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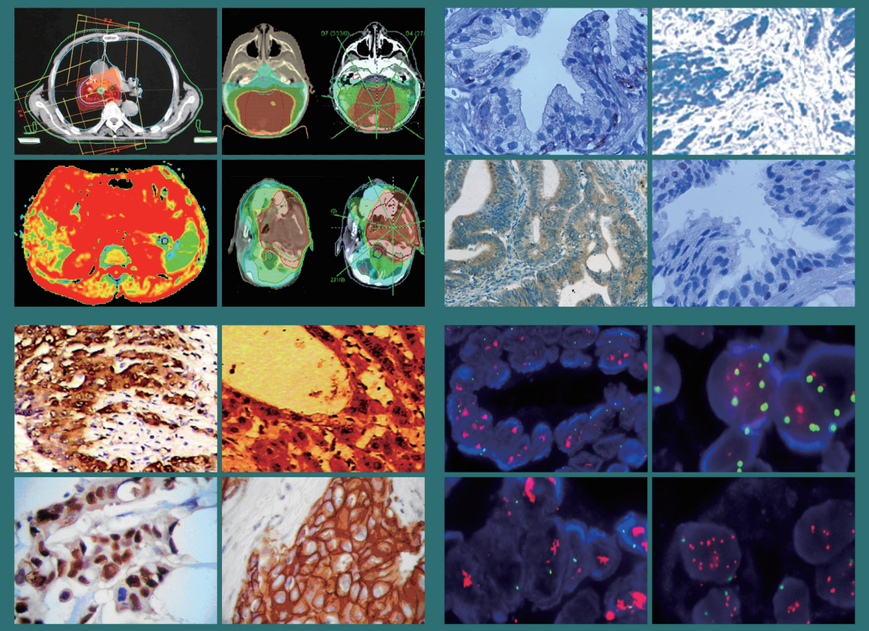

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EDITORIAL

Gastrointestinal cancer research in the era of precision medicine

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Lin Shen. M.D., Professor, Chief, Vice President, Peking University Cancer Hospital. Professor Lin Shen received her degree in oncology from Peking Medical University and specialized in gastrointestinal cancers. Her work focuses on precision medicine inadvanced gastric cancer, colorectal cancer with liver metastases, gastrointestinal stromal tumors, and other gastrointestinal malignancies. She is also interested in clinical trials and translational research, especially early stage clinical trials. Professor Shen has published over 130 papers and has hosted as a principal investigator or participated in over 80 global and domestic multicenter clinical trials. The results of several of these studies have provided evidence to standardize the clinical practice for gastrointestinal tumors in China. Now, Professor Shen is Chairman of Chinese Anti-cancer Drug Clinical Trials (ACTS), General Secretary of Gastric Cancer Committee and Vice-chairman of Colorectal Cancer Committee of Chinese Anti-Cancer Association (CACA), and also she is Chairman of Chinese Society of Multidisciplinary Team (CSMDT).

In the new era of precision medicine, increasing knowledge of the underlying signaling pathways and molecular defects involved in cancer growth or progression has enabled the discovery of several prognostic and predictive biomarkers. This in turn has led to the development of novel early diagnostic methods, accurate disease classification, therapeutic targets, and personalized therapy. Hence, we summarized the current status of gastrointestinal cancer-related biomarkers and treatment options.

Despite the continuous decline in both its incidence and mortality in recent decades, gastric cancer (GC) remains the fifth most prevalent malignancy; furthermore, the prevalence of GC in China is relatively higher than that in other countries. With the development of molecular biological techniques, molecular classifications of GC have emerged, and they show potential value in guiding precise and personalized therapy. Until now, 3 to 4 GC molecular classifications have been established. In particular, several biomarkers in immunotherapy have been explored. Based on the tumor microenvironment, a framework for classifying tumors according to tumor-infiltrating lymphocytes (TILs) and programmed death-ligand 1 (PD-L1) expression has been proposed. Here, we reviewed the development of molecular classifications and characteristics of different subtypes, and then discussed the application of molecular classification in clinical management, especially in immunotherapy.

Traditionally, *in vitro* cell lines derived from cancer cells and *in vivo* animal models are used to predict the clinical outcomes of novel drugs. Nevertheless, because of the lack of tumor heterogeneity, the differences in

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the tumor microenvironment, and the accumulation of genetic aberrations during passaging, cell line-based models have limitations for predicting therapeutic efficacies. In recent years, patient-derived tumor xenografts (PDTXs) have drawn increasing attention. They can faithfully recapitulate the histopathology, molecular characteristics, and therapeutic response of the original tumors. Despite this, although PDTX models can approximate the original tumor, they can never exactly replicate the *in vivo* environment. Unremitting efforts to combat the limitations of PDTXs may further broaden their application and strengthen their reliability in guiding individualized therapy. In our review, the establishment and characterization of PDTX models is discussed in detail.

Recent work by Le *et al* highlighted the efficacy of immunotherapy in deficient DNA mismatch repair (dMMR) colorectal cancer (CRC) patients who failed to respond to conventional therapy. Approximately 10%–15% of CRCs are caused by the hypermutation known as microsatellite instability (MSI), which is a consequence of dMMR. MSI CRC is associated with a lower stage at diagnosis and an improved stage-specific prognosis (though this remains controversial in stage IV patients). Current evidence supports the use of adjuvant chemotherapy with fluoropyrimidine plus oxaliplatin in stage III dMMR CRC. The distinctive genomic and tumor immunoenvironment characteristics of dMMR CRC have paved the way for tailored immunotherapies. It is expected that the mismatch repair status and other pathogenetic biomarkers will be implicated in various cancer types.

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) area heterogeneous group of tumors associated with variable presentations, growth rates, and prognoses; they can even have heterogeneous features within individual patients. The majority of GEP-NENs are nonfunctional. Therefore, the diagnosis of these neoplasms remains challenging. In addition, a lack of treatment options, limitations in present imaging modalities, and limited biomarkers make it difficult to manage NENs. Thus, novel biomarkers are necessary to provide modalities with high sensitivity and specificity for detecting the disease and predicting treatment effectiveness and prognosis. Genomic studies and molecular profiling have revealed a number of genomic alterations. Given that repeated biopsies are not always clinically feasible, the development of blood-based strategies to measure genetic changes in the circulating molecular signatures is relevant for the disease management strategy and analysis of treatment response and outcomes. Liquid biopsies, including the analysis of circulating tumor cells, circulating RNA, and microRNA, might potentially be of clinical relevance. All of these novel circulating biomarkers require urgent evaluation inclinical trials. We discuss novel circulating biomarkers, such as the NETest, circulating tumor cells, and microRNA in the review.

Researchers are increasingly aware that a macroscopic description of a disease cannot cover all of the characteristics of the various subtypes, while a driven gene can reveal similarities among a series of diseases. The development of molecular biological techniques helps us find new ways to re-recognize cancer. It is predicted that treatment patterns of gastrointestinal cancer will move toward personalized therapy based on molecular alterations, resulting in truly meaningful precision medicine.

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REVIEW ARTICLE

Advantages and limitations in the establishment and utilization of patient-derived xenografts in gastric cancer

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Abstract

Owing to the high genetic heterogeneity of tumors, small number of therapeutic strategies available, and frequent presentation of drug resistance, the prognosis for patients with advanced gastric cancer (AGC) are unsatisfactory. The utility of traditional cancer cell lines in translational research is limited by their poor correspondence to the genomic alterations and expression profiles that occur in actual patient tumors. In the last decade, increasing attention has been given to patient-derived tumor xenografts (PDTXs), which can faithfully recapitulate the histopathology, molecular characteristics, and therapeutic responses of the patient's tumor. However, the widespread development and utilization of PDTXs is restricted by factors such as the timeframe of establishment, lymphoma transformation during passaging, the immunodeficient microenvironment, and pharmacokinetic differences between mice and humans. In this review, we summarize the establishment and characterization of PDTX models for gastric cancer (GC). We then weigh the advantages and limitations of PDTXs when used to evaluate novel compounds, identify effective biomarkers, demonstrate resistance mechanisms, and predict clinical outcomes.

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Key words: patient-derived tumor xenograft (PDTX); gastric cancer (GC); preclinical research

In China, gastric cancer (GC) is the third leading cause of cancer-related deaths [1], and about 70% of patients with gastric cancer are diagnosed at an advanced stage [2]. For many years, fluorouracil-based combination chemotherapy has been the main treatment for advanced gastric cancer (AGC), with a clinical response rate of 40% and median survival duration being 10–14 months [3]. Despite the survival benefits that have been achieved by anti-cancer drugs (e.g., trastuzumab [4], ramucirumab [5], and apatinib [6]) that have specific targets, the prognosis for a patient with AGC is still grave, due to the limited molecular targets available and inevitable drug resistance. Therefore, exploring new therapeutic targets is an urgent goal in the development of improved treatments for GC. In preclinical studies, many targeted therapies (using drugs with specific targets) have shown excellent anti-tumor activity, but in most clinical trials, they have resulted in failure [7].

It is well known that suitable preclinical animal models are essential for subsequent clinical trials of human medical treatments. Traditionally, *in vitro* cell lines and *in vivo* animal models derived from cancer cell lines have been the most commonly used tools in predicting the clinical outcomes of novel drugs. Nevertheless, owing to the lack of tumor heterogeneity, differences in microenvironments, and accumulation of genetic aberrations during passaging, cell line-based models have limitations for predicting therapeutic efficacies [8]. In recent years, patient-derived tumor xenograft (PDTX) models have emerged as favorable preclinical models, because they are highly consistent with a patient's own physiological system in terms of biological characteristics and drug response [9–10]. In this review, we summarize the establishment and application of PDTX models for GC

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and elaborate on their benefits and limitations.

Establishment of the PDTX model

PDTX models are established by transplanting freshly resected tumors from human patients into immunodeficient mice. This enables the PDTX animal to preserve the histological morphology, architecture, and molecular features of the patient's original tumor tissues [11]. Increasing numbers of PDTXs have been successfully established for patients with melanoma, lung cancer, breast cancer, pancreatic cancer, and colorectal cancer [12–13]; these PDTXs accurately recapitulate the histopathological features, genomic alterations, and expression profiles of the patients' primary tumors [9, 14–15]. The establishment of PDTX models for GC patients has been reported in several studies [9, 16–28] (Table 1). In these studies, the PDTX was mainly derived from surgical tumor samples taken at an early stage. The stable engraftment rate varied from 5% to 100%, and was associated with the patients' gender [25], histological type [16, 28], procedure times [28], tumor sites [27] [metastatic tissue, 65% (51/79) vs. primary tissue, 27% (10/37), $P < 0.001$], and chemotherapy status [26] [prior to chemotherapy, 52.1% (37/71) vs. after chemotherapy, 21.9% (25/114), $P < 0.001$]. As has been shown for PDTXs of melanoma, the degrees of immunosuppression achieved in recipient mice can differ amongst multiple mouse strains, and this is a critically important factor for successful xenograft establishment [29]. In PDTXs of GC, mice displaying greater immunodeficiency appear to demonstrate a higher transplantation success rate [25, 28] [nude mice, 14/83 (16.9%) vs. non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, 32/119 (26.9%); nude mice, 8% (6/75) vs. NOD/Shi-scid/IL-2Rγnull (NOG) mice 10.5% (9/86)]. Recently, Chijiwa *et al* subcutaneously inoculated 116 surgically removed tumor tissues into NOG mice and established 61 PDTXs [27]. High transplantation rates were observed for tumors of the respiratory (67%), gastrointestinal (58%), and urological (57%) systems [27].

Increasing evidence has shown that the histopathological characteristics of PDTX models are highly consistent with their corresponding primary tumors, and these characteristics are stable over subsequent passages [26, 28]. Recently, a genomic landscape analysis of datasets derived from The Cancer Genome Atlas (TCGA), the Cancer Cell Line Encyclopedia (CCLE), and the Novartis Institutes for Biomedical Research Patient-Derived Tumor Xenograft Encyclopedia (NIBR PDXE) has revealed highly consistent mutation rates between PDTX cells and patient tumor cells [9]; this correspondence has also been specifically verified for GC [26, 28]. In general, PDTX models have retained the genetic expression profiles of their counterpart tumors, nevertheless, genes

Table 1 Summary of patient-derived xenografts of gastric cancer

Study	Year	Mouse strain	Engraftment stable rates	Latency period (day)	Tissue source	HER2 positive	Lymphoma transformation	Correlation with engraftment	PDX concordance with primary tissue
Nakatani [16]	1979	Nude mice	15/33 (45.5%)	NR	Surgery	NR	NR	Histologic type	Histological features
Yoshiyuki [17]	1990	Nude mice	8/32 (25%)	NR	Surgery	NR	NR	NR	NR
El-Rifai [18]	1998	Nude mice	8/NR	NR	Surgery	NR	NR	NR	Genomic features
Milne [19]	2007	Nude mice	3/60 (5.0%)	NR	NR	NR	NR	NR	Histological and genomic features
Jin [20]	2011	Nude mice	1/1 (100%)	NR	Surgery	NR	NR	NR	Histological features
Han [21]	2012	Nude mice	107/114 (94%)*	NR	Surgery	NR	NR	NR	Histological features
Zhang [22]	2013	NOD/SCID	20/NR	NR	Surgery	NR	NR	NR	NR
Chen [23]	2015	NOD/SCID	5/5 (100%)	NR	Surgery	80% (4/5)	NR	NR	Histological features
Huynh [24]	2015	NOD/SCID	8/NR	NR	NR	NR	NR	NR	NR
Zhang [25]	2015	Nude and SCID	49/207 (23.7%)	NR	Surgery	22% (7/32)	NR	Gender and histologic type	Histological and genomic features
Zhu [26]	2015	NOD/SCID	63/185 (34.1%)	65 (11–160)	Biopsies	21.6% (40/185)	1/63 (1.6%)	Chemotherapy status	Histological and genomic features, Chemosensitivity
Gao [9]	2015	NR	215/NR	NR	NR	NR	NR	NR	Genomic features and drug response
Chijiwa [27]	2015	NOG	3/10 (30%)	NR	Surgery	NR	8/61 (13.1%)	NR	Histological and genomic features
Choi [28]	2016	Nude and NOG	15/62 (24.2%)	94 (44–160)	Surgery	NR	5/15 (33.3%)	Histologic type and procedure times	Histological and genomic features

Note: *, 107/114 xenografts derived from 20 patients were established. NOD/SCID, Non-obese diabetic/Severe combined immunodeficiency; NOG, NOD/Shi-scid/IL-2Rγnull; NR, not reported

encoding cell adhesion molecules and immune system regulators have been downregulated [30–31]. This outcome is due to the PDTX being infiltrated with murine stromal components instead of human stromal elements [32].

Overall, the pathological, genomic, and transcriptomic features of PDTXs have been highly consistent with those of the human primary tumors from which they originated. Furthermore, PDTXs remain stable during passaging, which leads to better correspondence between xenografts and original patient tumors in terms of their respective responses to therapeutic chemotherapy [26, 33] and to targeted therapies [9, 23].

Preclinical and clinical utilization

Potential therapeutic targets and the exploration of biomarker efficacy

Traditionally, cancer cell lines were widely used in the process of screening for drug sensitivity [34–35]. Numerous efforts have been made to identify potential therapeutic targets. For example, a single study generated an entire pharmacogenomic landscape of likely functional processes and pathways that interact in cancers, based on integrated analyses of the genomic profiles of 11 289 tumors and 1001 cell lines [36]. A PDTX-based evaluation of those novel targets and compounds has been urgently needed.

In a feasibility study of PDTX clinical trials (PCTs), Gao *et al* [9] evaluated the responses of 62 single or combination therapies on 277 PDTXs. This PCT demonstrated that two pan-PI3K inhibitors (BKM120 and CLR457) and two combination treatments [(LEE011 + everolimus) and (LCL161 + paclitaxel)] had high response rates and increased progression-free survival (PFS); these results had strong implications for future clinical treatments. Other potential therapeutic strategies, such as using the antitumor activity of cetuximab against GC caused by EGFR dysregulation [22], using volitinib against GC caused by c-Met amplification [37], and using BEZ235 [38] and anti-HER3 antibodies in combination with trastuzumab [39] to treat epidermal growth factor receptor 2 (HER2)-positive GC, have been reported. Furthermore, the multikinase inhibitor regorafenib was found to reduce tumor angiogenesis and proliferation, induce apoptosis in xenografts of GC [24], and have antitumor activity consistent with the findings of the so-called "INTEGRATE" large-scale collaborative study [40].

Molecular mechanisms of therapy resistance

As the first approved drug targeting epidermal growth factor receptor 2 (HER2), trastuzumab induces antibody-dependent cellular cytotoxicity (ADCC) and blocks HER2-mediated signaling. This results in a response rate of 47.3% when combined with chemotherapy [4]. In contrast, subsequent clinical trials of lapatinib failed to demonstrate survival benefits [41–42]. Overall, the efficacy of these anti-HER2 therapies is limited by intrinsic and acquired drug resistance; this is caused by activation of HER2's downstream pathway, and by up-regulation of HER3 and IGF1R [43].

An ongoing clinical trial determined that up-regulation of HER2, at a high level, occurred in patients who were resistant to trastuzumab but sensitive to afatinib [44]. Pre-trastuzumab and pre-afatinib tissues were obtained to establish PDTXs, conduct protein mass spectrometry, and carry out next-generation sequencing to monitor proteomic and genomic alterations. Mutations of the following genes (resulting in the substitutions indicated) were identified both before and after treatment with trastuzumab: *ERBB3* (V104M), *RUNX1* (r174*), *CARD11* (P567T), *PTPRS* (V276M), and *MAGI2* (L114V). Additionally, the following mutations, as well as other alterations, might contribute to the acquired resistance of trastuzumab: *TP53* (R175H), *MYC* (R83W), *ALK* (L1162Q), and *MLL2* (E622*). Meanwhile, preclinical and clinical results suggested that amplification of EGFR might be associated with afatinib sensitivity in trastuzumab-resistant patients.

