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Molecular Signatures and Precision Medicine of Gastric Cancer

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Abstract

Gastric cancer (GC) is the fifth most commonly diagnosed cancer worldwide and the second leading cause of cancer-related deaths. Adenocarcinoma accounts for approximately 95% of all malignant gastric neoplasms. Most localized GC (stages II and III) are best treated with multimodality therapy with a 5-year survival in approximately 40% of patients; however, advanced GC only has limited treatment options with poor prognosis. GC is a highly heterogenous disease. Historically, many classification systems have been proposed, including anatomical classification (Borrmann classification, Siewert and Stein classification), histological classification (WHO classification, Laurén's classification), and extent of disease (early gastric cancer vs. advanced cancer). Originally proposed in 1965, the Laurén's classification divides GC into intestinal- and diffuse-types that are quite distinct in the histological features, epidemiology, and etiology.

Keywords: Gastric cancer; HER2; Microsatellite instability; PD-L1; CDH1; Cancer immunotherapy

Introduction

Intestinal-type tumors form glandular structures, often arise in patients with severe atrophic gastritis or persistent Helicobacter pylori infection and are strongly associated with intestinal metaplasia [1-5]. In comparison, diffuse-type GC displays poor cellular cohesion, poor differentiation, unresponsiveness to treatment, and worse prognosis [6]. The 2010 World Health Organization (WHO) classification scheme divides GC into papillary, tubular, mucinous, and poorly cohesive subtypes, in addition to a few less frequent histologic variants [7,8]. However, these histological classification schemes only have limited clinical utility to further stratify patients for chemotherapy, novel immunotherapy, and targeted therapies, and for predicting responsiveness and prognoses, due to the genetic heterogeneity and complexity of gastric cancers [5].

With mounting biological information from molecular profiling and expression analysis of gastric cancer in recent years, new comprehensive molecular classifications of gastric cancer have emerged. In 2014, The Cancer Genome Atlas (TCGA) project classifies the gastric cancer into four subtypes: tumors positive for Epstein-Barr virus (EBV+ GC), microsatellite unstable tumors (MSI), genomically stable tumors (GS), and tumors with chromosomal instability (CIN) [9]. In 2015, the Asian Cancer Research Group (ACRG) studies proposed a similar but not equivalent molecular classification that includes four subtypes: tumor with microsatellite stability (MSS)/ epithelial-mesenchymal transition (EMT), microsatellite-unstable tumors (MSI), microsatellite stable TP53-active (MSS/TP53+) GC, and microsatellite stable TP53-inactive (MSS/TP53-)[10]. The clinical utility of these new molecular classifications is still to be validated.

While the TNM stage (established by the depth of invasion of gastric wall (T), the involvement of lymph nodes (N) and the presence of distant metastasis (M)) remains the most important prognostic factor for GC, several molecular markers including HER2, the mismatch repair (MMR) genes, E-cadherin gene (CDH1), and the inhibitors of immune checkpoint factors (programed death ligand-1 (PD-L1), programed death-1 (PD-1)), have emerged with specific clinical utility in guiding gastric cancer surveillance, diagnosis, prognosis, and treatment [5]. This review will explore the growing relevance of these molecular signatures in the diagnosis and treatment of gastric cancer.

Literature Review

HER2 and gastric cancer

Clinical relevance of *HER2* amplification and overexpression in GC: The proto-oncogene *HER2,* also known as CerbB-2 and ERBB2, is located on chromosome 17q21 and belongs to the Epidermal Growth Factor Receptor (EGFR) family with tyrosine kinase activity. *HER2* plays a key role in regulating cell differentiation, proliferation, motility and signal transduction [11]. *HER2* receptor activates its downstream regulatory events by spontaneous homodimerization or heterodimerization with other EGFR family receptors [11-13]. *HER2* amplification and overexpression were first discovered in breast cancer [14]. Subsequently, *HER2* positivity has been observed in colorectal cancer, ovarian cancer, prostate cancer, lung cancer as well as gastric and gastroesophageal cancer [15,16]. We have found that strong HER-2 amplification and overexpression occurring more frequently in well to moderately differentiated tumors than in poorly differentiated tumors and patients with *HER-2/neu* gene amplification had decreased survival. A larger number of studies have shown that *HER2* overexpression are often associated with serosal invasion, metastases, higher disease stage, high frequent recurrence, and overall poor survival [17].

The overall reported frequency of *HER2* overexpression in GC ranges from 7% to 53.4% with a mean of 17.9% [18]. In the ToGA trial, 22% of gastric tumors were *HER2* positive, and *HER2* positivity differed significantly by histological subtype (intestinal 34%, diffuse 6%, mixed 20%) and the site of the tumor (32% GEJ and 18% gastric body) [19]. Another study reported that positive *HER2* amplification in 12.2% of the gastric and 24.0% of the gastroesophageal adenocarcinomas. HER-2 amplification was observed in 21.5% of the intestinal-type and 2% of the diffuse-type of GC, showing no association with age and gender, but strong association with poor survival of GC patients [20].

