$). All polyps not seen on white light were flat lesions.

Thirty-five of patients with duodenal polyposis had also polyps in the fundus, 4 had adenomas and 2 dysplastic areas in the antrum, thus FICE didn't change any Spigelman stage just defined with conventional endoscopy.

DISCUSSION

Duodenal adenomatous polyps are common manifestations of FAP found in 30% to 90% of patients, with a life time risk approaching 100%[5,6,43]. While rare in the general population (0.01%-0.04% of incidence at an average age of 65 years)[43], the risk of duodenal or periampullary cancer is increased several hundreds fold in FAP patients (estimated cumulative risk of 4.5% by age 57 and a median age at presentation of 52 years)[6,8]. Duodenal cancer is the second most common cause of disease-related mortality in patients with FAP, only the

Table 9 Features of duodenal polyps identified by white light endoscopy

	Kudo	Size (mm)	Paris CL	Histology	Spigelman
P1-P4	Ⅳ	11-20	Ⅱa	TA LGD	Ⅲ°
P5-P7	Ⅴ	6-10	Ⅱa	TA LGD	Ⅱ°
P8-P11	Ⅳ	5	Ⅱb	TA LGD	Ⅱ°
P12-P16	ⅢS	6-10	Ⅱa	TVA LGD	Ⅲ°
P17-P27	ⅢS	5	Ⅱa	TA LGD	Ⅱ°
P28-P34	ⅢS	5	Ⅱa	TA LGD	Ⅰ°
P35-P40	ⅢS	5	Ⅱb	TA LGD	Ⅱ°
P41-P43	ⅢS	5	Ⅰs	TA LGD	Ⅱ°
P44-P47	ⅢS	5	Ⅰs	TA LGD	Ⅰ°
P48-P50	ⅢS	11-20	Ⅱa	TA LGD	Ⅱ°
P51-P58	ⅢL	5	Ⅱb	TA LGD	Ⅱ°
P59-P65	ⅢL	5	Ⅱa	TA LGD	Ⅱ°
P66-P68	ⅢL	6-10	Ⅱa	TA LGD	Ⅱ°
P69-P72	ⅢL	6-10	Ⅱa	TA LGD	Ⅲ°
P73-P79	ⅢL	6-10	Ⅱb	TA LGD	Ⅱ°
P80-P84	Ⅱ	5	Ⅱa	TA LGD	Ⅲ°
P85-P91	ⅢL	5	Ⅱb	IN	Ⅱ°
P92-P94	ⅢS	5	Ⅱa	IN	Ⅰ°

IN: Inflammatory; TA: Tubular adenoma; LGD: Low grade dysplasia.

Table 10 Features of duodenal polyps identified by fujinon intelligent color enhancement

	P95-P97	P98-P100	P101-P102	P103-P105
Kudo	Ⅳ	Ⅳ	Ⅴ	Ⅳ
Size (mm)	5	6-10	6-10	5
Paris CL	Ⅱa	Ⅱb	Ⅱb	Ⅱb
Histology	TA LGD	TA LGD	TA LGD	TA LGD
Spigelman	Ⅰ°	Ⅱ°	Ⅱ°	Ⅱ°

TA: Tubular adenoma; LGD: Low grade dysplasia.

second to advanced and metastatic colorectal cancer. A regular and careful program of endoscopic surveillance is worthwhile in identifying early pre-malignant lesions.

Gastric polyps, particularly fundic polyps, are considered always non-neoplastic lesions, also in FAP and non-FAP patients; nonetheless high rate of their prevalence (20%-88%)[6,9-11] and several cases of dysplasia in FGPs in FAP have been recently reported, with rate of incidence up to 54%[9-11,18].

Chromoendoscopy, both traditional and virtual, has been proven to be a good tool to increase polyps identification rate and to predict their histology[29-32]. Only one study was published on the use of FICE in the evaluation of duodenal lesions[44]. This study was conducted using a double balloon enteroscopy on patients with duodenal lesions. In this study only two FAP patients were included and FICE enhanced mucosal pattern of these polyps, and it correlated with the increase of detection of more lesions.