A recent study revealed that lapatinib epigenetically induces the upregulation of transcription factor c-Myc, which in turn reduces the sensitivity of breast cancer cells to lapatinib. This negative feedback loop could be suppressed by combining lapatinib with an epigenetic inhibitor [45]. Interestingly, upregulation of c-Myc also has a role in the acquired resistance to c-Met inhibitors that occurs in GC; a combined treatment consisting of c-Myc inhibitors and c-Met blockade was found to exert synergistic antitumor activity in MET-amplified PDTXs [46]. Whether c-Myc functions as a downstream effector in the drug resistance to anti-HER2 therapy that has been observed in GC remains to be explored, using suitable xenografts.

Another important determinant in cancer cell resistance against chemotherapy and other potential target therapies is the activation of the PI3K/AKT pathway. Li *et al* [47] demonstrated that PDTXs containing a pathway-activating mutation of PIK3CA responded to the AKT inhibitor AZD5363. They also showed that the combination of AZD5363 and taxotere could overcome the intrinsic resistance to taxotere observed in a PDTX where the tumor suppressor protein PTEN had been disrupted.

Co-clinical trials and mouse avatars

Tremendous efforts have been made to facilitate the transfer of scientific breakthroughs from one system into another: in this case, from the mouse models (of various human diseases) in which breakthroughs have been

achieved, to the realm of human medical treatment. In an era of precision medicine, the concept of co-clinical trials and the use of "mouse avatars" have come into being, with the aim of integrating mouse-based biomedical techniques into clinical guidance. The "co-clinical trial project" utilizes genetically engineered mouse models (GEMMs) to guide ongoing human clinical trials; the validity of this method was proved by Pandolfi *et al* while researching acute promyelocytic leukemia (APL) [48]. In contrast, the "mouse avatar" technique exploits the capability of PDTX models to faithfully recapitulate the characteristics of a specific patient's tumor, facilitating the evaluation of multiple novel drugs or drug combinations for efficacy against that tumor [49]. Both of these initiatives emphasize the real-time integration and analysis of preclinical and clinical data, which contributes to the stratification of responders, prioritization of drug combinations, demonstration of resistance mechanisms, and exploration of biomarkers. Whereas GEMMs are usually engineered based on the well-defined driver mutations of particular cancers, PDTXs can preserve the accumulated effects of multiple mutations that have not yet been individually cloned and identified.

In 2012, Morelli *et al* [50] conducted a phase I trial that evaluated the efficacy of 22 drugs on xenografts derived from a 29-year-old patient with advanced adenoid cystic carcinoma (ACC). On the basis of the preclinical data, the patient was then given a combination treatment of figitumumab (an anti-IGF1R monoclonal antibody) and PF00299804 (a pan-EGFR inhibitor) for four cycles, followed by figitumumab alone (due to the severe diarrhea related to PF00299804). This resulted in a minor response in the patient's rapidly growing liver lesion [50]. Other studies have demonstrated that mouse avatars can faithfully replicate clinical outcomes in the treatment of small cell lung cancer [51], advanced sarcoma [52], colorectal cancer [53], and HER2-positive, trastuzumab-refractory esophagogastric (EG) cancer [44].

Limitations and corresponding solutions

Despite a PDTX model's resemblance to a specific instance of a human disease, several limitations to this method need to be recognized.

The period from model establishment to clinical guidance

Widespread use of mouse avatars to treat GC is limited by the time and cost required to establish stable xenografts. In terms of clinical decision-making timeframes, the period from stable mouse avatar engraftment to preclinical data collection may be too long to be of practical use. Although transplantation procedure optimization (e.g., through the use of support matrix materials and the minimization of *ex vivo* procedure time) and high-throughput screening based on genomic profiles would reduce the time needed to complete the process, many significant improvements must be made before we see the universal application of mouse avatars to the treatment of GC. However, using PDTX models not only to generate treatment recommendations for the individual donor of the specific PDTX, but also to identify novel therapeutic strategies and biomarkers that can be applied to the treatment of future patients experiencing tumors with molecular characteristics similar to those of a given PDTX, is a concept with more significant clinical impact.

Lymphoma transformation during the passaging of PDTX

When the tumor-burden becomes too great for a PDTX mouse, researchers "passage" that tumor into the next generation of PDTX mice. It is noteworthy that lymphoma transformation accounted for 1.6% (1/63) to 33.3% (5/15) of the transplantable GC PDTXs observed during the passaging of tumor tissue. This kind of lymphoma mainly originates from patients who had epithelial tumors and who presented pathological characteristics of B-cell lymphoma [54–55], especially in tissues infected with the Epstein-Barr virus (EBV) [26–28]. This phenomenon has been reported in PDTX models for various primary cancers inoculated into NOD/SCID [56], NSG [57], and NOG [58] strains of mice, but not for those inoculated into nude mice. One possible explanation is that EBV infected the B-cells in donors, then remained latent until implantation into immunocompromised mice; however, natural killer cells in nude mice can interfere with the reactivated EBV, thus resulting in their resistance to lymphoma transformation in PDTXs [57]. The identification and reduction of lymphoma transformation are critical factors to improving the successful engraftment rate of PDTXs. This could be facilitated by: (1) the detection of EBV-infection and inflammatory infiltration of the primary tissues, before inoculation; (2) histopathology diagnosis during passaging; and (3) blockade strategies using rituximab [58].

The immunodeficient microenvironment

The necessity of using immunodeficient hosts in the establishment of PDTXs results in the rapid loss of human stromal elements and human functional immune system elements. This restricts the therapeutic response of the PDTX to immunomodulatory agents. However, the emergence of humanized mice has helped to address this obstacle. Patient-matched immune systems could be reconstructed in the xenograft environment by mobilizing hematopoietic stem and progenitor cells (HSPCs) [59]

or mature circulating peripheral blood mononuclear cell (PBMCs) [32] from the patient. Admittedly, this is a great challenge because inappropriate immune responses against human or murine tissues might be induced [32].

Divergence in pharmacokinetic and metabolic profiles

There are vast differences between the pharmacokinetic and metabolic profiles of the murine system vs the human system. Wong *et al* [60] reported treatment efficacies in humans that were consistent with those in xenografts that had received the same treatment. Instead of employing the maximum tolerated dose (MTD) in mice, which might lead to overestimations of therapeutic responses, dosages were calculated according to the pharmacokinetic parameters of humans. Furthermore, whether certain novel compounds are safe for human should be taken into considerations. Currently, several software packages – including Cloe® PK (Cyprotex), PK-Sim5® (Bayer Technology), and GastroPlus™ (Simulations Plus, Inc.) – allow for the comparison of pharmacokinetic parameters and extrapolation of PDTX model testing results to clinical trials [49].

Conclusions and future prospects

In summary, while PDTX models can closely approximate very specific human pathologies, they are not perfect replications of those pathologies. As interest in and experience with PDTXs has increased over the past decade, databases with clear histopathological and molecular backgrounds have been created, such as the PDX collection of the EurOPDTX Consortium (http://europdx.eu/pdx-collection.html) and the repository of PDX models maintained by the Jackson Laboratory (http://jaxservices.jax.org/invivo/PDTX.html). Those resources significantly facilitate the clarification of tumor biology, evaluation of drug efficacy, demonstration of drug resistance mechanisms, and identification of biomarkers [61]. Ongoing efforts to overcome the limitations of the PDTX method may broaden its application further and strengthen its reliability as a guide for individual patient therapies.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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REVIEW ARTICLE

Gastric molecular classification and practice in immunotherapy

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Abstract

Gastric cancer (GC) is a highly heterogeneous malignancy with a high incidence worldwide; the prevalence of GC is relatively higher in China than in other countries. Treatment of advanced GC has been slow to develop due to lack of a proper classification system to guide clinical practice. With the development of molecular biology techniques, the molecular classification of GC has been established and may have applications in guiding precise and personalized therapy. To date, three or four molecular classifications for GC have been recognized; these include Singapore, the Cancer Genome Atlas (TCGA) Research Network, and Asian Cancer Research Group (ACRG) classifications. Here, we review the development of molecular classifications and characteristics of different subtypes, and discuss the applications of molecular classifications in clinical practice, with a focus on immunotherapy.

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Gastric cancer (GC) is the third leading cause of global cancer-related mortality, and the prevalence of GC in China is higher than that in other countries around the world, with morbidity and mortality rates of 42.5% and 45%, respectively [1]. According to cancer statistics for 2015 published by China's National Cancer Center, GC ranks second of all tumors in estimated morbidity and mortality rates and ranks first for both parameters in rural areas [2].

Treatment methods for advanced GC have been slow to develop, and chemotherapy remains the backbone of therapeutic strategies. In contrast to melanoma, non-small cell lung cancer, and breast cancer, targeted therapies and immunotherapies have not yet been extensively applied. This discrepancy could be related to the lack of proper molecular classifications to guide clinical practice. Traditional clinicopathological classification systems have mainly included Lauren and World Health Organization (WHO) classifications. These classifications depend on cell and tissue morphology observed under a microscope and they are influenced by many subjective factors. Moreover, classifying results cannot accurately reflect the biological behaviors of tumors [3]. With the development of genomics, transcriptomics, proteomics, and metabolomics, molecular classification of GC has emerged and it shows potential in guiding precise, personalized therapy.

Molecular classification

The concept of tumor molecular classification was first proposed in the 1990s and referred to classifying tumors using information obtained from comprehensive molecular analysis [3]. After this concept was proposed, hundreds of thousands of studies have investigated molecular classifications in different types of tumors, generating huge amounts of data and facilitating the establishment of several different molecular classifications. To date, three or four GC molecular classifications have been recognized.

In 2011, Tan et al. identified two intrinsic GC subtypes (G-INT and G-DIF) by analyzing the gene expression profiles of 37 GC cell lines and validated these subtypes in primary tumors from 521 patients in four independent cohorts. They found that two intrinsic subtypes were associated with patient survival and response to chemotherapy [4].

In 2013, Lei *et al* compared gene expression patterns among 248 gastric tumors and identified three major subtypes (proliferative, metabolic, and mesenchymal). The subgroups exhibit differences in molecular and

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genetic features and response to therapy and have also been shown to be associated with Lauren and Tan classifications. Cancer cells of the metabolic subtype are more sensitive to 5-fluorouracil, whereas cancer cells of the mesenchymal subtype include cells with features of cancer stem cells and are particularly sensitive to phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) inhibitors *in vitro*, providing important insights into clinical treatment strategies [5].

In 2014, the Cancer Genome Atlas (TCGA) Research Network published their molecular classification in Nature [6]. After comprehensive molecular evaluation of 295 primary gastric adenocarcinomas using single nucleotide polymorphism array somatic copy-number analysis, whole-exome sequencing, mRNA sequencing, miRNA sequencing, array-based DNA methylation profiling, and reverse-phase protein arrays, they proposed a molecular classification dividing GC into four subtypes: tumors positive for Epstein-Barr virus (EBV+), microsatellite unstable tumors (MSI), genomically stable tumors (GS), and tumors with chromosomal instability (CIN). The clinical and molecular characteristics are summarized in Table 1.

In 2015, the Asian Cancer Research Group (ACRG) published a new molecular classification in Nature Medicine [7]. The group procured 300 primary GC tumor specimens, and, through analysis of data from next-generation sequencing, they classified GC into four subtypes: mesenchymal-like type (epithelial mesenchymal transition [EMT]), microsatellite-unstable type (MSI), p53 (TP53)-active type (MSS/p53+), and TP53-inactive type (MSS/p53–). ACRG also validated these subtypes in independent cohorts in order to provide a consistent and unified framework for further clinical and preclinical translational research. Characteristics of the four subtypes are summarized in Table 2.

Practice in immunotherapy

Current approaches to GC management largely consist of endoscopic detection, followed by gastrectomy and chemotherapy (CT) or chemoradiotherapy (CRT) in a neoadjuvant or adjuvant setting. For advanced GC, the efficacy of CT and CRT is not satisfactory. One of the key reasons for observed heterogeneity in response to treatments is a one-size-fits-all approach to treatment. With the development of next-generation sequencing and bioanalysis techniques for large datasets, we are entering the age of precision medicine [8]. The aim of precision medicine is to improve efficacy and reduce adverse reactions through screening patients for genes, biomarkers, phenotypes, and social psychological characteristics [9]. Traditional clinicopathological classifications cannot fully and accurately reflect the biological heterogeneity of tumors; therefore, the appropriateness of some treatment strategies is unclear. The development and establishment of molecular classifications may provide a solid foundation for precision treatment.

Targeted therapy and immunotherapy are the two important components of precision medicine and show some overlap. After the ToGA study demonstrated that trastuzumab in combination with chemotherapy for human epidermal growth factor receptor 2 (HER2)-positive advanced GC as a first-line regimen was superior to chemotherapy alone [10], several receptor tyrosine-kinase (RTK)-targeted drugs were investigated in GC. However, the majority of these studies yielded negative results. A retrospective analysis suggested that one important reason for the failure is the absence of biomarker-driven trials or the methodology of biomarker selection [11]. In summary, tumor cell-targeted therapies have not been sufficiently established in GC, and further studies are needed.

As has been observed in other cancer types, the use of immunotherapy approaches may improve outcomes in patients with GC. Generally speaking, immunotherapy includes active immunotherapy (e.g., cancer vaccines and immunogenes), passive immunotherapy (e.g., adoptive immune cell transfer and some monoclonal antibodies), and nonspecific immunomodulator therapy (e.g., cytokines and checkpoint inhibitors) [12–14]. Checkpoint inhibitor therapy is currently a research hotspot that is relatively mature and well recognized, and molecular classification may have great potential value in guiding clinical practice in this field. Using genomic technology in GC in an effort to improve our understanding and the stratification of GC on a genetic and molecular level, TCGA classification has revealed that patients with EBV-positive and MSI subtypes may be the appropriate population for immunotherapy approaches. EBV-positive tumors, possibly derived from viral stimulation, may show amplification of genes that encode the immunosuppressant proteins programmed death ligand (PDL) 1 and 2. KEYNOTE-012 and CheckMate-032 trials have suggested a trend toward improved response rates and progression-free survival (PFS), with higher levels of PD-L1 overexpression, although the validity of PD-L1 as a robust biomarker of response should be confirmed in additional studies [12, 15, 16]. The MSI subgroup is characterized by gene promoter hypermethylation and displays a high mutational load, including alterations in major histocompatibility complex (MCH) class I genes.

To date, checkpoint inhibitors targeting PD-1, PD-L1, and T lymphocyte antigen (CLTA)-4 have had a major impact on clinical practice. These three types of checkpoint inhibitors have been evaluated in multiple tumor types with confirmed responses in GC.

Table 1 TCGA classification and characteristics

Subtype	Clinicopathological characteristics	Percentage (%)	Molecular characteristics
EBV+	Tend to be male, present in the gastric fundus or body	9	Mutations in *PI3KCA* (80% nonsilent), *ARID1A*, *BCOR*; rare *TP53* mutations; EBV-CIMP; higher prevalence of DNA hypermethylation; *CDKN2A* ($p16^{INK4A}$) hypermethylation; lack *MLH1* hypermethylation; *JAK2*, *ERBB2*, *CD274 (PD-L1)*, *PDCD1LG2 (PD-L2)* amplification; PD-L1/2 overexpression; immune cell signaling
MSI	Tend tobe female, diagnosed at relatively older ages	22	MSI-high status; hypermutations (*PIK3CA*, *ERBB3*, *ERBB2*, *EGFR*, *B2M*, *HLA-B*); lack targetable amplification; no *BRAF* V600E hmutation; hypermethylation; *MLH1* silencing (*MLH1* promoter hypermethylation); gastric-CIMP; mitotic pathway
GS	Diagnosed at an earlier age, diffuse histology	20	Mutations in CDH1, RHOA; CLDN18-ARHGAP fusion; cell adhesion; few other clear treatment targets.
CIN	Elevated frequency in the geal gastroesopha junction/cardia, intestinal histology	50	Genomic amplification of RTKs, RTK-RAS activation; elevated phosphorylation of EGFR; recurrent amplification of the gene encoding ligand VEGFA; *TP53* mutation (71%); elevated p53 expression.

PI3KCA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; ARID1A: AT-rich interactive domain-containing protein 1A; BCOR: BCL-6 corepressor; CIMP: CpG island methylator phenotype; CDKN2A: cyclin dependent kinase inhibitor 2A; MLH1: mutL homolog 1; JAK2: Janus kinase 2; ERBB2: erb-b2 receptor tyrosine kinase 2; PDCD1LG2: programmed cell death 1 ligand 2; PD-L1/2: programmed dead ligand 1/2; EGFR: epidermal growth factor receptor; B2M: beta-2-microglobulin; HLA-B: human leukocyte antigen-B; CDH1: cadherin 1; RHOA: Ras homolog A; CLDN18: Claudin-18; ARHGAP: Rho GTPase activating protein; RTKs: receptor tyrosine kinases; VEGFA: vascular endothelial growth factor A

Table 2 ACRG classification and characteristics

Subtype	Clinicopathological characteristics	Percentage (%)	Molecular characteristics
EMT	Diffuse-type predominant (> 80%); younger age; poorer prognosis; higher chance of recurrence (63%); most recurrence for peritoneal seeding	15.3	Lower number of mutation events; less CDH1 expression; no RHOA mutations
MSI	Intestinal subtype (60%); predominantly in the antrum, diagnose at early stage; best prognosis; lower chance of recurrence (23%); higher percentage of liver-limited metastasis recurrence	22.7	Loss of MLH1; DNA hypermutation; hypermutation, such as mutations in KRAS, PI3K-PTEN-mTOR pathway, ALK, and ARID1A genes; enrichment of PIK3CA H1047R mutations
MSS/p53+	Moderate prognosis; more frequent EBV infection	26.3	p53 activation; relatively higher prevalence of mutations in APC, ARID1A, KRAS, PIK3CA, SMAD4
MSS/p53–	Moderate prognosis	35.7	Highest prevalence of TP53 mutations; recurrent focal amplifications in ERBB2, EGFR, CCNE1, CCND1, MDM2, ROBO2, GATA6, and MYC with corresponding increases in mRNA and protein expression levels

CDH1: cadherin1; RHOA: Ras homolog gene family, member A; MLH1: mutL homolog 1; KRAS: kirsten rat sarcoma viral oncogene homolog; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN: phosphatase and tensin homolog; mTOR: mammalian target of rapamycin; ALK: anaplastic lymphoma kinase;ARID1A: AT-rich interactive domain-containing protein 1A; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; APC: amino acid-polyamine-organocation; SMAD4: mothers against decapentaplegic homolog 4; ERBB2: human epidermal growth factor receptor 2; EGFR: epidermal growth factor receptor; CCNE1: cyclin E1; CCND1: cyclin D1; MDM2: mouse double minute 2 homolog; ROBO2: Roundabout homolog 2; GATA6: GATA-binding factor 6

Anti-PD-1 drugs

Pembrolizumab is a highly specific, humanized monoclonal IgG4 antibody against PD-1. Its activity has been and is being investigated in many phase I to III clinical trials, including GC cohorts, investigating drug therapy or use in combination with chemotherapy or other monoclonal antibodies targeting HER2 or vascular endothelial growth factor receptor 2 (VEGFR2) (ClinicalTrials.gov identifier: NCT02335411 [KEYNOTE 059], NCT02370498 [KEYNOTE 061], NCT0249458 [KEYNOTE 062], NCT02443324, NCT02563548, NCT02318901) [17]. In the phase Ib KEYNOTE 012 trial, 39 patients treated with pembrolizumab showed an overall response rate (ORR) of 22% by investigator review, with a 6-month PFS of 24% and median duration of response of 6 months [15]. Another monoclonal antibody against

PD-1, nivolumab, was investigated in the phase Ib/II CheckMate 032 trial, in which the activity of single-agent nivolumab or nivolumab plus ipilimumab (an anti-CTLA-4 monoclonal antibody) was explored in multiple tumor types, with the initial report on the GC cohort presented at GI ASCO 2016 [16, 18]. Irrespective of PD-L1 status, patients were treated with single-agent nivolumab, and the ORR was 14%, with a median duration of response of 7.1 months. Further analysis showed that the ORRs in patients with PD-L1-positive (≥ 1% cutoff) and -negative tumors were 27% and 12%, respectively [12]. Two trials of nivolumab are currently ongoing (ClinicalTrials.gov identifier: NCT01928394 and NCT02267343).