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In addition, intertumoral heterogeneous *HER2* expression is far more common in gastric carcinomas than in breast carcinomas [21]. The discordant HER2-positive results were observed between paired biopsy and resection specimens and between paired primary and distant metastases in HER2-positive GC cases [18,22]. Other factors that also affect *HER2* status in GC include disease stages, interpretation of IHC results, old paraffin blocks, and antibodies used for IHC [23].

Trastuzumab is a monoclonal antibody directed against *HER2* and was first introduced for the treatment of HER2-positive advanced breast cancer, with significant reduction in recurrence and improvement in survival [14]. In the ToGA trial, patients with unresectable HER2-positive GC treated with chemotherapy and trastuzumab showed a statistically significant improved median overall survival of 4.2 months in a post-hoc analysis [16,24-26]. The proven benefits of trastuzumab in the ToGA trial made it the first molecular targeted agent approved as a standard treatment in gastric cancer. Other HER2-targeted agents including pertuzumab, lapatinib, trastuzumab-emtansine, and afatinib have been tested; and the efficacies of these agents were either unsatisfactory or similar as trastuzumab [27-31]. Taken together, *HER2* amplification and overexpression is not only a negative prognostic marker, but also a targeted therapeutic marker for gastric cancer. Therefore, it is imperative to determine the *HER2* status in advanced gastric or gastroesophageal junction adenocarcinoma in order to select patients who may benefit from trastuzumab treatment.

Genomic alterations associated with HER2-positive GC: In addition to amplification/overexpression of HER2, a variety of genetic variations were also identified in the HER2+ GC. These include hot spot mutations most frequently found in TP53 (54%) followed by CDKN2A (4%), KRAS (2%), KIT (2%), and PIK3CA (2%), and concomitantly co-amplification of CCNE1 (8%), PIK3CA (8%), KRAS (2%), CDK4 (2%), and CDK6 (2%) [32]. All HER2+ GC with concomitant CCNE1 amplification tends to progress more rapidly after trastuzumab-based chemotherapy [32]. On the other hand, only 17.4% of the TP53 inactive GC subgroup displayed focal amplifications in oncogenes such as HER2. A small subset of these HER2+ and TP53- GC also harbor concomitant amplification of EGFR and/or MET and are associated with aggressive behavior [33]. Hence, combination therapy should be tested when enrolling these patients into anti-*HER2* therapies.

***HER2* testing in GC:** Because of the differences in *HER2* expression, scoring, and outcomes in GC relative to breast carcinoma, guidelines for *HER2* testing in GC have been established by College of American Pathologists (CAP), Society for Clinical Pathology (ASCP), and American Society of Clinical Oncology (ASCO). *HER2* testing should be performed for all patients with advanced GC who may benefit from HER2-targeted therapy. Testing can be performed on biopsy or resection specimens (primary or metastasis) or FNA specimens (cell blocks) prior to the initiation of trastuzumab therapy. *HER2* status should be evaluated by IHC testing first, followed by ISH when IHC result is 2+ (equivocal) and evaluated in areas with strongest intensity of *HER2* expression by IHC. Ruschoff/Hofmann method should be applied to score *HER2* IHC and ISH results for GC [34]. *HER2* IHC results are scores by a four-tier *HER2* scoring system as 0, 1+, 2+, and 3+, with scores of 0 and 1+ considered negative, 3+ as positive, and 2+ as equivocal [35]. *HER2* positivity or overexpression by IHC is defined as strong complete, basolateral and lateral membranous reactivity in at least 10% stained tumor cells for resection specimens and a small single cluster of cells (or at least five cells) for biopsy specimens. *HER2* positivity or amplification by ISH is defined as a ratio of *HER2* signal to CEP17 signal of ≥ 2.0 after at least 20 non-overlapping nuclei of tumor cells are evaluated for *HER2* probe and CEP17 probe signal enumeration. If IHC is 2+ and there are three or more CEP17 signals, on average, with a ratio <2, then presence of more than six *HER2* signals, on average, is interpreted as positive for *HER2* amplification by ISH/FISH [25,34].

Mismatch repair (MMR) system and gastric cancer

Microsatellite instability and the mismatch repair genes system: Microsatellites are short DNA sequences consisting of repetitive arrangements (usually 10 to 60 times) of one to six nucleotides. Microsatellites are randomly distributed throughout the genome [9,36-38]. Correct replication of these highly repetitive DNA sequences is maintained by the mismatch repair (MMR) system, comprised of several proteins encoded by MLH1, MSH2, MSH6, and PMS2 genes [39-41]. The Msh2 and Msh6 protein form a heterodimer and interacts with Msh2/Msh3 to detect the replication error. The mismatched nucleotide sequence can be removed and resynthesized with the subsequent recruitment of the Mlh1/Pms2 [40]. Dysfunction of MMR proteins lead to insertions and/or deletions in the microsatellite regions during DNA replication, a phenomenon known as microsatellite instability (MSI) [42,43]. The dysfunction is usually caused by the mutations in the coding region, promoter methylation, or loss of heterozygosity [40,44,45]. It has been shown that MSI tumors are associated with 100- to 1000-fold increased mutation rates throughout the genome when compared to microsatellite stable (MSS) tumors [44-46]. The repetitive sequences of microsatellite DNA are particularly vulnerable to replication errors and can be used as a marker to evaluate the function of the MMR system [42].