However, neither previous studies using traditional chromoendoscopy nor FICE, were conducted in evaluation of gastric polyps in FAP patients. To the best of our knowledge, our study is the first that has assessed the role of FICE in FGPs dysplasia identification and in the gastroduodenal polyps characterization in FAP subjects.

In agreement with the literature's data, the prevalence of gastric polyps was relatively elevated (90.8%), while duodenal polyps were diagnosed in 68.4% of patients, slightly lower than the reported literature value.

In the majority of FAP subjects (62/76; 83.3%), gastric polyps were so numerous that they carpeted the fundic mucosa, making difficult identifying dysplasia by random biopsies on the basis of the total number of polyps, as indicated in a recent study conducted by Bianchi *et al*[9]. Consequently, having an endoscopic technique able to target fundic biopsies is important to overcome this issue. Moreover, Bianchi *et al*[9] reported a prevalence of dysplasia in fundic polyps of 42%, while we have found only fundic gland polyps without displastic or adenomatous areas, although we have followed their sampling method. A possible explanation to this marked mismatch, could lie in the size of the polyps removed: we did found only subcentimetric polyps, while Bianchi *et al*[9] have demonstrated that the risk of dysplasia correlated with polyp size. No polyps removed had suspected superficial features according to Kudo classification, while Bianchi *et al*[9] did not adopted any classification to describe mucosal and vascular pattern; consequently we don't know if their removed polyps had or not a suspected pattern.

FICE pointed our attention on 10 fundic polyps, that seemed suspected for harboring adenomatous tissue;

however histological results did not confirmed this suspect and all of polyps resulted fundic gland polyps. In this case, FICE has not increased the identification rate of dysplasia or adenomatous tissue in fundic polyps.

Prevalence of patients with antral adenomas was about 21.1% (16/76), more than reported in the Western World data, but consistent with Japanese findings. The use of FICE could explain our result, since it has increased the identification rate of antral adenomas compared to white light, with a difference near to statistical significance (P = 0.0857).

The very low specificity of the method (12.0%) could be explained by the presence of phlogosis (in fact almost all false positive harbored flogistic areas) able to distort the mucosal and vascular pattern, specifically enhanced by virtual chromoendoscopy.

Therefore, FICE allows to identify a greater number of adenomas to the detriment of a greater number of biopsies. Anyway this approach didn't determine a different timing in the surveillance program, but changed the attention on the antral evaluation during the following endoscopies. In duodenal bulb FICE was able to identify more adenomas than traditional endoscopy (P = 0.03). Furthermore, all patients with FICE-identified adenomas had polyps in the duodenum too, thus the identification of bulbar adenomas didn't modify surveillance timing.

Taking into account also bulbar polyps, duodenal adenomas prevalence in FAP patients was 68.4%, with low Spigelman stages (9.2% stage Ⅲ e 0% stage Ⅳ). In duodenum, FICE has allowed to see a greater number of adenomas than white light (P < 0.001), without no modifications of Spigelman stage neither identification of high grade dysplasia.

Among FICE identified polyps, 4 lesions were suspected for high grade dysplasia, but three were inflammatory polyps at histopathological examination and one was a tubular adenoma with low grade of dysplasia. Other 7 polyps (Kudo IV) had an increased capillary density but they were tubular adenomas with low grade of dysplasia.

Finally, in duodenum, FICE increased the polyps detection rate but didn't change any Spigelman stage determined with conventional endoscopy. These data are in agreement with the little size and the absence of high grade dysplasia. Moreover this method wasn't able to modify FAP patients' prognosis, polyps' surveillance program and their therapeutic management. We did not find any relationship between the presence of gastric polyps, duodenal polyposis and high Spigelman stage (P = 1).

Adenomas were 435 and 81 of them were diagnosed only by FICE that was able to identify a significative higher number of adenomas (P = 0.0062). Overall, FICE has specificity, sensitivity,positive and negative predictive values higher than traditional endoscopy referring to adenomas (96.0% *vs* 7.1%; 98.8% *vs* 80.2%; 90.3% *vs* 44.9%; 98.8% *vs* 27.6%, respectively; P < 0.0001). Conversely, it wasn't possible to correlate for high grade dysplasia due the absence of dysplastic lesions according to the histopathological examination.