Anti-PD-L1 drugs

Three anti-PD-L1 antibodies, i.e., atezolizumab (MPDL3280A), dervalumab (MEDI4736), and avelumab (MSB0010718C), were evaluated in GC and showed confirmed responses [19–21]. In a phase Ib trial, avelumab in two GC cohorts achieved ORRs of 15% with a median PFS of 11.6 weeks and 7.3% with a median PFS of 14.1 weeks [21].

Anti-CTLA-4 drugs

Tremelimumab and ipilimumab are two anti-CTLA-4 monoclonal antibodies tested in several trials. In a small phase II trial, tremelimumab was used as second-line therapy for GC, but the ORR (5%) and median overall survival (OS; 4.8 months) did not meet the expected results [22]. A phase Ib/II trial to combine tremelimumab and dervalumab in refractory GC is ongoing (ClinicalTrials.gov identifier: NCT02340975). In a recently completed phase II trial, ipilimumab was used as a maintenance drug after first-line CT and showed a shorter PFS (2.9 versus 4.9 months) but longer OS (16.8 versus 12.1 months), but the longer OS did not reach statistical significance (NCT01585987). The results of the CheckMate-032 trial for the combination of ipilimumab and nivolumab and the NCT01928394 trial for the combination of ipilimumab and nivolumab for refractor GC have not yet been published [16, 23].

Discussion

From 2011 to 2015, GC molecular classifications were rapidly established; TCGA and ACRG classifications are the most commonly used classifications and provide important information for molecular diagnosis, personalized therapy, and development of targeted and immunotherapy drugs. Molecular classifications can permit stratification of patients according to genomic and proteomic information, providing insights into clinical guidance. In theory, it is more reasonable to develop targeted therapies for diseases with the same molecular aberrations than to treat cancers with similar morphologies using CT. Thus, we predict that traditional treatment strategies based on tumor phenotypes will be replaced by precision medicine based on features of genomic aberrations [3].

Targeted therapy in GC has only succeeded in a few trials; this lack of efficacy can be attributed to the highly heterogeneous nature of GC caused by protein expression, gene amplification, and gene mutations and to insufficient selection of patient groups by biomarkers. As immunotherapy gradually becomes a major research focus, similar problems will arise. Immune responses are dynamic, and there is still a lack of consensus on optimal assaying techniques, such as for adequate definition of PD-L1 positivity, with trials using differing antibodies and staining cut-off points [24]. Several studies have discussed immune-related gene expression signatures as promising biomarkers [25–26]. Based on the tumor microenvironment, a framework for classifying tumors according to tumor-infiltrating lymphocytes (TILs) and PD-L1 expression has been proposed [27]. However, all of these ideas must be evaluated in further studies.

Additionally, problems associated with resistance mechanisms to targeted therapies and immunotherapies, alterations in molecular phenotypes, activation of bypass signaling, advantages and disadvantages of monoclonal antibodies and small molecular inhibitors, concomitant and combined medicines for targeted therapy and immunotherapy or traditional therapy, and understanding and targeting of the tumor microenvironment are all linked to molecular changes. Thus, further studies are required to obtain critical genomic, transcriptomic, proteomic, and metabolomic data.

A macroscopic description of diseases will not cover all the characteristics of different groups, although a driver gene can exhibit similarities in a series of diseases. The development of molecular biology techniques may help us to identify new methods to recognize and diagnose sickness. We predict that treatment patterns for GC and all other tumors may be replaced with personalized therapies based on molecular classifications, allowing the realization of truly meaningful precision medicine.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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REVIEW ARTICLE

Circulating biomarkers for nonfunctional gastroenteropancreatic neuroendocrine neoplasm: Where do we stand?

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Abstract

Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) encompass a heterogeneous group of tumors associated with variable presentations, growth rates, and prognoses. The majority of GEP-NENs are nonfunctional, and their diagnosis remains challenging given the often subtle and variable clinical manifestations of these tumors. As a consequence, GEP-NENs are often recognized at an advanced stage; indeed, most patients with nonfunctional GEP-NENs exhibit metastatic disease at diagnosis. Lack of treatment options as well as limitations in currently available imaging modalities and biomarkers make it challenging to manage NENs. Thus, novel biomarkers are needed to provide high sensitivity and specificity for minimum disease detection and to predict treatment efficacy and prognosis. Although tissue-based biomarker data can provide such information, circulating biomarkers such as NETests, circulating tumor cells, and microRNAs, are superior owing to their easy accessibility and the ability for repeated real-time sampling.

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Key words: neuroendocrine neoplasm; biomarker; circulating tumor cells; NETest; microRNA

Neuroendocrine neoplasms (NENs) constitute a heterogeneous group of tumors, and the tumor phenotype can range from indolent to almost completely unregulated growth, resulting in aggressive invasion and metastasis [1]. GEP-NENs are the most common sites, accounting for 55%–70% of all NENs [2]. The incidence of all GEP-NENs has increased markedly from 2.48 per 100,000 persons in 1994 to 5.86 per 100,000 in 2009 [3]. GEP-NENs can be defined as functioning or nonfunctioning depending on the presence of a syndrome related to inappropriate hormone secretion. Most GEP-NENs are nonfunctional and lack specific manifestations; thus, they are often diagnosed at a late stage at which metastatic progression is observed. Two critical unmet needs are the inability to establish an early and accurate diagnosis and the evaluation of NEN therapeutic responses. The latter is mainly based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria, which is difficult to assess in indolent lesions. Overall, the current criteria for the assessment of NEN progression and therapeutic responses is suboptimal. Given the limited accuracy of currently available biomarkers, NEN-specific biomarkers are necessary to ensure scientific and clinical value.

Biomarkers are classified into three categories by the National Institutes of Health. Type 0 biomarkers suggest the natural history of the disease, type I biomarkers reflect interventional effects, and type II biomarkers are surrogate clinical endpoints [4]. Ideal biomarkers should be multidimensional, providing information on the specific diagnosis, proliferative and metastatic capacity, presence of residual lesions, and therapeutic responses [5]. Recent molecular studies have investigated the genomic landscapes of these tumors. These studies have resulted in the identification of mutations and expression anomalies in genes and pathways, such as the ATRX-DAXX, multiple endocrine neoplasia type 1 (MEN1), and phosphoinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways, as well as epigenetic alterations, such as DNA methylation, histone modification, chromatin remodeling, and extension of alternative telomerase activation mechanism [6–9]. These discoveries in turn may lead to new and better prognostic biomarkers and

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additional candidates for targeted therapies. However, it is still unclear whether such molecular biomarkers may be correlated with biological behaviors and clinical features. Compared with tissue biopsies, sampling the blood is minimally invasive and allows for dynamic monitoring of molecular changes in the tumor rather than relying on a static time point. In this review, we summarize the approach for rational validation of potential circulating candidates that may be involved in nonfunctional GEP-NENs management. We also focus on recent advances in our understanding of the roles of these biomarkers as diagnostic/prognostic factors and the optimal therapeutic approaches for management of GEP-NENs.

Current biomarkers

Current neuroendocrine tumor biomarkers include chromogranin A (CgA), neuron-specific enolase (NSE), pancreatic polypeptide, gastrin, and 5-hydroxyindoleacetic acid (5-HIAA). However, these biomarkers are not sufficient for accurate identification of the primary tumor site and prediction of prognosis. Immunohistochemistry for CgA is not sufficiently specific for the diagnosis of NENs and may be negative in poorly differentiated neuroendocrine carcinomas (NECs). Moreover, chromogranin B is more prevalent in colorectal and appendix NENs [10]. Ki67 antigen, a proliferation-related protein, is associated with biological behavior, treatment responses, and prognosis. However, morphologically well-differentiated NENs can have high Ki67-labeling indices (20%–50%). In fact, about 40% of grade 3 GEP-NENs are well differentiated and could be better designated as "NET G3". Notably, studies have shown that NET G3 lesions have a less aggressive phenotype and exhibit sensitivity to platinum-based chemotherapy compared with poorly differentiated high-grade NECs [11]. Alternative evaluation methods, such as circulating biomarkers, have been investigated. The best known circulating biomarker is plasma CgA. Increased CgA is generally considered to be sensitive (60%–90%) and accurate once an NEN has been identified [12]. Moreover, an early decrease in CgA after treatment is positively correlated with survival rate [13]. However, measurements are usually nonspecific (10%–35% specificity) since CgA is elevated in other conditions, including neoplasia, renal failure, cardiac and inflammatory diseases, and proton pump inhibitor (PPI) administration [14]. A standard cutoff value does not exist, and variations occur in measurements across different laboratories. Thus, novel biomarkers associated with accurate diagnosis and assessment of treatment and prognosis are needed. Current mono-analyte blood-based biomarkers for diagnosis and follow-up of NENs do not achieve satisfactory metrics of sensitivity and specificity.

Multi-analyte assays with algorithmic analytics and NETest

Specific mono-analytes, which define the secretory status of a tumor, have been proven to be useful in diagnosis but are disappointing in the assessment of disease progression. Current scientific analyses of biomarker identification have focused on the development of multi-analyte assays with algorithmic analytics (MAAAs). This strategy facilitates the coupling of integral aspects of disease represented by individual markers into a mathematical algorithm that provide multidimensional clinical and pathobiological information inaccessible in a mono-analyte approach [15–16]. MAAAs have been used to identify circulating NET transcripts and have shown that blood measurements are correlated with tumor tissue transcript analysis. The latter is segregated into six gene clusters, which differentiate SD from PD [17]. The MAAA-derived values allow for a sensitive, noninvasive approach for detection of NENs. Such an approach may be rendered even more informative in combination with assessments provided by integration with objective data obtained from imaging and nuclear medicine scanning.

Blood-based multi-analyte algorithm analysis polymerase chain reaction (PCR)-based tests (NETests) show potential for GEP-NEN management. NETest assays are not affected by factors unrelated to NEN disease and exhibit a high specificity of 97% and sensitivity of 98%. For example, the NETests are not elevated in patients receiving PPIs, making them superior to plasma CgA [18]. Under such conditions, NETests may be more reflective and specific in comparison with mono-analyte tests. Moreover, NETests provide additional information that can be used for the detection of disease recurrence and prediction of the therapeutic response of stabilized analogs of somatostatin (SSA) and peptide receptor radionuclide therapy (PRRT) [19]. Considering the relatively indolent and slow progressive behaviors of GEP-NENs, the RECIST criteria and mono-analyte markers are not sufficient to accurately assess residual lesions and recurrence. In addition, NETests are considered as useful in assessment of the adequacy of operative resection and radiofrequency ablation [20]. Therefore, NETests have the potential for precise determination of residual disease, minimal disease detection, and recurrence for patients after R0 resection. However, due to the limited data and short follow-up period, additional studies are needed to establish the most accurate timing of blood collection and other metrics in the prediction of residual/recurrent disease.

MicroRNA (miRNA)

miRNAs are short, noncoding RNAs of approximately 21–23 nucleotides that can interfere with protein expres-

sion either by inducing cleavage of their specific target mRNAs or inhibiting their translation. miRNAs have been shown to regulate a rapidly increasing list of complex biological processes, including cell proliferation, the cell cycle, and apoptosis. miRNAs play an important regulatory role in tumor development and progression, suggesting a wide spectrum of novel diagnostic and therapeutic opportunities. Because of the rarity of the tumor and lack of cell lines, few studies have reported the miRNA signatures of GEP-NENs, and most studies have concentrated on neuroendocrine tumors located in the pancreas and small intestine. Unique miRNAs expression profiles have been shown to be associated with different types and subsets of GEP-NENs. Roldo [21] investigated the global miRNA expression patterns in the normal pancreas and pNENs; the results demonstrated that the upregulation of miR-103/107, associated with a lack of miR-155 expression, was greater in pNENs than in the normal pancreas. The data showed that miRNA expression could distinguish pNENs from normal pancreas tissues. Moreover, overexpression of miR-21 was found to be associated with high rates of pNEN tumor proliferation and liver metastasis. miR-133a was shown to be downregulated during progression from primary to metastatic SI-NENs, suggesting that this miRNA may have an important role in tumor development and progression with utility for prognosis [22]. Li [23] characterized nine miRNAs in well-differentiated SI-NENs, five (miR-96, miR-183, miR-196a, and miR-200a) were upregulated during tumor progression, whereas four (miR-31, miR-129-5p, miR-133a, and miR-215) were downregulated.

miRNAs have potential applications as novel diagnostic and predictive biomarkers. Additional studies are needed to clarify the roles and mechanisms of miRNA expression in biological behavior and may improve early detection rates and accurate assessment of prognoses. In contrast to DNA or mRNAs, miRNAs have long half-lives in vivo and are stable *in vitro*, enabling miRNA profiling techniques to be extremely sensitive, objective, and standardized, even in formalin-fixed tissues. Indeed, miRNAs can be extracted from various specimen types, including fresh or formalin-fixed paraffin-embedded (FFPE) samples, and body fluids, such as plasma, serum, urine, and sputum [24].

Recently, miRNAs have been shown to be differentially expressed and have roles in the regulation of oncogenes or tumor-suppressor genes. The modulation of miRNAs may affect tumor proliferation, and this approach could be transferred to the clinic setting [25]. The therapeutic application of miRNAs involves two strategies. The first is directed against gain-of-function and aims to inhibit oncogenic miRNAs using miRNA antagonists. The second strategy, miRNA replacement, involves the reintroduction of a tumor-suppressive miRNA to restore a loss of function [26]. However, the association between miRNA concentrations in sera and tissues is weak. Both up- and downregulation of miRNA expression have been noted in NENs, suggesting that the use of this marker could be more complex than expected. Moreover, the roles of miRNAs in various genetic networks and regulatory pathways need to be analyzed in larger cohort neoplastic and normal tissues. From a therapeutic standpoint, adequate assessment of the functional effects after miRNA inhibition and antagonism *in vivo* are critical for the clinical application of anti-miR-based therapies.

Circulating tumor cells (CTCs)

CTCs are known to shed into the peripheral blood from solid tumors and therefore provide a less invasive and easily accessible source of tumor material that can be collected in a serial fashion. The presence and persistence of CTCs have been associated with decreased progression-free and overall survival in patients with metastatic breast, colorectal, and prostate cancer [27]. Currently, CTCs have been reported in blood samples from a number of patients with metastatic GEP-NENs (43% in the midgut and 21% in pNENs). In addition, CTCs are associated with progressive NENs and could be used as prognostic markers [28].

The CellSearch platform detects CTCs with high sensitivity and specificity and is the only system approved by the US Food and Drug Administration. The CellSearch platform requires the cellular expression of epithelial cell adhesion molecule (EpCAM), and the majority of NENs exhibit strong expression of EpCAM. Khan [29] analyzed 176 patients with metastatic NENs and showed that 49% of patients had at least one CTC in 7.5 mL blood. CTCs remain significant when other prognostic markers, including grade, tumor burden, and CgA levels, are considered. A liver metastatic burden of over 25% has been shown to be correlated with increased CTCs.

Studies have shown that CTCs in NENs may be heterogeneous. The heterogeneity may have important implications as mutations may arise when cells are shed from the primary tumor or could occur in the circulation; the latter may represent an escape mechanism from therapy. The CTCs of NENs correlate with prognosis and even have a role in adjuvant therapy through reflecting the response to chemotherapy. For example, in patients with GEP-NEN treated with SSA therapy, expression of somatostatin receptor 2 (SSTR2) and SSTR5 can predict treatment response [30]. Thus, the molecular characterization of CTCs could potentially assist in understanding NET metastasis and resistance to therapy in addition to their utility as biomarkers. However, additional studies are needed to determine how to cluster the entire spectrum of CTCs accurately and how to analyze specific subtypes. Moreover, the relationships among pathological and prognostic information need to be verified. CTCs

are not sensitive to detect of different types of NENs and are not specific for subgroups of NENs; thus, these cells should be evaluated in future studies.

Other circulating biomarkers

Recent studies have discovered more potential circulating biomarkers and solidified the potential utility of these approaches to more precisely define tumor dynamic behaviors. Circulating tumor DNA (ctDNA) is shed into the bloodstream by cells undergoing apoptosis or necrosis, and the load of ctDNA correlates with tumor staging and prognosis [31]. Moreover, recent advances in the sensitivity and accuracy of DNA analysis have allowed for genotyping of ctDNA for somatic genomic alterations. However, lower-stage tumors and even advanced cases involving low-level micrometastatic disease have reduced numbers of ctDNA fragments. In 2016, the European Neuroendocrine Tumor Society (ENETs) [32] reported three novel potential biomarkers in serum. High levels of DcR3 and TFF3 were found to be correlated with poor survival in SI-NENs, and DcR3 was shown to be a marker of liver metastasis. TFF3 and Mindin are sensitive, specific, novel diagnostic biomarkers of SI-NENs found circulating in the serum. However, the molecular mechanisms of those circulating biomarkers remain unclear, and additional studies are needed to validate these markers for clinical applications.

Conclusions

GEP-NENs are heterogeneous tumors that exhibit different characteristics based on disease subtype and have heterogeneous features within individual patients. Considering the flaws of current biomarkers, identification of efficient molecular profiling and liquid biopsy techniques is critical for providing valuable information for diagnosis, classification, monitoring of treatment responses, and determining prognoses in patients with GEP-NENs. Genomic studies and molecular profiling have revealed a number of genomic alterations. Such analyses can identify prognostic and predictive genetic alterations, though these approaches are not currently used to inform the initial treatment decisions. The relationships between tumor behaviors and specific genes, however, remain unclear. Given that repeated biopsies are not always feasible clinically, the development of blood-based strategies to measure changes in circulating molecular signatures is relevant for disease management and analysis of treatment response and outcomes. Such approaches, including analyses of CTCs, circulating RNA, NETests, and miRNAs, may be clinically relevant. Compared with mono-analyte biomarkers, NETests can define multiple variables that represent tumor growth and are applicable in the assessment of multidimensional information for monitoring tumor response to therapy and defining ambiguous clinical scenarios, such as stable disease or mixed responses. Furthermore, NETests may predict treatment response early in the course of therapy, which allows for real-time modification of treatment regimens. miRNAs have primarily been studied in the context of pNENs and SI-NENs, which have the potential for early diagnosis and therapeutic applications. CTCs are associated with progressive behaviors and have roles in adjuvant therapy. All these potential benefits of novel circulating biomarkers will have to be evaluated in appropriately designed clinical trials.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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REVIEW ARTICLE

Implications of the mismatch repair-deficient status for the management of colorectal cancers

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Abstract

Although the majority of colorectal cancer (CRC) cases develop through the CIN pathway, approximately 15% of cases are caused by the hypermutation known as microsatellite instability (MSI) that is a consequence of deficient (d) DNA mismatch repair (MMR). dMMR CRCs have distinct phenotypic characteristics compared with microsatellite stable (MSS) tumors. MSI CRC is associated with an earlier stage at diagnosis and improved stage-specific prognosis, although this is controversial in stage IV patients. Current evidence supports the use of adjuvant chemotherapy with fluoropyrimidine plus oxaliplatin for stage III dMMR CRC. The distinctive genomic characterization and expression profiling of dMMR CRC paves the way for tailored immunotherapies. This is supported by recent studies that highlighted the efficacy of immunotherapy in dMMR CRC. Here, we describe the molecular aspects of the MMR system and discuss the associations of MMR-deficient/MSI-H status with clinical management, especially for patients with metastatic CRC.

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Key words: colorectal cancer (CRC); mismatch repair; microsatellite instability; immunotherapy

A recent study by Le DT, *et al*, highlighted remarkable responses of cancers with microsatellite instability (MSI) to anti–PD-1 inhibitors in patients who had failed conventional therapy. This finding made us re-evaluate the significance of MSI in colorectal cancer (CRC) [1]. Approximately 15% of CRCs arise from the MSI pathway that is a consequence of deficient (d) DNA mismatch repair (MMR). The frequency of MSI varies according to the tumor stage, with the highest rates among early stage cancers, with the rate decreasing with progression to locoregional and distant metastases [2]. dMMR CRCs possess many unique characteristics that make them distinguishable from other CRCs. They are notable for greater survivability, although conflicting results have been observed in stage IV patients. dMMR cases do not benefit from fluoropyrimidine-based therapy in early-stage disease as compared to proficient DNA mismatch repair (pMMR) CRCs. Nowadays, the surging interest in cancer immunotherapy, particularly checkpoint blockade, has led to a further focus on MSI tumors, which are notable for their substantial T cell infiltrates. In this review, we first summarize the clinicopathological and molecular features of the MMR system, then discuss the implications of dMMR/MSI-H status in clinical management, especially for patients with metastatic CRC.

Microsatellite instability

Microsatellites are short tandem repeat sequences that occur throughout the genome and are used as markers of dMMR. DNA polymerases are more prone to make mistakes in these regions. MSH2, MSH6, MLH1, and PMS2 are mismatch repair proteins involved in DNA repair. Following DNA replication, the MMR machinery slides along the DNA and targets mismatches for correction when it encounters them, and loss of any of the MMR repair proteins can result in frameshift mutations of microsatellites, namely, MSI.

The Cancer Genome Atla (TCGA) revealed that the MSI-H frequency in CRCs was approximately 13% [3], and details are shown in the following section. Patients with MSI due to germline mutations in one of the *MMR* genes are defined as having Lynch syndrome. Lynch syndrome accounts for approximately 3–4% of all CRCs and one-third of all cases of dMMR/MSI-associated CRC. Patients with Lynch syndrome have an elevated risk for cancers

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Table 1 MSI-H frequency in digestive system cancers

Tumor type	Frequency		Study
	n	%	
Gastric cancer	295	22	TCGA [7]
Colorectal cancer	1066	13	Hampel H, *et al* [8]
Hepatocellular carcinoma	37	16	Chiappini F, *et al* [9]
Ampullary carcinoma	144	10	Ruemmele P, *et al* [10]
Esophageal adenocarcinoma	76	7	Farris AB 3rd, *et al* [11]
Pancreatic ductal adenocarcinoma	338	0%–2%	Laghi L, *et al* [12]

of the ovaries, kidneys, bladder, stomach, small bowel, bile ducts, and brain, with the highest increase in risk for endometrial cancer (60% of women) and CRC (80% of patients) [4]. Sporadic MSI cancers develop in a background of dense promoter hypermethylation of cancer-specific genes, known as the CpG island methylator phenotype (CIMP), and they are associated with a somatic *BRAF* p.V600E mutation [5] that serves to distinguish them from Lynch syndrome cases. Less commonly, they may arise from biallelic somatic inactivation of the genes encoding an MMR component [6].

MSI-H frequency differs across cancer types. Table 1 showed the MSI-H frequency in digestive system cancers. Pancreatic ductal adenocarcinomas have a very low proportion of MSI-H cases while gastric cancer has the highest frequency.

Clinicopathological features of deficient MMR CRCs

CRC patients with dMMR tumors have distinct clinical and pathologic features that make them distinguishable from other CRCs, such as proximal colon predominance, poor differentiation, and/or mucinous histology [13]. In addition, dMMR CRC patients have a greater inflammatory state with higher serum C-reactive protein levels, dense tumor infiltrating lymphocytes (TILs), and higher platelet counts than pMMR CRC patients, as well as worse prognostic inflammatory scores based on these factors [14].

Tumors with dMMR are more common among stage II cases (almost 20%), and are relatively less common among metastatic CRCs (4%) [15]. Significantly, it has been established that dMMR CRC patients have overall better survival outcomes and are less likely to have metastases than pMMR CRC patients [16]. However, studies indicate that the better prognosis of dMMR CRC is more apparent in earlier stage tumors [17]. When a dMMR CRC metastasizes or relapses, this advantage disappears and they fare no better, if not worse, than pMMR metastatic CRC patients [18]. As we mentioned above, sporadic dMMR tumors carry somatic mutations in the *BRAF* oncogene in approximately half of cases. *BRAF* V600E shows an independent negative prognostic association with survival in microsatellite stable (MSS) CRC [19], but associations with the combination of MSI and *BRAF* have not been thoroughly investigated. Several recent studies stratified CRC patients based on MSI and *BRAF* status into three prognostic groups: MSI/*BRAF*-wild type or mutant (best prognosis), MSS/*BRAF*-wild type (intermediate prognosis), and MSS/*BRAF* mutant [20–21], although other studies have reached conflicting results [22], and no consensus exists to date on the best prognostic subgroupings.

Genomic characterization and expression profiling of dMMR CRCs

The TCGA network project revealed that CRCs could be split into three major groups—hypermutated (13%), ultramutated (3%), and those with chromosomal instability (84%) [3]. The hypermutated category has a high mutation rate of 12–40 mutations/Mb. dMMR in the hypermutated cancers results from acquired hypermethylation of the MLH1 promoter in almost all cases, leading to the silencing of expression of MLH1 and non-functioning mismatch repair, which is again in accordance with the previously discussed findings. Almost all of these tumors showed CIMP characteristics, with several other specifically tested genes also demonstrating promoter methylation. A small number of cancers show either inherited (Lynch syndrome/HNPCC) or somatic *MMR* gene mutations.

An international expert consortium [18] recently reached a consensus to describe four consensus molecular subtypes (CMS) after analysis of 18 different CRC gene expression datasets, including data from TCGA in conjunction with molecular data on mutations and (somatic copy number aberrations) SCNAs for a subset of the samples. CMS1 (MSI-immune, 14%) CRCs are hypermutated because of defective MMR with MSI and MLH1 silencing and accordingly are CIMP-high with frequent *BRAF* mutations, while having a low number of SCNAs. This equates with the previously well-characterized sporadic MSI CRC subgroup. Gene expression profiling revealed evidence of strong immune activation (immune response, PD-1 activation, NK cell, Th1 cell, and cytotoxic T cell infiltration signatures) in CMS1, consistent with pathological descriptions of prominent tumor-infiltrating $CD8^+$ cytotoxic T lymphocytes. Patients with the CMS1 subtype have a very poor survival rate after relapse. Recently, Becht *et al* [23] reported that CRC molecular subgroups and microenvironmental signatures are highly correlated. They retrospectively analyzed the composition and the functional orientation of the immune, fibroblastic, and angiogenic microenvironment of 1388 CRC tumors

from three independent cohorts using transcriptomics. The CMS1 subgroup is characterized by overexpression of genes specific to cytotoxic lymphocytes. These distinct immune orientations of the CRC molecular subtypes pave the way for tailored immunotherapies.

Treatment of MSI metastatic CRC

Predictive value of MMR status in stage II/III CRCs

Adjuvant chemotherapy in stage II tumors is controversial [24]. Limited data are currently available on the potential benefit of chemotherapy in high-risk stage II dMMR CRC. Preclinical studies have shown that dMMR tumor cells are susceptible to oxaliplatin despite displaying resistance to 5-FU [25]. The preponderance of evidence also suggests that 5-FU-based adjuvant chemotherapy is ineffective in patients with stage II dMMR tumors [26]. In the recent AGEO Study [27], the authors reported that patients with high-risk stage II dMMR CRC tended to have better outcomes with oxaliplatin-based adjuvant chemotherapy compared with surgery alone. These results need to be interpreted with caution because of the small number of patients in that subgroup. In the subgroup analysis, the disease-free survival benefit of oxaliplatin-based chemotherapy was statistically significant in multivariable analysis only in stage III cases (hazard ratio = 0.41, 95% confidence interval = 0.19 to 0.87, $P = 0.02$), consistent with the MOSAIC Study [28]. AGEO is the largest study of dMMR CRC patients, and it showed a statistically significant improvement in disease-free survival with oxaliplatin-based adjuvant chemotherapy in comparison with surgery alone in stage III patients.

MMR status and its role in the management of metastatic CRC

We have mentioned that dMMR CRCs have a greater inflammatory state, exhibited by higher serum levels of C-reactive protein and dense tumor infiltrating lymphocytes (TILs). A recent study refined these classic observations by showing that the mismatch repair–deficient tumor microenvironment strongly expresses several immune checkpoint ligands, including PD-1, PD-L1, CTLA-4, LAG-3, and IDO, which indicates that their active immune microenvironment is counterbalanced by immune inhibitory signals that resist tumor elimination [29]. Based on the results of the current and previous studies, Le DT, *et al* [1] hypothesized that dMMR/MSI CRC would have a significant clinical response to pembrolizumab (humanized anti-PD-1 antibody) treatment and a phase II clinical trial has shown strikingly positive effects in patients with MSI metastatic CRCs. As expected, whole-exome sequencing of tumor tissue revealed an average of 1,782 somatic mutations in cancers with MSI versus 73 somatic mutations in cancers without MSI. Le DT, *et al* are continuing to enroll new CRC patients in this original study. In addition, a phase III study for metastatic CRC patients has been initiated. The subjects will receive either 200 mg IV pembrolizumab (every 3 weeks for up to 35 doses) or IV mFOLFOX6/FOLFIRI-based standard therapy (every 2 weeks; NCT02563002) [30]. Multiple clinical trials studying the response of dMMR/CRC patients to pembrolizumab combined with other therapies are also underway now. For example, though limited data is available regarding the role of CTLA4 in CRC and whether anti-CTLA4 antibody therapy would be beneficial for dMMR CRC or any CRCs in general [31], a current study co-administering nivolumab (human anti-PD-1 monoclonal antibody) and ipilimumab (human anti-CTLA-4 monoclonal antibody) has been initiated for dMMR and pMMR CRC patients (NCT02060188); a treatment regimen which has been found to be more efficacious than either agent alone in melanoma trials [32–33].

Aside from pembrolizumab, other immune checkpoint inhibitors, such as the human anti-PD-L1 monoclonal antibody durvalumab, are being tested for efficacy against dMMR/MSI CRC (NCT02227667). Another dMMR CRC study is administering a combination of standard chemotherapy with the PD-L1 inhibitor, atezolizumab (800 or 1,200 mg IV every 2–3 weeks; NCT01633970). While there are no published findings on the efficacy of durvalumab or atezolizumab in CRC patients, it can be assumed that the researchers hope to find similar benefits in dMMR CRC patients as was seen in the pembrolizumab trial [34].

Testing of DNA mismatch repair and microsatellite instability

dMMR tumors can be identified by immunohistochemistry (IHC) showing lack of one or more of the MMR proteins in the tumor tissue. IHC testing does lack some sensitivity because of cases where the protein is intact but not functional. The National Cancer Institute Workshop recommended five necessary microsatellite markers to determine MSI, including two mononucleotide loci (BAT-25 and BAT-26) and three dinucleotide loci (D2S123, D5S346, and D17S250). These regions are amplified within both tumor and normal tissue via fluorescent multiplex polymerase chain reaction (PCR) and their size assessed by capillary electrophoresis [35]. Either IHC or MSI testing can be used, as both tests have a false-negative rate of 5–10% [36].

On the basis of the MSI status, CRCs can be classified into three groups, as shown in Table 2 [37]. MSI-H corresponds to dMMR, whereas MSI-L and MSS indicate pMMR. Loss of MMR protein detected by IHC has been

Table 2 Criteria for interpretation

	5 loci analyzed	> 5 loci analyzed	Interpretation
No.of markers	≥ 2	≥ 30 – 0%	MSI-H
Exhibiting instability	1	< 30 – 40%	MSI-L
Length changes	0	0	MSS or MSH-L

shown to be highly concordant with DNA-based MSI testing with a good sensitivity (> 90%) and an excellent specificity (100%) [38].

Stadler ZK *et al* [39] used the numeric mutational load of a multigene panel to identify MMR status. Thirteen percent of the patients (n = 28) exhibited MMR-D by IHC. Using the 341-gene assay, 100% of the 193 tumors with < 20 mutations were MMR-proficient. Of 31 tumors with ≥ 20 mutations, 28 (90%) were MMR-D. The three remaining tumors were easily identified as being distinct from the MMR-D tumors with > 150 mutations each. With a mutational load cutoff of ≥ 20 and < 150 for MMR-D detection, sensitivity and specificity were both 100% (95% confidence interval, 93% to 100%). A cutoff for mutational load can be identified via multigene next-generation sequencing tumor profiling, which provides a highly accurate means of screening for MMR-D using the same assay that is used for tumor genotyping.

Future directions

The promising findings from the dMMR CRC pembrolizumab clinical trials has boosted interest in immunomodulatory therapies for targeted treatment of this important CRC subtype. The next step in drug development for PD-1 inhibitors is to assess immunotherapy across tumor types. Mismatch repair testing is or will soon be integrated into standard of care algorithms. In addition, If the mechanism proposed for the efficacy of MSI-guided immunotherapy is correct, the ultimate biomarker for immunotherapeutic response is not MSI or even the mutational burden but the presence of immunogenic neoepitopes [40]. Neoantigen-based vaccinations are being studied in another clinical trial (NCT01461148) that is recruiting patients with surgically resected MSI CRC with lymph node metastases or metastasis to one or more distant organs.

It is expected that mismatch repair status and other pathogenetic biomarkers will be readily implicated in various cancer types.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

Expression and significance of carcinoembryonic antigen, cancer antigen 153, and cyclooxygenase-2 in breast cancer*

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Abstract

Objective This study aimed to evaluate serum and nipple discharge levels of carcinoembryonic antigen (CEA) and cancer antigen 153 (CA153) and tissue cyclooxygenase-2 (COX-2) expression in breast cancer cases and associations of these proteins with breast cancer metastasis.

Methods The immunohistochemical *Ultra Sensitive™ S-P* method was used to detect COX-2 expression in 77 cases of invasive breast carcinoma. Of these cases, 52 exhibited CEA and CA153 in both serum and nipple discharge (electrochemiluminescence method), and associations of these biomarkers with breast cancer prognosis were studied. Sixty cases of benign breast lesion were selected as a control group. Overall survival of breast carcinoma patients was evaluated. COX-2 expression was evaluated relative to clinicopathological features and CEA and CA153 levels, and its role in invasiveness was investigated.