The FICE identified lesions (106/468; 22.6%) were mostly flat (67.9%; P = 0.029) and small (all below 1 cm). According to already published data, FICE was particularly able to identify polyps with these features. It isn't clear if this ability might have clinical and procedural consequences.

In summary, in our study, FICE, like traditional endoscopy, could not identify any adenoma at risk of malignant transformation probably as a consequence of patients features (*e.g.,* favorable genotype, recent EGD).

Nonetheless FICE significantly increases adenoma detection rate (P = 0.0062) but does not change any Spigelman stage and thus does not modify patient' s prognosis, surveillance program and treatment strategies. Probably a careful patient selection, an accurate histological examination, a concomitant use of lateral viewing endoscope, could make FICE gain that role who everybody expects in FAP patient.

COMMENTS

Background

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited syndrome characterized by the development of colorectal cancer by the age of 40 years in nearly 100% of individuals. The use of colon endoscopic surveillance and prophylactic colectomy have strongly reduced mortality in FAP patients leading to the introduction of surveillance strategies for the prevention of other extracolonic malignancies (*e.g.,* in the duodenum and in the stomach). Duodenal adenomatous polyps are common manifestations of FAP found in 30% to 90% of patients. Duodenal cancer is the second most common cause of disease-related mortality in patients with FAP, only the second to advanced and metastatic colorectal cancer. Gastric polyps, particularly fundic polyps, are considered always non-neoplastic lesions, also in FAP and non-FAP patients; nonetheless high rate of their prevalence (20%-88%) and several cases of dysplasia in FGPs in FAP have been recently reported, with rate of incidence up to 54%.

Research frontiers

The observation of the pit and capillary patterns of the mucosal glands and the mucosa, respectively, by chromoendoscopy might predict the histology of mucosal lesions.

Innovations and breakthroughs

Chromoendoscopy is a staining method that uses biocompatible dye agents which accumulate within crypt orifices during endoscopy. Chromoendoscopy has difficulty in achieving complete and even coating of the mucosal surface with the dye, requires the extra cost for the of the dye spraying equipments and extra time to perform the procedure. Fujinon Intelligent Color Enhancement (FICE™, Fujinon Corp, Saitama, Japan) is a spectral estimation technique based on arithmetically processing of a white-light image captured by a video endoscope and sent to the spectral-estimation matrix-processing circuit. Preliminary studies suggested that FICE successfully achieves enhancements of real-time observations of mucosal and microvascular patterns and may discriminate between adenomatous and non-adenomatous polyps and it may identify the presence of dysplasia. In the study, FICE, like traditional endoscopy, could not identify any adenoma at risk of malignant transformation probably as a consequence of patients features. However FICE significantly increases adenoma detection without changing patient's prognosis, surveillance program and treatment strategies. Probably a careful patient selection, an accurate histological examination, a concomitant use of lateral viewing endoscope, could make FICE gain that role who everybody expects in FAP patient.

Applications

The timing of endoscopic and histology surveillance is currently a great challenge. Spectral estimation by fujinon intelligent color enhancement (FICE) may identify dysplasia and discriminate between adenomatous and non-adenomatous polyps.

Terminology

FAP is an autosomal dominant inherited syndrome who invariably develops to colorectal cancer by the age of 40 years in nearly 100% of individuals. Several endoscopic imaging techniques have been proposed to enhance the detail of these patterns. Among these, chromoendoscopy is a staining method applied in endoscopy that uses biocompatible dye agents which accumulate within crypt orifices. FICE is a modern endoscopic spectral estimation technique that successfully enhances the observation of mucosal and micro-vascular patterns.

Peer-review

The presented results, obtained with 76 FAP patients, indicate that FICE assay offers considerable advantage over traditional chromoendoscopy to discriminate between adenomatous and non-adenomatous polyps. The authors, however, caution that the application of FICE to FAP patients while helpful in prediction the histology of the mucosal lesion and significantly increases the detection of adenomas, do not change the prognosis and treatment.

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