Results Among cases of invasive breast cancer, 72.7% (56/77) were COX-2 immunopositive, compared to 16.7% of benign lesions (χ^2 = 66.745, P = 0.000) percentage of positive cells. COX-2 overexpression in breast cancer correlated positively with histological grade (II vs III; χ^2 = 4.064, P = 0.043), lymph node metastasis (χ^2 = 9.135, P = 0.003), and distant metastasis (χ^2 = 8.021, P = 0.003). However, COX-2 expression did not correlate with age (≤ 50 vs 50 years) or tumor size (≤ 5 vs > 5 cm) (χ^2 = 0.081, P = 0.776 and χ^2 = 3.702, P = 0.054, respectively). Among breast cancer patients, COX-2 overexpression in tumors also correlated with shorter overall survival (P < 0.05). In brief, increased COX-2 expression correlates with worse prognosis and shorter overall survival. Malignant lesions were associated with significantly higher serum and nipple discharge levels of biomarkers, relative to benign lesions (P < 0.05). These biomarkers were present at significantly higher levels in nipple discharge than in serum (P < 0.05). Furthermore, significantly higher nipple discharge levels of CEA and CA153 were observed in COX-2-positive breast carcinoma patients, compared to COX-2-negative patients (P <0.05). Shorter overall survival in cancer patients group related to COX-2 overexpression in tumors (P < 0.05).

Conclusion The study suggests that COX-2 overexpression correlates with poor clinicopathological parameters in breast cancers and might be an important biological marker of invasion and metastasis. The findings of the present study suggest that combined detection of COX-2 tissue expression and CEA and CA153 in serum and nipple discharge could facilitate clinical monitoring and diagnosis of metastasis in patients with breast cancer.

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Key words: breast cancer; carcinoembryonic antigen (CEA); cancer antigen 153 (CA153); cyclooxygenase 2 (COX-2); prognosis

Breast cancer is the most common form of cancer among women, and the annual incidence of related morbidity is increasing worldwide [1–2]. In recent years, China has seen a relatively high incidence of breast cancer, with continued progress toward a peak incidence; currently, a large number of patients are expected to die of breast cancer complications or serious organ metastasis each year [2–3]. Pathogenesis of breast cancer is not yet

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fully clear. Many studies have found that abnormal expression of molecules such as estrogen receptors, progesterone receptors, human epidermal growth factor receptor-2, and Ki-67 can serve as biological indicators, thus guide clinical diagnoses, treatments, and prognostic determinations [4]. Several other molecular biomarkers, such as cyclooxygenase-2 (COX-2), carcinoembryonic antigen (CEA), and cancer antigen 153 (CA153) have also been confirmed to contribute to the evolution of malignancy [2, 4–5]. Cellular conditions such as hypoxia lead to the increased COX-2 expression [5–6]. COX-2 is an inducible enzyme that interferes with tumor development and angiogenesis via the inhibition of apoptosis, which is mediated through suppression of the proapoptotic Bax protein and anti-apoptotic bcl-2 protein overexpression [5–6]. COX-2 overexpression is a frequent feature of malignant disease and is commonly associated with a poor prognosis.

Body fluid components are easily detected and therefore serve as ideal diagnostic biomarkers. Serum biomarkers such as CA153 and CEA are considered as prognostic factors and can be evaluated during follow-up [2–3]. Serum protein markers are advantageous because they can be easily used to construct a multiplex tumor-associated autoantibody assay [3, 7]. Nipple discharge evaluation and management can be undertaken with minimal difficulty by performing a careful history and examination and following a logical thought process to link the type of nipple discharge with a suitable mode of treatment [2, 7]. To date, the precise implications of CA153 and CEA screening of both serum and nipple discharge and COX-2 screening of breast cancer tissue, as well as the correlations of these markers with breast carcinoma invasiveness and metastasis, have not yet been investigated. In this study, we evaluated expression of the potential biomarker COX-2 in a panel of mammary tissues and CEA and CA153 levels in both serum and nipple discharge to explore the above markers and associated clinicopathological parameters in breast cancer.

Materials and methods

Patients

The present study was approved by the Rizhao local ethical committee. Written informed consent was obtained from all patients before their participation in the current study. Clinical and pathological information was documented at the time of surgery. Each biopsy slide was subjected to pathological reading, and an overall pathological diagnosis was determined for each subject. Mammary tumor samples were obtained from patients after surgical removal at Rizhao People's Hospital. The samples were fixed in 10% neutral formalin and embedded in paraffin. Hematoxylin and eosin (HE)-stained sections were subjected to pathological diagnosis according to the current World Health Organization (WHO 2012) diagnostic criteria for mammary tumors. All diagnoses were revised by 2 pathologists using the same guidelines to ensure consistency. Tumor grades were determined using modified Bloom–Richardson scores. Grades were obtained by summing the scores for tubule formation, nuclear pleomorphism, and mitotic count (possible scores: 1, 2, or 3). The final scores ranged between 3 and 9 and were divided into 3 grades: I, 3–5 points; II, 6–7 points; and III, 8–9 points. The pathologists were blinded to the clinical histories of cases and the results of immunohistochemical staining assays. A pathological reading was determined for each biopsy slide, and an overall pathological diagnosis was determined for each subject. After revision by 2 pathologists, 77 malignant tumor samples collected at Rizhao People's Hospital from June 2014 to June 2016 were selected for immunohistochemical and survival analyses. The patients ranged in age from 32 to 77 years (mean age: 53.6 years). Sixty cases of benign breast disease (age range: 21–67 years, mean: 43.3 years) were selected as a control group. Patients diagnosed with invasive breast cancer had not received hormone endocrine therapy, anti-neoplastic chemotherapy, or radiotherapy during the last 6 months.

Measurement of CEA and CA153 in serum and nipple discharge samples

All samples were collected before any treatments were initiated. For biomarker analysis, 3 ml of heparinized blood and 0.2 mL of nipple discharge were drawn per individual. The nipple was first cleaned with alcohol swabs to remove cellular debris. Nipple discharge was expressed by manual breast compression, and droplets were collected in an Eppendorf tube. The tube was then stored in a dedicated refrigerator at 4°C. Samples were transported to the laboratory department within 8 h after collection. Viscous samples were diluted up to 20-fold with normal saline before centrifugation and storage at 4°C. Commercial reference control sera were used for quality control and calibration. CEA and CA153 were detected using an electrochemiluminescence method (E-601; Roche, Germany) in the clinical laboratory at Rizhao People's Hospital. In our laboratory, the cut-off values for CEA and CA153 were 3.40 ng/mL and 25.00 U/mL, respectively, in serum and 9.8 ng/mL and 35.00 U/mL, respectively, in nipple discharge. Patients were classified into 2 groups by histological grade: grade II and grade III. Patients were also classified by the levels of CEA and CA153 in peripheral blood or nipple discharge: those with normal levels and those with high levels.

Immunohistochemical evaluation of COX-2 in tumor tissues

Immunohistochemical methods were used to detect COX-2 expression. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were then deparaffinized and rehydrated using standard procedures. Serial sections (3–4 μm) were deparaffinized in xylene and hydrated through a graded series of ethanol. The specimens were finally washed in phosphate-buffered saline (PBS) within 5 min and examined under a binocular dissecting microscope. Immunoreactions were processed using the Ultra Sensitive™ S-P Kit (Maixin-Bio, China) according to the manufacturer's instructions, and signals were visualized using a DAB substrate, which stained the target proteins yellow. Negative controls were obtained by substituting PBS for the primary antibody. Tissues known to express COX-2 were used as positive controls. COX-2 positivity was indicated by cytoplasmic staining. The number of COX-2 positive cells was classified semiquantitatively according to the positive rate, and the distribution score was defined by the estimated percentage of positive cells in 5 fields at 400 × magnification and color intensity. In brief, a score was assigned to represent the estimated proportion of positive tumor cells on an entire slide. For each histological section, the percentage of positive cells was scored as: 0, < 5% stained cells; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, > 75% stained cells. For staining intensity, values from 0 to 3 were attributed as follows: 0, negative (-); 1, weak staining (light yellow); 2, moderate staining (tan); and 3, strong staining (dark brown). Scores corresponding to the percentage of positive cells and staining intensity were multiplied to obtain a total immunoreactive score (IRS; possible range: 0–12). Samples with an IRS of 0–4 were considered negative; those with an IRS > 4 were considered positive.

Statistical analysis

SPSS version 17.0 statistical software (SPSS Inc., USA) was used for data analysis. Numerical data were assessed using the χ^2 test or Fisher's exact test, as appropriate. CEA and CA153 levels are expressed as means and standard deviations (mean ± SD). As the data related to these markers did not exhibit Gaussian distributions, the nonparametric Mann–Whitney U-test was used to determine differences between the benign and malignant groups. Correlations of COX-2 staining with CEA and CA153 expression were assessed using a Spearman's rank correlation coefficient test. Relationships of this dichotomous variable with other clinicopathological correlates were established using the χ^2 test or Fisher's exact test, as appropriate. Analyses were performed using GraphPad Prism V5.0 software (GraphPad Software Inc., USA). Values were considered statistically significant at a P value < 0.05.

Results

Comparison of CEA and CA153 levels in malignant and benign groups

Fig. 1 showed the serum and nipple discharge levels of CEA and CA153 in the malignant and benign groups. The biomarker levels in serum (Fig. 1a) and nipple discharge (Fig. 1b) were significantly higher in the malignant group, than the benign group ($P < 0.05$). Furthermore, in the malignant group, the levels of both biomarkers were significantly higher in nipple discharge than in serum ($P < 0.05$; Fig. 1c).

COX-2 expression and relationships with biological parameters

Cytoplasmic COX-2 expression was detected in both breast cancer and benign breast lesion tissues. The COX-2 expression findings in the benign and malignant groups

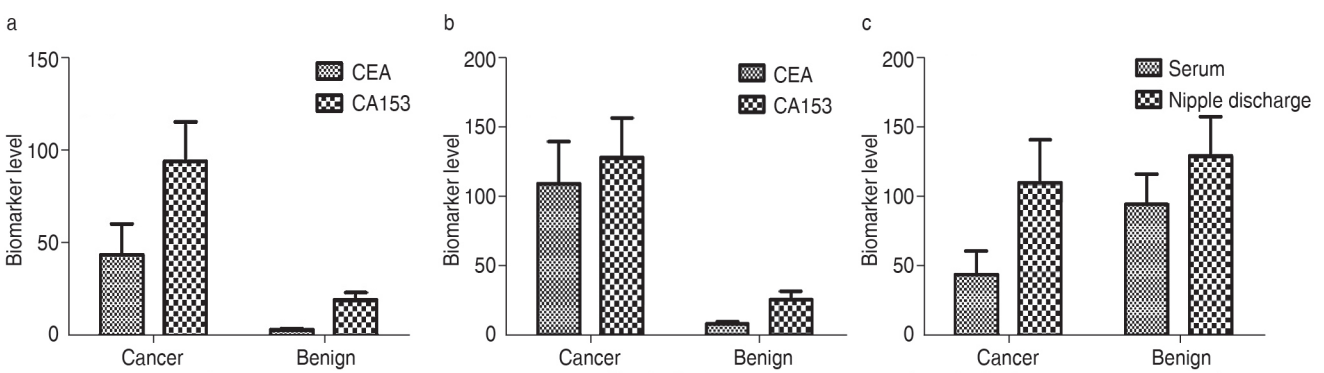

Fig. 1 Comparison of biomarker levels among groups. (a) Comparisons of serum biomarker levels and (b) nipple discharge biomarker levels between malignant and benign groups; (c) comparison of serum and nipple discharge biomarker levels in the malignant group.

Table 1 COX-2 expression in different groups

Groups	*n*	COX-2		χ^2 value	*P* value
		-	+		
Breast carcinoma	77	21	56	82.020	0.000
Benign leision	60	50	10		

Table 2 Relationships between COX-2 expression and biological parameters of breast cancer

Biological parameters	*n*	COX-2		χ^2 value	*P* value
		Negative	Positive		
Age at diagnosis (years)					
≤ 50	36	12	34	0.081	0.776
> 50	41	9	22		
Tumor size (cm)					
≤ 5	65	15	50	3.702	0.054
> 5	12	6	6		
Histoligical grade					
II	57	19	38	4.064	0.043
III	20	2	18		
Lymph node metastasis					
Present	50	8	42	9.135	0.003
Absent	27	13	14		
Distant metastasis					
Present	22	1	21	8.021	0.005
Absent	55	20	35		

were shown in Table 1. In benign tissues, COX-2 was weakly expressed in only 15.4% (10/60) of samples. In contrast, immunohistochemical analysis revealed some degree of positivity in 72.7% (56/77) of the examined malignant tumors, and the COX-2 positive rate was higher among breast cancers than among benign lesions (χ^2 = 82.020, P < 0.01).

Table 2 described the relationships between COX-2 expression and clinicopathological parameters of breast cancer. COX-2 overexpression correlated significantly with histological grade (II vs III; χ^2 = 4.064, P = 0.043), lymph node metastasis (χ^2 = 9.135, P = 0.003), and distant metastasis (χ^2 = 8.021, P = 0.003). However, COX-2 expression did not correlate with age at diagnosis (≤ 50 vs > 50 years) or tumor size (≤ 5 vs > 5 cm) (χ^2 = 0.081, P = 0.776 and χ^2 = 3.702, P = 0.054, respectively).

Comparison of nipple discharge biomarkers levels by COX-2 expression

Fig 2 illustrates the comparison of CEA and CA153 levels in nipple discharge from COX-2-positive and -negative cases of breast carcinoma. The respective CEA and CA153 levels were 126.42 ± 34.18 and 134.45 ± 32.57 in the COX-2-positive group, and 72.89 ± 33.41 and 98.76 ± 35.19 in the COX-2-negative group. The levels of CEA and CA153 in nipple discharge were significantly higher in COX-2-positive breast carcinoma patients, compared to COX-2-negative patients (P < 0.05 for both). COX-2 overexpression in tumors also correlated with significantly shorter overall survival among cancer patients (P < 0.05). These results demonstrate that increased COX-2 expression is related to worse prognosis and shorter overall survival (data not shown).

Follow-up

The follow-up durations ranged from 3 months to 2 years, and the levels of above-mentioned indicators were detected through dynamic blood draws. Patient survival was monitored from January 2014 to August 2016 via telephone communication and periodic visits to Rizhao People's Hospital. Overall survival was defined as the period (months) between surgical tumor resection and death related to the malignant process. Patients who died of any other cause were not included in this analysis. Patients were censored if the follow-up period was < 6 months. Efficacy assessments were performed at 6-week intervals. Progressive disease (PD) and stable disease (SD) were assessed after the start of adjuvant treatment, and treatment responses and disease progression were investigated according to the modified Response Evaluation Criteria in Solid Tumors (RECIST version 1.0). A complete response was recorded when the tumor had disappeared completely, and a partial response was recorded when the largest diameter of the tumor shrank by > 30%; "any response" was recorded for any degree of response or a decrease in size without mention of the tumor dimensions. SD was recorded for cases of no sign of recurrent disease within 6 months or changes in tumor size, and PD was recorded if a tumor size increase of any degree was observed. Progression-free survival (PFS) was calculated from the first date of study drug administration until PD or death from any cause. We evaluated heterogeneity in PFS among patients according to treatment before study entry (i.e., no local treatment vs surgical or local radiotherapy vs whole-breast

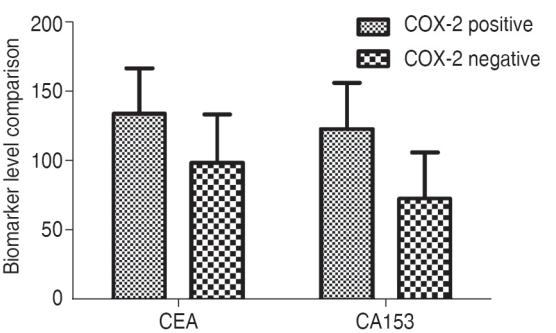

Fig. 2 Biomarker levels in nipple discharge from COX-2 positive and negative breast carcinoma patients.

radiotherapy), because some patients with no evidence of disease might have artificially influenced PFS. Follow-up information was available for 63 of the patients. Patients were classified into COX-2-negative and -positive groups. Of the evaluable patients, 42 achieved SD, and 21 achieved PD. The overall response rate was 37.2%, with a median PFS of 26 months [95% confidence interval (CI) = 11.1–23.6] and a median overall survival of 65 months (95% CI = 40.7–70.2). In the univariate analysis, patients with detectable CEA and CA153 in nipple discharge had significantly increased risks of disease progression (hazard ratio [HR] = 1.61) and death (HR = 1.53; $P < 0.05$). In the univariate and multivariate analyses, COX-2-positive patients who expressed both CEA and CA153 in nipple discharge and serum had significantly increased risks of disease progression (HR = 1.62) and death (HR = 1.66; $P < 0.05$). Kaplan–Meier survival curves further demonstrated survival differences among patients by COX-2 expression status and combined (nipple discharge + serum) biomarker status ($P < 0.05$).

Discussion

Breast cancer is a major public health issue, which accounts for 23% of all cancers in women worldwide, and has an incidence more than twofold higher than cancer at any other site [1–3]. In recent years, the incidence of breast cancer has increased significantly worldwide. Accordingly, numerous studies have sought to determine the most effective ways to evaluate and treat breast cancer, assess therapeutic effects, correctly evaluate prognosis, and identify postoperative recurrences. Mammography is an important diagnostic method mainly used for breast cancer screening. The resolution and calcification detection rates associated with mammography can be further improved by computer assistance [8]. However, the ability of this technique to diagnose early breast cancer is limited [3, 8] and it is mainly used to diagnose advanced stages of the disease. A previous report demonstrated the clinical diagnostic significance of serum CEA, CA153, and CA125 levels and provided details regarding the management of breast cancer recurrence and metastasis [2–3, 7]. The associations of high serum CEA and CA153 levels with poor prognoses have been validated [2–3, 7]. However, the use of serum tumor markers for breast cancer diagnosis is somewhat limited by factors such as relatively limited sensitivity and specificity in stand-alone assays, as levels of these markers reflect tumor burdens. Nipple discharge is a common complaint among women [2–3, 7, 9]. In patients with early or localized breast cancer, serum CA153 levels do not clinically facilitate diagnosis [7].

Nipple discharge evaluation and management are relatively simple in the context of a careful history and examination and a logical thought process that links the type of discharge with a suitable mode of treatment. However, nipple discharge may be a sign of serious abnormality within the breast. Discharge is classified as normal or abnormal, depending on features such as laterality, cycle variation, quantity, color, or presentation [3, 7, 9–10]. Nipple aspiration has been described as a quick, painless, and noninvasive method for collecting breast epithelial cells and extracellular fluid from the breast ductal and lobular epithelium [11]. However, the ability to obtain adequate fluid has consistently been associated with the following 4 factors: age between 35 and 50 years, earlier age at menarche, non-Asian ethnicity, and history of lactation [6–7]. The nipple aspiration fluid collection rate among native Chinese women is relatively lower than that among women of non-Asian ethnicity. In the present attempt to validate whether biomarkers in nipple discharge might serve as novel breast cancer biomarkers, we examined the levels of CEA and CA153, which are known breast cancer tumor markers, in both serum and nipple discharge samples from patients with benign and malignant breast lesions, as well as COX-2 expression in tissues, to explore the significance and combined predictive value of these markers in breast cancer cases and for determining the prognosis of breast papillary cancer cases. Our study revealed that CEA and CA153 levels were higher in nipple discharge than in serum, and the combined detection of CEA and CA153 in both nipple discharge and serum was significantly higher than that in serum or nipple discharge alone. The clinical results from our study groups revealed that CEA and CA153 in nipple discharge could serve as novel biomarkers of breast cancer prognosis [3]. The human mammary gland comprises discrete ductal alveolar systems in which the breast epithelium exfoliates cells and secretes fluids into the luminal compartment of the gland. Nipple discharge is located in or originates from mammary ducts, where benign and malignant breast tumors are generally found. Nipple discharge in the ducts of non-lactating women contains concentrated proteins secreted from the breast ductal epithelium. These unique cellular and biochemical components, which reflect the true alveolar-ductal system microenvironment, has led to nipple discharge being recognized as a potential gold mine of biomarkers for early breast cancer diagnosis. In addition, biochemical compounds of physiopathological interest are found at higher concentrations in breast ductal secretions than in matched serum samples.

Prognosis is directly related to factors such as tumor size, lymph node involvement, and the presence of distant metastasis. Large tumors with lymph node involvement or distant metastases indicate a poor prognosis and worse overall and disease-free survival [1–2, 4].

COX-2 has been investigated in the context of several human cancers and was found to correlate with disease evolution. In our study, only 15.4% of benign

lesions weakly expressed COX-2; in contrast, breast cancer tissues were more likely to exhibit some degree of COX-2 positivity. Among breast cancer cases, we observed positive correlations of COX-2 expression with a histological high grade (III), lymph node metastasis, and distant metastasis. Our results also revealed that patients with elevated COX-2 expression had shorter survival times. The strong correlations of COX-2 expression with breast cancer prognostic factors suggested that increased COX-2 expression was associated with worse prognosis, as observed in our survival analysis. We suggest that differences in COX-2 expression in breast cancer patients are related to variations in tumor behavior, thus confirming the association between COX-2 expression and disease aggressiveness. In addition to shorter overall survival, the positive correlation of COX-2 expression with breast carcinoma was verified, thus suggesting a worse prognosis. In our study, we observed shorter survival among patients with higher levels of COX-2 expression in tumors. Therefore, COX-2 inhibitor anti-inflammatory drugs could potentially be used to treat mammary tumors. Our results demonstrate correlations of increased COX-2 expression with worse prognosis and shorter overall survival. These findings suggest that COX-2 overexpression in breast carcinoma might be an important biological marker of invasion and metastasis, and the combined detection of COX-2 could yield better early markers for clinical metastasis monitoring of patients with breast cancer.

Conclusions

Our results demonstrate that increased COX-2 expression correlated with a worse prognosis and shorter overall survival. Our study suggests that COX-2 overexpression correlates with poor clinicopathological parameters in breast cancer patients and might be serve as an important biological marker of invasion and metastasis. Further, our findings suggest that COX-2 overexpression may be considered a negative prognostic marker of breast cancer. The combined detection of COX-2 with CEA and CA153 in both serum and nipple discharge provides a better early marker for the clinical monitoring of metastasis in patients with breast cancer.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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REVIEW ARTICLE

Milk fat globule epithelial growth factor VIII (MFG-E8) sustains survival of cancer cells by prompting tumor angiogenesis and suppressing host immunities*

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Abstract

Milk fat globule epithelial growth factor VIII (MFG-E8) is a novel adhesion protein mainly produced by macrophages and dendritic cells; it is expressed in most of the human tissues and functions to prompt cancer progression and survival. MFG-E8 contains a signal sequence for secretion, two epidermal growth factor (EGF)-like domains at the NH2 terminus and two discoidin domains with blood-clotting factor V/factor VIII (C1 and C2) at the COOH terminus. The second EGF domain contains an arginine-glycine-aspartic (RGD) integrin-binding motif that engages $\alpha_v\beta_5$ integrins to facilitate cell adhesion and induce integrin-mediated signal transduction. Integrin $\alpha_v\beta_3$ associates with VEGF receptor 2, engagement of integrins can promote angiogenesis, which plays key roles in growth, proliferation, and survival of cancer cells. VEGF stimulates the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on angiogenic vasculature, thereby potentiating effects of VEGF receptor engagement. Mice expressing a mutant form of $\alpha_v\beta_3$ integrin are unable to undergo tyrosine phosphorylation, confirming the important role that this integrin plays in pathological angiogenesis and providing important mechanistic insights. The C-terminus discoidin-like domains promote binding to membrane phospholipids, functioning close to VEGF like angiogenesis. MFG-E8 is an opsonin for apoptotic cells, and it acts as a bridging protein between apoptotic cells and phagocytes. It also influences cell immunities by altering $CD4^+$ and/or $CD8^+$ cells. Antibody or small peptide works with MFG-E8 at different functional sites or interacts with EGF-like domains and/or discoidin-like domains may play an important role in anti-angiogenesis or immune restoration. Altering the structures and/or functions of MFG-E8 and/or its domains is promising for development of novel anti-cancer strategies.

Key words: milk fat globule epithelial growth factor VIII (MFG-E8); carcinoma; target therapy; angiogenesis; apoptosis

List of abbreviations: milk fat globule epithelial growth factor VIII (MFG-E8); vascular endothelial growth factors (VEGFs); fibroblast growth factor (FGF); tripeptide Arg-Gly-Asp (RGD); granulocyte/monocyte colony-stimulating factor (GM-CSF); cyclin-dependent kinase inhibitor 1 (P21WAF1/CIP1); B-cell lymphoma 2/Bcl-2 associated X protein (Bcl-2/Bax); platelet-derived growth factor receptor β (PDGFRβ); tumor cells proliferation rate index (Ki-67); toll-like receptor (TLR)

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Research on cancer pathways targeting therapeutic approaches is imperative and a promising new hope in cancer management. Cancer angiogenesis, which is regulated by angiogenic and anti-angiogenic factors, is a prerequisite for solid tumor growth, proliferation, and metastasis; its biological process has been developed in cancer treatment [1–2]. The angiogenic factors include vascular endothelial growth factors (VEGFs), and

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fibroblast growth factors (FGF1, FGF2). These angiogenic factors promote signal transduction and endothelial cell proliferation by engaging trans-membrane receptor tyrosine kinases on endothelial cells, prompting new blood vessel formation, and cancer cell proliferation and survival.

Milk fat globule epithelial growth factor VIII (MFG-E8), is a newly discovered glycoprotein with VEGF-like structures and functions [3], which is mainly produced by macrophages and dendritic cells. It is an opsonin for apoptotic cells and it acts as a bridging protein between apoptotic cells and phagocytes. It is highly expressed in breast cancer and melanoma, and contains two N-terminus EGF-like domains and two C-terminus discoidin-like domains. The second EGF repeat includes the integrin-binding motif Arg-Gly-Asp (RGD) which can interact with integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, where the discoidin-like domains promote binding to membrane phospholipids, making MFG-E8 a "bridging factor" in cell apoptosis as its main function [4]. MFG-E8 binding to integrins regulates a variety of signaling pathways, prompting VEGF-dependent angiogenesis by activating Akt in mice with pancreatic cancer [5], enhancing PDGF-PDGFR beta signaling mediated by integrin-growth factor receptor crosstalk [6], and inducing the expression of the transcription factors Twist and Snail, promoting motile mesenchymal phenotype in melanoma cells [7]. It also prompts proliferation of colon epithelial cells through integrin-mediated cellular signaling, and treatment with an siRNA targeting α_v-integrin reduced the proliferation of Colon-26 cells that was stimulated with recombinant MFG-E8 [8]. MFG-E8 is highly expressed in patients with cholangiocarcinoma (CCA), which was significantly characterized with a poor differentiation, pathological advanced stage, and metastasis of CCA [9].

The two discoidin-like domains promote binding to membrane phospholipids and removal of apoptotic cells by macrophages, clearing epithelial cells in involution, maintaining intestinal epithelium and facilitating vascularization [10–11]. MFG-E8 promotes tumor progression in oral squamous cell carcinoma and it might be involved in the clearance of apoptotic SCC cells by living SCC cells [6, 12]. It plays a key role in T reg cell homeostasis, by influencing $CD4^+$ and $CD8^+$ effector T cell activation and function, and regulating tumor immunity balance and tolerance [13]. Interaction on RGD or C1 and/or C2 functions to regulate cancer angiogenesis, immunities, proliferation, and survival or help to develop new cancer treatment modalities or agents.

MFG-E8 structure and expression

Milk fat globule epithelial growth factor VIII (MFG-E8), a 66-kDa glycoprotein, was initially identified in mice, and then isolated from the mammary gland of several other mammalian species, such as bovines and humans [3]. It is abundantly expressed in the mammary glands, spleen, lymph nodes, brain, and lungs; however, its expression is low in the liver and small intestine [10, 14]. MFG-E8 contains a signal sequence for secretion, two epidermal growth factor (EGF)-like domains at the NH2 terminus and two discoidin-like domains with blood-clotting factor V/factor VIII (C1 and C2) at the COOH terminus [3, 15]. The second EGF domain contains an RGD integrin-binding motif that engages $\alpha_v\beta_5$ integrins to facilitate cell adhesion and to induce integrin-mediated signal transduction. Integrin $\alpha_v\beta_3$ associates with VEGF receptor 2 [16], and engagement of integrins can enhance endothelial cell survival and migration [17]. VEGF also stimulates the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on angiogenic vasculature, thereby potentiating the effects of VEGF receptor engagement. Recent studies in mice, expressing a mutant form of $\alpha_v\beta_3$ integrin that is unable to undergo tyrosine phosphorylation, confirm the important role that this integrin plays in pathological angiogenesis for providing important mechanistic insights [18]. Pharmacological inhibitors of VEGF and FGF receptor kinases and antibodies that bind to these growth factors or their receptors have been extensively studied clinically and in animal models of cancer.

Two mRNA MFG-E8 variants were found in mice by Oshima [19] – 66 kDa long form of MFG-E8 (MFG-E8L) containing a 37 amino acid proline/threonine rich (P/T rich) domain (between the second EGF domain and the first dicoidin domain), and 53 kDa short form of MFG-E8 (MFG-E8S) that lacks the P/T-rich domain [19–20]. The expressions of the two variants show spatial and temporal specificity but similar biological activity. MFG-E8S is distributed widely, whereas MFG-E8L has been found in activated mice macrophages, immature dendritic cells, skin Langerhans cells, and epidermal keratinocytes [21–23].

MFG-E8 contributes to phagocytes and removal of apoptotic cells

Apoptosis is an ordinary process of cell suicide that, unlike necrosis, does not elicit inflammation. Recently, it has been shown that if the removal process of apoptotic cells fails, apoptotic cells undergo post apoptotic secondary necrosis and release inflammatory cytokines.

The function of MFG-E8 is associated with promoting removal of apoptotic cells by macrophages, clear epithelial cells in involution, maintenance of intestinal epithelium, and facilitating vascularization [10–11]. The MFG-E8 production from macrophages is increased by granulocyte/monocyte colony-stimulating factor (GM-CSF) and fractalkine (CX3CL1) [24–25]. Its expression is downregulated in autoimmune disease [10], Alzheimer

disease, atherosclerosis, and sepsis [26–28], but it is upregulated in some disease conditions and cancer [7, 29].

Apoptotic cells were quickly removed by phagocytic cells such as monocytes, macrophages, and dendritic cells. The apoptotic cells express "eat me" signals, i.e., express phosphatidylserine (PS) on the external plasma membrane surface; MFG-E8 and the C-terminus discoidin domains mediate attachment to PS on apoptotic cells and the RGD motif of N-terminal domains engages $\alpha_v\beta_3/\alpha_v\beta_5$ integrins expressed on the advancing phagocytes [30–32]. This process downregulates cyclin-dependent kinase inhibitor 1 (P21WAF1/CIP1) expression and increases the B-cell lymphoma 2/Bcl-2 associated X protein (Bcl-2/Bax) ratio, which prompts endothelial cell survival by decrease in apoptosis [33]. MFG-E8 acts as a "bridge molecule" in the process of phagocytosis, recognizing and binding the apoptotic cells to the phagocyte. Decreased MFG-E8 level by sepsis or inflammation could be detrimental to apoptotic cell clearance [22]; however, high levels of it can inhibit cell engulfment [34]. This phenomenon complicated MFG-E8 in the involvement of disease pathogenesis and progression. MFG-E8-deficient mice develop an autoimmune phenotype that is characterized by autoantibody production, splenomegaly, and histological evidence of glomerulonephritis [10].

MFG-E8 prompts cancer angiogenesis

MFG-E8 contains two epidermal growth factor (EGF)-like domains, which contain an RGD sequence at the NH2 terminus. This EGF-like structure confers MFG-E8 with angiogesis-like function, and this angiogenesis promoting activity was attributed to enhancement of VEGF-induced Akt phophorylation and endothelial cell survival in a $\alpha_v\beta_3/\alpha_v\beta_5$ integrin-dependent manner [35–36]. At least 8 integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$) of its family, especially $\alpha_v\beta_3$, contributed to endothelial cell migration, proliferation, and apoptosis. It plays an important role in new blood vessel formation via junctional adhesion molecule 1/A (JAM-1/A) [37], increasing frequencies of blood vessels and myofibroblasts in wound area of wild type mice and reducing frequencies in knockout mice [38–39]. It was also found to enhance MFG-E8 expression in the dermis and granulation tissue with localization near blood vessels during wound healing. MFG-E8 accumulated around $CD31^+$ endothelial cells and co-localized with α-smooth muscle antibody$^+$ (αSMA^+) or platelet-derived growth factor receptor β^+ ($PDGFR\beta^+$) in human and murine dermis affects angiogenesis via actions on pericyte precursors (PCs) as well as endothelial cells (ECs) [36, 38]. In both xenograft tumors and clinical specimens of melanoma, MFG-E8 expression was increased near blood vessels where bone marrow-derived mesenchymal stromal cells (MSC) could be found, and MFG-E8 increased tumor angiogenesis by increasing VEGF and endothelin-1 expression in MSC and by enhancing M2 macrophage polarization [40].

Neutzner *et al* [5] found that MFG-E8 promoted tumorigenesis in Rip1-Tag2 transgenic mice. MFG-E8 mRNA was easily detected in pancreatic tumors and in cell lines derived from these tumors, but were not readily identified in normal pancreas. MFG-E8 deficient mice exhibited aggregate tumor burdens that were 2-fold lower and angiogenic islets that were ¼ lower than those in corresponding control wild type mice.

MFG-E8 derived from either tumor or host myeloid cells, promoting B16 melanoma growth *in vivo* through coordinated Akt and Twist signaling in the tumor microenvironment in C57Bl/6 mice. The vascularity of B16 melanoma growing and tumor cells proliferation rate index (Ki-67) in the MFG-E8 knockout mice were both reduced by 30%–50% compared to that of tumors in wild-type (WT) mice [36]. $\alpha_v\beta_3$ integrin overexpression in the vertical growth phase melanoma promotes cancer progression through coordinated $\alpha_v\beta_3$ integrin signaling in tumor cells, vascular elements, and infiltrating myeloid cells [41–43]. This promotes signal transduction and endothelial cell proliferation by engaging trans-membrane receptor tyrosine kinases on endothelial cells, eventually leading to new blood vessel formation and cancer cell proliferation and survival [35].

MFG-E8 suppresses host cellular and humoral immunities

The two discoidin domains are closely related to immune responses, which, like blood-clotting factor V/factor VIII (C1 and C2) at the COOH terminus, are the binding sites for apoptotic cell. Anti-tumor immune response was observed by using the purified exosomes. The immunosuppressive effects include T-cell killing due to enrichment of Fas ligand on tumor exosomes, NK cell inhibition or inhibition of monocyte differentiation in dendritic cells, possibly mediated by TGF [44]. The C1C2 domain in lactadherin can target other amino acid sequences to exosomes, fused to interleukin 2 or GM-CSF. The fusion protein can be secreted by live cells associated with exosomes [45]. Fusing C1C2 domain to a chicken egg ovalbumin (OVA) antigen in OVA-secreting tumor cell lines could induce more efficient anti-tumor immune responses and cause tumor cells to grow slower than tumor cells secreting the same antigen without fusion [46]. MFG-E8 may also affect the quality and magnitudes of antigen-specific responses by modulating apoptotic cell engulfment and processing [24]. It mediates and interacts with sentinels of apoptotic death signals, such as FADD and pro-apoptotic Bcl-2 family members. It interferes with the TNF and IL-6 mediated inflammatory signal

cascade and Rip kinase activities in dying tumor cells [13, 47]. It plays a key role in T reg cell homeostasis; combined use of anti-MFG-E8 antibodies with chemotherapy enhances cross-presentation of tumor antigens on dendritic cells, and markedly increases $CD4^+$ and $CD8^+$ effector T cell activation and function, resulting in high levels of surface CD44, INF-γ production, and tumor-specific cytotoxicity [24, 48]. MFG-E8-secreting tumor showed impaired IFN-γ production and CD107a immobilization by influencing $CD8^+$ cells with dense infiltrate of $CD4^+$ $FoxP3^+$ T regs [48–49]. It elicits STAT-3 activation, which promotes tumor cell survival and immune suppression; knockdown of MFG-E8 with shRNAs decreased phosphorylated Fak, phosphorylated Src, and phosporylated Akt levels, and increased apoptosis [43]. MFG-E8 altered IgG2a and IgG2b antibodies which might contribute to tumor destruction through Fc-dependent cytotoxicity [24, 48], it also helped dendritic cells to regulate the delicate balance between immunity and tolerance by fine-tuning recognition of dying cells [13]. MFG-E8 suppresses T cell activation/proliferation and inhibits Th1, Th2, and Th17 subpopulations while increasing regulatory T cell subsets. Neutralizing MFG-E8 substantially abrogates these effects, whereas addition of recombinant MFG-E8 to differentiated embryonic stem cells restores immunosuppression. Furthermore, MFG-E8 suppresses T cell activation and regulates T cell polarization by inhibiting protein kinase Cθ phosphorylation through the $\alpha_v\beta_{3/5}$ integrin receptor [50].

Discussion

MFG-E8 is found in almost every organ in humans. It is highly expressed in advanced stages of breast cancer, oral squamous cell carcinoma, colorectal cancer, and metastatic melanoma associated with a poor prognosis [12, 43, 51]. It is also highly expressed in triple negative phenotype or lower ErbB2 amplification breast cancer, but ER and/or PR levels reversed MFG-E8 expression. MFG-E8 expression decreases during tumor progression in ER^+ and $erbB2^+$ human breast cancers, but is highly increased in progressed triple negative breast cancers [52]. MFG-E8 was present in high levels in triple negative (ER^-, PgR^-, $erbB2^-$) breast cancer cell lines and in serum of patients. Transcription of MFG-E8 was controlled by p63 or TP63, and is a target gene of P63/P73 [53–54]. Abnormalities in p63/p73 regulation are important features of triple negative (basal) breast cancers. P63 gene regulates MFG-E8 expression, and MFG-E8 knockdown sensitizes triple negative breast cancers to cisplatin treatment [54]. MFG-E8 is expressed in triple negative breast cancers as a target gene of the p63 pathway, but may serve a suppressive function in ER^+ and $erbB2^+$ breast cancers.

Dendritic cell and tangible and tumor-associated macrophages (TAMs) produce large amount of MFG-E8 [43, 55]. Through a study on cancer stem cells and TAMs, Jinushi *et al* [56] found that MFG-E8 modulates oncogenic signals and triggers chemotherapy drug resistance in cancer stem/initiating cells by inducing Stat3 phosphorylation and smoothened expression, and downregulates sonic hedgehog pathways (Shh). MFG-E8 in association with IL-6 plays a critical role in mediating human cancer stem cell tumorigenicity. Its processes include several pathways, like PI3K/Akt, STAT3/SOCS3, β-catenin, mTOR, and TGF-β/FOXO, that regulate anti-cancer drug responsiveness and cancer cell efferocytosis [10, 57–58].

Yamazaki *et al* [12] reported that MFG-E8 promotes tumor progression in oral squamous cell carcinoma (SCC) and that it might be involved in the clearance of apoptotic SCC cells by living SCC cells by studying surgical samples of oral SCC and carcinoma *in situ*. MFG-E8 expression was correlated with clinicopathological features such as tumor size, pathological stage, locoregional recurrence, and scattering invasion pattern. By IHC staining, MFG-E8 was enhanced in apoptotic SCC cells, and some of which were apparently engulfed by the neighboring SCC cells. Transient MFG-E8 knockdown by siRNA in ZK-1 cells decreased cell proliferation and invasiveness and increased cell death.

Administration of anti-MFG-E8 antibodies alone achieved modest tumor destruction in colon adenocarcinoma in mice [48]. Combined use of cytotoxic agents (gemcitabine, 5-Fu, or CPT-11) with anti-MFG-E8 antibodies in mice colon adenocarcinoma model showed enhanced caspase-3 activation and anti-tumor effects compared with that after chemotherapy or antibody alone, and it also resulted in a loss of mitochondrial membrane potential [48]. MFG-E8 also plays an important role in T cell and dendritic cell function by influencing the cytokine productions. Anti-MFG-E8 antibodies attenuate tumor cell resistance to cytotoxic treatments, likely because of the inhibition of Akt activation. Some degree of intrinsic tumor cell sensitivity to the cytotoxic agent appears necessary for this enhancement. Efficient cross presentation of immunogenic antigens serves a key role in achieving a multiple array of anti-tumor immune responses by DC-targeted vaccines; and the activation of innate immune signals mediated by TLR, NLR, and/or CD40 may sense DC to facilitate cross presentation of immunogenic tumor antigens and triggering specific T cell responses [13, 59].

An additional mechanism by which anti-MFG-E8 antibodies might increase tumor cell killing, particularly in conjunction with anti-VEGFR-2 antibodies, may involve a more robust inhibition of the tumor blood supply, as MFG-E8 is required for VEGF-induced

angiogenesis. Moreover, knockdown of MFG-E8 in MC38 carcinoma cells exposed to chemotherapy also reduces VEGF production. Fens *et al* [60] found that RGD-modified erythrocytes show specific binding and internalization by tumor endothelial cells *in vivo* and *in vitro*, and widespread necrosis in tumors with a typical viable rim and central core necrosis in mice with B16.F10 melanoma. This phenomenon possibly by endothelial cell damage induced by degradation of erythrocytes and released large amounts of iron, causing intoxication of the endothelial phagocytes.

MFG-E8 not only prompts cancer vascular angiogenesis, but also regulates multi cancer pathways (p63/p73, PI3K/Akt, STAT3/SOCS3, β-catenin, mTOR, TGF-β/FOXO, etc.), which makes cancer cell resistant to chemotherapy and host immunity suppression. The multi-domain structure of MFG-E8 allows production of truncated proteins that are stable and that could be used as therapeutic agents. Truncated forms of MFG-E8 (such as the entire NH2 terminus) may bind to $\alpha_v\beta_3$ with higher affinity than RGD containing peptides that have little secondary structure and thus are more effective inhibitors. Additionally, MFG-E8 gene knockout influences tumor regeneration, development, and progression; the influences of MFG-E8 gene blockade in immunity also needs further concurrent study. It is likely that MFG-E8 fragments will also inhibit Del1: $\alpha_v\beta_3$ interactions, blocking at least one of the compensatory pathways that are apparently upregulated in MFG-E8 gene knockout tumors.

Conclusion

MFG-E8 is present widely in human tissues and can easily be targeted by its special multi-domain structures. It can be used as a marker of tumor progression in melanoma, cancer burden in metastatic breast cancer and its role in other types of cancer will be revealed in the future. Treatment modalities may focus on developing small molecules as inhibitors targeting $\alpha_v\beta_3/\alpha_v\beta_5$ integrin to influence cancer angiogenesis, targets on C1 and/or C2 domains to restore host cellular or humoral immunities, and/or to elicit new anti-cancer agents by coupling MFG-E8 antibodies. Development of MFG-E8-based therapeutics on cancer will be focused in the future studies.

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Conflicts of interest

The authors indicated no potential conflicts of interest.

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CASE REPORT

Middle lobe torsion after right upper and lower lobectomy: repositioning of lobar torsion using a 3-cm uniportal video-assisted thoracoscopic surgery

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Abstract

We aimed to describe a method for repositioning of right middle lobar torsion by using a 3-cm uniportal video-assisted thoracoscopic surgery (VATS) approach. Middle lobe torsion occurred after right upper and lower lobectomy in a 74-year-old man. Immediate re-exploratory thoracotomy using the 3-cm uniportal VATS approach was performed. The torsion was corrected, and the lobe was anchored to the anterior chest wall with Prolene stitches. The patient recovered well postoperatively with daily improvements in chest radiographic findings. Follow-up examination was performed using fiberbronchoscopy, which revealed an unobstructed right middle lobe bronchus and sticky yellow sputum. Follow-up chest computed tomography was performed 3 months after the primary surgery and revealed increased expansion of the right middle lobe. We repositioned the right middle lobe successfully by using the 3-cm uniportal VATS approach, but more cases are needed to confirm the feasibility of the approach. Lobectomy remains the primary treatment option for such cases.

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Key words: lobe torsion; 3-cm uniportal; video-assisted thoracoscopic surgery (VATS)

Lobar torsion is a rare but life-threatening complication of elective pulmonary resection. As the fissure between the middle and lower lobes is often not well developed, the right middle lobe is the most common site of torsion after lobectomy. Traditionally, treatment includes re-exploratory thoracotomy with resection of the affected lobe. We present a case of right middle lobe torsion after right upper and lower lobectomy that was treated surgically using a 3-cm uniportal video-assisted thoracoscopic (VATS) approach.

Clinical summary

A 74-year-old man with right upper lobe and hilus pulmonis masses was referred to our institution for lung resection. Preoperative computed tomography (CT) and the values of lung cancer markers were consistent with the diagnosis of lung cancer. The patient underwent right upper and lower lobectomy with the 3-cm uniportal VATS approach. A systematic lymph node dissection was also performed. The fissures were well developed, and the inferior pulmonary ligament was partially mobilized. He was extubated immediately after surgery and was transferred to the ward as per routine practice. Postoperative chest radiography revealed satisfactory expansion of the right middle lobe (Fig. 1).

On the first postoperative day, the patient maintained stable vital signs without systemic upset. He was coughing up sticky yellow sputum; fiberbronchoscopic suction was performed to enhance his recovery.

On postoperative day 5, the patient suddenly became tachypneic and tachycardic, but reported having cough with less sputum. Physical examination revealed loss-of-breath sounds on the right lung field. Fiberbronchoscopic examination revealed right middle lobar bronchus stricture with reduced sputum. The bronchoscope could not pass through the right upper and lower lobes, and the bronchial stumps were intact. Emergency CT was performed and revealed right middle lobe atelectasis (Fig. 2).

Immediate re-exploratory thoracotomy was performed by using the 3-cm uniportal VATS approach. At surgery, the right middle lobe had undergone a 180° clockwise torsion at its bronchovascular pedicle. The lobe was a little dusky and edematous but still viable. The torsion was corrected, and the lobe was anchored to the anterior chest wall with Prolene sutures. The patient recovered

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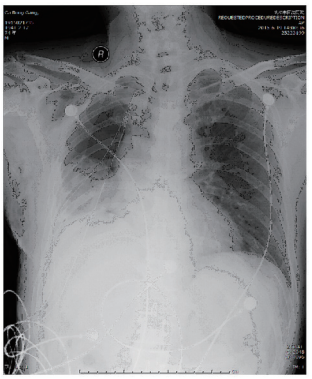

Fig. 1 Postoperative chest radiography revealed satisfactory expansion of the right middle lobeChest radiograph.

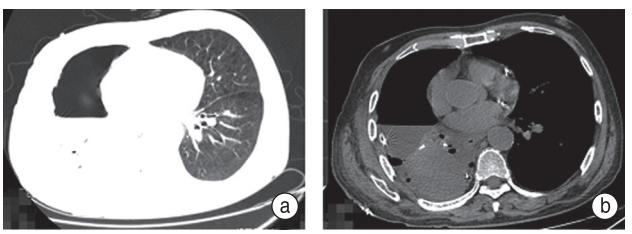

Fig. 2 Chest computed tomographic images revealed right middle lobe atelectasis. (a) Pulmonary window; (b) Mediastinal window

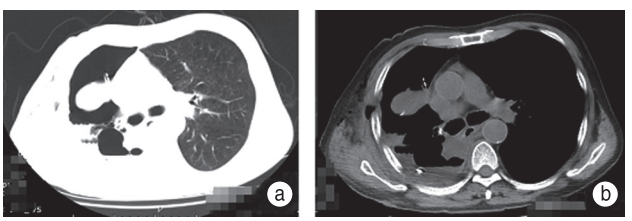

Fig. 3 Chest computed tomographic images revealed reexpansion of right middle lobe. (a) Pulmonary window; (b) Mediastinal window

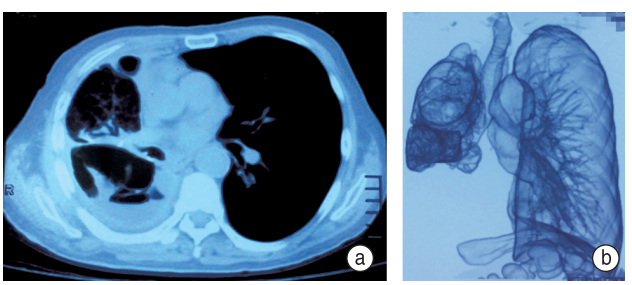

Fig. 4 Chest computed tomographic images: 3 months after the primary surgery showed increased expansion of the right middle lobe. (a) Pulmonary window; (b) 3D Reconstruction

well postoperatively with daily improvements in chest radiographic findings. Follow-up examination with fiberbronchoscopy was performed and revealed an unobstructed right middle lobar bronchus and sticky yellow sputum (Fig. 3).

Follow-up chest CT performed 3 months after the primary surgery showed increased expansion of the right middle lobe (Fig. 4).

Discussion

Lobar torsion refers to a rotation of the bronchovascular pedicle that results in airway obstruction or vascular flow interruption [1]. It is a rare complication that may occur after trauma, any thoracic procedures, and lobectomy [2–5]. It usually involves the right middle lobe after right upper or lower lobectomy, especially in the presence of a complete fissure, and necessitates anchoring of the middle lobe to the remaining lobe to prevent torsion. [6] However, other lobes can also be involved. Isolated reports in the English literature have documented 35 cases of lobar torsion occurring after thoracotomy, with 25 of these occurring after pulmonary resection [7–10].

The true incidence of lobar torsion after pulmonary resection is difficult to determine. The signs and symptoms may present notably with fever, tachycardia, and loss-of-breath sounds over the affected lung field. Radiographic and fiberbronchoscopic examinations are essential to confirm the typical radiographic findings, including homogenous consolidation on plain radiography and absence of contrast enhancement in the affected lobe on CT. A careful bronchoscopic examination may reveal abnormally tight and obstructed orifice of the affected lobe. Suturing the middle lobe to the lower lobe after a right upper lobectomy has been suggested to possibly prevent lobar torsion. In our case, right upper and lower lobectomy was performed to retain the middle lobe only, so lobe torsion occurred much more easily.

After a diagnosis of lobar torsion is established, treatment must begin immediately. Delayed surgical treatment may result in increased risk of mortality. The current treatment of lung torsion involves thoracotomy with lung resection of the nonviable lobe [11]. However, in our case, right upper and lower lobectomy had been performed earlier; performing the middle lobectomy would have been the same as performing a pneumonectomy. Therefore, we did our best to avoid such a case. At surgery, the right middle lobe was a little dusky and edematous but viable. Thus, the torsion was corrected, and the lobe was anchored to the anterior chest wall with Prolene sutures.

Although the incidence of lobar torsion after lung resection is low, delayed diagnosis may contribute to severe complications. For cases of lobar torsion occurring after thoracoscopic lobectomy, emergency thoracoscopy should be performed. Surgeons should be aware of the possibility of lobar, segmental, or

pyramid torsion occurring after lung resection. However, for remaining lobes that are mobile after lobectomy, fixation to the diaphragm is recommended to prevent lobar torsion. In our case, we repositioned the right middle lobe successfully by using the 3-cm uniportal VATS approach, which has never been reported before. Although the uniportal VATS access may cause less pain and further improve cosmesis, even experienced VATS surgeons can find the limited access challenging because of the cramping of instruments and difficulty in achieving stapling angles [12] during the procedure. We investigated many methods to overcome the difficulties, including the following: crossing the instruments; selecting thinner and curved instruments; and changing the posture to minimize obstruction of the view by lobes. In addition, establishing a team with high skill in lobectomy is needed in order to make the uniportal VATS approach easier to perform. Therefore, lobectomy and many other operations can be completed with the application of the 3-cm uniportal VATS approach.

In the present case, we successfully repositioned the torsion lobe by using the 3-cm uniportal VATS approach. However, more cases are needed to confirm the feasibility of the approach, and lobectomy remains to be the primary treatment option.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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CASE REPORT

Two cases of chronic myelomonocytic leukemia combined with monoclonal gammopathy of undetermined significance and a literature review

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Abstract

To describe myelodysplastic syndrome (MDS)/myeloproliferative neoplasm (MPN) combined with monoclonal gammopathy of undetermined significance (MGUS) in order to investigate the potential association between these 2 diseases. Two cases of confirmed chronic myelomonocytic leukemia (CMML) combined with MGUS were reported. In addition, prior publications of cases with combined MDS or MPN with MGUS were reviewed. The first case was of a 77-year-old man whose routine blood tests showed abnormal hemogram results. The diagnosis was CMML combined with IgM monoclonal gammopathy, and the disease course was 4 years. The CMML gradually progressed and the patient presented with anemia, thrombocytopenia, autoimmune hemolysis, and an increase in the number of immature cells in the bone marrow. Although the MGUS caused fluctuations in the concentrations of IgM, no IgM-associated organ damage was observed. Eventually, this patient died from a lung infection. The second case was of a 78-year-old man who sought treatment because of fever and a cough. An increase in the number of monocytes was discovered in the peripheral blood. Bone marrow smear results suggested obvious active granulocytes and an increase in the percentages of promyelocytes, myelocytes, and metamyelocytes. Unhealthy granulocytes and immature monocytes could also be observed, and the percentage of monocytes was increased. In addition, serum IgG levels were increased, and immunofixation electrophoresis results showed IgG-κ type M proteins. The diagnosis was CMML combined with IgG monoclonal gammopathy. These diseases were stable and follow-up was conducted for 1 year after diagnosis. The cases in this study combined with those that were reviewed in the relevant literature indicate that the presence of these 2 diseases in the same patient might not be a coincidence. The development of the 2 diseases in case 1 was different, and we speculate that they might have had different clonal origins. Whether CMML is a risk factor for MGUS and the role of clonal plasma cells in the occurrence and development of MDS and MDS/MPN requires further studies on a larger number of cases.

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Key words: myeloproliferative neoplasms (MPN); myelodysplastic syndrome (MDS); monoclonal gammopathy of undetermined significance (MGUS)

Chronic myelomonocytic leukemia (CMML) constitutes a group of clonal diseases that originate from committed hematopoietic myeloid stem cells or from pluripotent stem cells. Monoclonal gammopathy of undetermined significance (MGUS) is a common pre-malignant plasma cell disease. These diseases are both common neoplastic diseases in elderly people. Although these 2 neoplastic diseases originate from different types of committed hematopoietic stem cells, case reports of patients with both of these diseases are not rare. It is unclear if these 2 diseases present together coincidentally or are causally related. The current report describes 2 cases of CMML combined with MGUS and presents a review of the relevant literature.

Case report

Case 1

The patient in this case was a 77-year-old man. He was admitted to the hospital on July 15th, 2011 because of an increase in white blood cells (WBCs) for more than 1 year and gum bleeding for 3 days. The patient first sought

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treatment at a local health care institution in October 2010 because of an upper respiratory infection. Routine blood tests revealed WBC count: 11.4×10^9/L, hemoglobin (Hb): 132 g/L, and platelet (PLT) count: 71×10^9/L. The patient did not seem concerned at that time.

After admission in 2011, physical examination demonstrated that the patient was lucid and was in good spirits. No signs of anemia were present, the skin featured scattered petechiae and ecchymosis, there were no palpable superficial lymph nodes, and no abnormalities were found in the heart, lungs, or abdomen. A full abdominal computed tomography (CT) scan showed mild fatty liver, a cyst on the right kidney, and some pelvic fluid. Routine blood tests found a WBC count of 34.7×10^9/L with a differential count of 51.9% neutrophils, 10.8% lymphocytes, and 36.6% monocytes; Hb: 108 g/L; PLT: 26×10^9/L.

A blood smear showed metamyelocytes: 0.01%, rod neutrophils: 0.05, lobed neutrophils: 0.60, lymphocytes: 0.18, and monocytes: 0.16. Moreover, small clusters of platelets, but no eosinophils or basophils, were observed. Routine urine tests showed that protein was ±. Complete immunologic and serologic tests showed IgA: 1.9 g/L, IgG: 7.59 g/L, IgM: 48.44 g/L, IgE: 15 IU/ml, and the erythrocyte sedimentation rate (ESR) was 90 mm/hr. Blood biochemistry showed globulin: 48.1 g/L, total bilirubin concentration: 24.3 μmol/L, indirect bilirubin: 20.9 μmol/L, creatinine: 135 μmol/L, uric acid: 452 μmol/L, and the electrolyte concentrations were normal. Serum protein electrophoresis revealed the presence of immunoglobulin M protein.

A bone marrow smear showed clear active proliferation of nucleated cells and an increased granulocyte/erythrocyte ratio. The percentage of cells with a granulocyte lineage was 62%, of which myeloblasts, promyelocytes, myelocytes, metamyelocytes, rod neutrophils, lobed neutrophils, and eosinophils accounted for 0%, 1.2%, 12.4%, 13%, 21%, 12.4%, and 1.6%, respectively. Monocytes accounted for 12.4%, and among these, immature monocytes accounted for 6.4%, while mature monocytes accounted for 6%. A total of 16 megakaryocytes appeared on the whole smear, and the distribution of platelets was scattered. The percentage of lymphocytes was 13.4%. No abnormal cell morphology was observed. Iron staining showed that extracellular iron staining was positive (+). The intracellular iron positivity rate was 21%, the neutrophil alkaline phosphatase (NAP) positivity rate was 5%, and the NAP score was 5 points (the reference score in our laboratory is 7–63 points).

Bone marrow immunology typing showed that 0.7% of the immature cell population was of the myeloid lineage; in addition, 77% of cells of the granulocyte lineage had maturation defects. A bone marrow biopsy showed active bone marrow hyperplasia, active granulocyte lineage proliferation, scattered primitive immature cells, and an increased percentage of mature granulocytes. In addition, increased numbers of cells of plasma cell lineage were observed on histologic sections, and some regions contained aggregated clusters.

Quantitative polymerase chain reaction (qPCR) results for the BCR-ABL fusion gene were negative, tests for the *JAK2* V617F gene mutation returned negative results, and the karyotype of the chromosomes was normal. The diagnosis was 1. CMML combined with MGUS, 2. chronic renal insufficiency, and 3. hyperuricemia.

To alleviate his symptoms, the patient received treatment that included hemostasis, anti-inflammation, and renal protection; after his symptoms improved, he was discharged from the hospital. After that, the patient was admitted several more times because of recurrent dizziness and fatigue, coughing, asthma, and gum bleeding.

The results of a second exam showed that the patient's renal function was stable. The numbers of white blood cells and monocytes had increased, and the patient was anemic. The results of routine blood tests from February 2013 showed WBC: 10.57×10^9/L with a differential count of 28.9% neutrophils, 20.3% lymphocytes, and 47.8% monocytes; Hb: 69 g/L; and PLT: 31×10^9/L. Blood biochemistry showed globulin: 54.9 g/L, total bilirubin level: 52.1 μmol/L, and indirect bilirubin level: 49.3 mmol/L. A Coombs test returned positive results. The level of IgM was 68.51 g/L.

The results from the bone marrow puncture smear showed active proliferation. In addition, the percentage of immature monocytes had increased to 28%; in these cells, each had a large cell body with an irregular shape, the cytoplasm was abundant, the nuclei were irregular, the nuclear chromatin was fine and granular, and most of the cells did not have a nucleolus. Only 10.5% of the cells were mature monocytes. Proliferation of cells with a granulocyte lineage was clearly evident. Myeloblasts, promyelocytes, myelocytes, metamyelocytes, rod neutrophils, and lobed neutrophils accounted for 10%, 5%, 2.5%, 1.5%, 7.5%, and 22.5%, respectively. Abnormalities such as nuclear atypia and a decrease in the number of cytoplasmic granules could also be observed. Proliferation of cells of the erythrocyte lineage was decreased; the number of lymphocytes was also decreased and they accounted for only 7.5% of the cells. Thirty-nine megakaryocytes were present on the whole section, and the number of platelets was decreased.

A thoracic CT showed inflammation in the superior lobe of the left lung and in the inferior lobe of the right lung as well as emphysema. After the patient received oral hydroxyurea and prednisone as well as treatment for his symptoms (anti-infection, kidney protection, activating blood circulation to dissipate blood stasis,

blood perfusion, and improvement of myocardial blood supply), the patient's condition improved. However, the patient ultimately died in December 2014 of a lung infection combined with cardiopulmonary insufficiency.

Case 2

The patient was a 78-year-old man. He was admitted to the hospital on February 9, 2014 because of coughing for 10 days and fever for 2 days. Ten days before admission, the patient developed an upper respiratory infection and experienced a cough. He was treated with azithromycin and levofloxacin. The effects of these drugs were poor, and the patient then experienced fatigue and a fever of 39.4℃.

Physical examination upon presentation showed that the patient was lucid and in poor spirits. There were no obvious signs of anemia and both lungs exhibited coarse breath sounds and did not exhibit dry or wet rales; the heart rate was 90 beats/min, and atrial fibrillation without a murmur was detected. After admission, a thoracic X-ray showed bronchitis. Electrocardiography showed rapid atrial fibrillation, a complete right bundle branch block, and partial lead ST-T changes.

Routine blood tests found WBC: 7.93×10^9/L with a differential count of 58.9% neutrophils, 10.2% lymphocytes, and 29.6% monocytes; red blood cells (RBCs): 3.2×10^{12}/L; Hb: 108 g/L; and PLT: 137×10^9/L. Blood biochemistry results showed that the albumin, globulin, creatinine, and uric acid levels were 34.6 g/L, 44.5 g/L, 122 μmol/L, and 492 μmol/L, respectively. The ESR was 101 mm/h. A blood smear showed that the percentage of mature monocytes was significantly increased and accounted for 36% of the white blood cells. The ferritin concentration was 1203 ng/ml, and tests for serum folate and vitamin B12 revealed normal levels.

A bone marrow smear showed active bone marrow hyperplasia, proliferation of cells of the granulocyte lineage, and an increase in the percentage of promyelocytes, myelocytes, metamyelocytes, and monocytes. Additionally, unhealthy granulocytes could be observed; 0.4% of the total cells were immature monocytes, while 17.2% were mature monocytes. Complete serology showed IgA: 0.23 g/L, IgG: 27.8 g/L, and IgM: 0.26 g/L.

Immunofixation electrophoresis showed the presence of IgG-κ type M proteins; M proteins accounted for 28.2%, with levels of free light chain κ: 9.31 mg/L (range: 3.3–19.4 mg/L), λ: 11.7 mg/L (range: 5.7–26.3 mg/L), and κ/λ: 0.79 (range: 0.26–1.65).

The patient had a history of hypertension for more than 20 years and a history of coronary heart disease and atrial fibrillation. He had received a pacemaker and had a history of gallstone surgery. A review of the patient's routine blood tests from 2012 and 2013 showed that the patient had a normal white blood cell count and an increased percentage of monocytes, which fluctuated between 15%–35%.

The final diagnosis included the following: 1. CMML combined with MGUS, 2. bronchitis, and 3. hyperuricemia. The patient received anti-infection treatment, which was ineffective after 5 days, with the patient continuing to exhibit a high fever, poor appetite, and fatigue. The patient was then treated with an intravenous injection of methylprednisolone, and his body temperature returned to normal. However, the patient again exhibited signs of a fever 4 days later. After he received cyclophosphamide combined with methylprednisolone, the symptoms of fever and fatigue gradually disappeared. Treatment with these drugs was stopped after 2 months.

A clinical follow-up was performed recently. His disease was stable, and he did not complain of any discomfort. Routine blood tests, complete immunology tests, and immunofixation electrophoresis have been performed regularly, and the tests did not show any significant changes.

Discussion

MDS is composed of a group of clonal diseases that have the following clinical presentation: ineffective hematopoiesis in the bone marrow and a high-risk of transformation into acute myeloid leukemia. Before 2008, CMML had been classified as a subtype of MDS. In recent years, the World Health Organization (WHO) decided that the French-American-British (FAB) classification system did not adequately reflect the clinical prognosis and biological behaviors of patients; therefore, CMML was classified into an independent, larger disease group (MDS/MPN). In fact, according to the new classification system, CMML includes previous MDS-CMML (more emphasis on dyshematopoiesis) and MPN-CMML (more emphasis on bone marrow proliferative changes) [1]. MGUS is a common pre-malignant plasma cell disease. MGUS may be divided into 3 different subtypes: non-IgM type MGUS, IgM type MGUS, and light chain type MGUS. Non-IgM type MGUS is further divided into IgG, IgA, double clone, and the rare IgD and IgE types [2]. All types typically evolve into multiple myeloma (MM), AL amyloidosis, or other diseases at a rate of 1% per year.

Although the diseases mentioned above originate from different types of committed hematopoietic stem cells, case reports of the presence of these 2 diseases in a single patient are not rare. In 1986, the Spanish researcher Costa [3] first reported a case of CMML combined with lymphoma. Since then, researchers from different countries also reported cases of CMML combined with lymphoma [4–5]. A prospective study by Economopoulos [6] showed that 5 out of 61 patients with CMML had combined MGUS, 8.2%,

much higher than the 1–3% in the general population. However, Cesana[7] studied disease transformation of 1231 patients with either MGUS or smoldering MM and showed that only 0.4% of cases had combined MPN. This leads to the question of whether MGUS combined with MDS or MDS/MPN are coincidental or whether these 2 diseases are causally related.

Mailankody *et al* [8] retrospectively analyzed secondary hematological malignancies in 8740 cases of MM and 5652 cases of MGUS that occurred in Sweden between 1986 and 2005. In cases of MGUS, the risk of development of MPN/polycythemia vera (PV) was increased 5-fold. Compared with MGUS, the risk of development of AML/MDS in patients with MM was even higher, and patients with the IgG/IgA type were more prone to the development of acute myeloid leukemia (AML)/MDS than were patients with IgM type gammopathy. However, the incidence of MPN/PV was not increased significantly in patients with MM.

These results are not consistent with those of a study with a small sample size conducted by Duhrsen *et al* [9], which showed that the number of cases of plasma cell tumors combined with MPN/PV was significantly increased. The authors admitted that the high risk of incidence obtained in their study might have been because of detection bias. For example, the clinical data of patients with MGUS were obtained from the Swedish Inpatient Registry; therefore, they could not account for some patients who sought treatment for other diseases and who were diagnosed with MGUS after they underwent MGUS-related examinations. In addition, routine blood tests during the clinical follow-up of patients with MGUS might lead to the discovery of MDS at an early stage.

To exclude bias associated with targeted examinations for other diseases, Mayo Clinic [10] performed a survey on local healthy residents above the age of 50 years. Their results showed that the development of MDS in patients with MGUS was increased 2.4-fold compared with the control group; moreover, no patients with MGUS developed acute lymphoid leukemia (ALL). These results are consistent with the results of the Swedish report. Because the number of patients with AML in the cohort study was small, the study could not confirm whether MGUS was associated with AML. Compared with the Swedish study, which showed that the development of MDS in patients with MGUS was 8.1 times that in the controls, the number was significantly decreased. The researchers thought that the reason for this was that the subjects who were enrolled in the Swedish study were mostly patients who had clinical symptoms and went to the hospital for a serum protein electrophoresis examination; therefore, some patients who did not have clinical symptoms were missed.

The findings of the Mayo Clinic study, which differed from those of the Swedish study, did not show that patients with IgA/IgM MGUS were prone to the development of MDS or AML. Although conclusions from these 2 large studies had some differences, they both found that the incidence of MDS in patients with plasma cell diseases was significantly increased compared with that in the general healthy population.

It is unclear whether these 2 types of hematological tumors in the same individual arise from the same stem cells or have different cellular origins. The observation by Shvidel *et al* [11] was that the disease progression of CMML was different from the progression of lymphoma. Therefore, they thought that these tumor types did not arise from a common abnormal stem cell population. Zagaria [12] reported 1 case of MGUS in a patient who developed anemia and thrombocytosis after 2 years. The patient was diagnosed with MDS with a 5q deletion. After the first diagnosis of MGUS and before the development of MDS, the presence of a MYD88 mutation was discovered in the bone marrow. To clarify the origins of these 2 clones, allele-specific PCR combined with fluorescence in situ hybridization was used to detect the molecular cytogenetic changes in hematopoietic stem cells. All of the CD34+ hematopoietic stem cells had the 5q deletion; however, no mutations in the MYD88 gene were observed. At the cytogenetic and molecular levels, these results showed that these 2 tumors were independent of each other. Anelli [13] also reported that patients with polycythemia vera with the *JAK2* V617F mutation developed IgG type lymphoplasmacytic lymphoma (LPL) after several years. The detection of the *MYD*88 L265P mutation and the *JAK2* V617F mutation confirmed that these 2 diseases were two independent clonal diseases that co-existed in the same person.

However, some studies also reported that leukemia cells in patients with CMML expressed both monocyte and NK cell markers together; therefore, it is possible that these tumor cells might have originated from common progenitor monocyte and NK cells [14]. During treatment for a nose bleed following a respiratory infection, it was discovered that the first patient (case 1) in the current study had CMML combined with IgM MGUS. The disease course lasted more than 4 years, and the CMML gradually progressed; the clinical presentation included anemia, thrombocytopenia, autoimmune hemolysis, and an increase in immature cells in the bone marrow. Although MGUS is associated with fluctuations in IgM values, no IgM-associated organ damage was observed. For example, lymphadenopathy, hyperviscosity, hypercalcemia, and kidney and bone damage did not significantly worsen during disease transformation as compared with when the patient was first diagnosed. These results also suggest that these tumors had different clonal origins; if 2 types of tumor clones were present, CMML might have been the

dominant clone.

Because MGUS and MDS or MDS/MPN primarily occur in elderly people who typically do not exhibit clinical symptoms, MGUS is usually discovered incidentally when patients seek treatment for other diseases. The diagnosis of MDS or MPN/MDS is usually confirmed because of the presence of clinical symptoms induced by abnormal blood cells. Therefore, when a patient presents with these 2 diseases simultaneously, it is difficult to know which disease developed first. Malhotra *et al* [15] retrospectively analyzed 90 patients with MPN who were treated at their medical center within the past 5 years. Immunofixation electrophoresis results from the blood and urine of a total of 32 patients were examined. Out of these, 15 were diagnosed with MGUS/MM; however, the majority of patients were diagnosed with MPN at the same time or before the MGUS/MM was diagnosed, and only 3 patients were diagnosed first with MGUS/MM followed by MPN. None of these 3 cases featured an increase in the number of red blood cells or platelets, which indicates that the diagnosis was delayed relative to the actual course of disease development. The 2 elderly patients described in this article were discovered to have both CMML and MGUS during treatment for other diseases; therefore, no treatment-related factors were involved. Because immunoglobulin detection was not performed initially, this test could not confirm which disease developed first.

Studies have shown that monocytes and nurse-like cells derived from monocytes are important components of the tumor microenvironment that play important roles in the maintenance and protection of tumor cell growth in the blood [16]. Whether CMML may be a risk factor for MGUS and the function of clonal plasma cells in the occurrence and development of MDS or MDS/MPN requires further study.

Yoshida *et al* [17] reported 14 cases of MDS combined with MGUS at their medical center within the past decade; this article is currently the clinical report with the largest number of cases of MDS combined with MGUS. These cases accounted for 10.2% of MDS patients, significantly higher than the number of people of the same age in a control population with anemia. When the patients were diagnosed with MDS, the majority of them were also diagnosed with MGUS, which suggests that MGUS was not associated with treatment and that MGUS could be present in patients with any MDS WHO classification subtype. Among the 12 patients, 11 patients had low-risk chromosome karyotypes; combined with other risk factors, the clinical outcomes of the MDS combined with MGUS group were not significantly different from those in the MDS group, which suggests that MGUS did not influence the prognosis of the patients with MDS. Case 1 in this article had a 4-year-long disease course. The patient's disease did not transform into acute leukemia, and he eventually died from a lung infection. Compared with the median survival period of 20–40 months of patients with CMML without MGUS, the survival period was not decreased; therefore, this observation supports the idea that MGUS might not have an adverse effect on the prognosis of patients with CMML.

The elderly are susceptible to both MGUS and MDS/MPN. After a review of the relevant literature, a limited number of studies showed that the incidence of secondary MDS/MPN in patients with MGUS was higher than that in the general population. The association between these 2 diseases and the mutual influence on each of their prognoses require further studies of a larger number of clinical cases. In addition, these results suggested that in future work, routine blood tests should be the focus for patients with MGUS. Finally, an analysis of serum M protein should not be ignored in patients with MDS/MPN.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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