Clinico-pathological features of MSI-GC

Gastric cancer with microsatellite instability (MSI-GC) represents a distinct subtype of GC as defined by both TCGA and ACRG studies, and MSI-GC is associated with elevated mutation rates in genes of oncogenic signaling pathways such as *PIK3CA, ERBB3, ERBB2,* and *EGFR* genes [9,10,47,48]. In addition, frequent mutations have been observed in genes regulating cell cycle regulation and apoptosis such as *TGFβ RII, IGFIIR, TCF4, RIZ, BAX, CASPASE5, FAS, BCL10,* and *APAF1*, and in genes in maintaining genomic integrity such as *MSH6, MSH3, MED1, RAD50, BLM, ATR, and MRE11* [42]. Surprisingly, BRAF V600E mutation which was frequently reported in sporadic colon cancer caused by MSI, has not reported in MSI-GC [48].

The prevalence of MSI-GC was reported to be 8.5% to 37.8% and can be observed in either sporadic GC or in the setting of Lynch syndrome [42,44,45,49,50]. It is more frequently associated with old age, intestinal histotype, female gender, the distal stomach, earlier tumor stages, multiple synchronous gastric cancers, and better overall and tumor-specific survival [10,51,52]. In the sporadic setting, hypermethylation of MLH1 promoter was observed in over 50% of MSI GCs while mutations in MLH1 and MSH2 have been reported in 12–15% of this GC subgroup [53]. In the setting of Lynch syndrome, dysfunction of the MMR system is caused by autosomal dominant mutations mostly in MLH1 and MSH2, less frequently in PMS2 and MSH6, and rarely due to epigenetic silencing of MSH2 [54]. Unlike the sporadic MSI-GC, patients with Lynch syndrome are at increased risk of developing various cancers at a younger age [40,44,45].

Most MSI-GC showed unique histological features including highly pleomorphic tumor cells with large vesicular nuclei, trabecular, nested, micro alveolar, or solid growth pattern, and abundant tumor-associated inflammatory stroma consisting of either polymorphs and/

or lymphocytes [55]. However, some MSI-GC could completely lack these histological features and are indistinguishable from MSS-GC. Evaluation of the microenvironment of the MSI-GC showed increased tumor-infiltrating lymphocytes (TIL) and high level and frequency of expression of immune checkpoint factors including PD-L1, LAG-3, IDO, and CTLA4 [56,57]. It has been hypothesized that the higher mutational rate of microsatellite instability-high (MSI-H) tumors results in increased expression of neoantigens, which in turn, recruit and activate the TILs, inducing an intense immune response as well as the expression of immune checkpoint factors [53,57,58].

MSI status affects the survival and response to chemotherapy in GC: MSI-H colorectal carcinomas usually show a better prognosis when compared to microsatellite instability-low (MSI-L) tumors and should not receive adjuvant chemotherapy with fluoropyrimidine after resection [59,60]. Similarly, MSI-GC showed a 37% mortality risk reduction and improved median OS compared to MSI-L or MSS- GC patients [60]. Results from the MAGIC trial showed that MSI and MLH1 deficiency in patients treated by surgery alone led to better outcomes, while it had a negative prognostic effect in those treated with chemotherapy [61-63]. Moreover, MSI-GC demonstrated higher 3-, 5-, and 10-year disease-specific survival rates compared to MSS patients even with positive resection margins (R+) [61,62]. Interestingly, a prospective genomic-profiling research showed that metastatic MSI-GC on standard cytotoxic therapy progressed more rapidly with a significantly shorter progression-free survival compared with MSS patients [63]. However, when these fast-progressing patients were treated with anti-PD-1 antibodies either as a single therapy or in combination with anti-CTLA4 antibodies, almost 50% of the patients showed durable immunotherapy responses [64]. Targeting immune checkpoints with monoclonal antibodies has recently become the promising strategy for treatment of several tumors [58,65,66]. In the Keynote-012 trial, 17% of all patients with advanced GC were determined to be MSI-H GC and 50% of these patients reached partial response to pembrolizumab, even in patients without detectable *PD-L1* expression [67-69]. Similar results were also observed in the KEYNOTE 059 trial, where patients with MSI-H group of gastric or gastroesophageal junction advanced adenocarcinoma showed an impressive reported ORR of 57% with the response duration ranging from 5.3 to 14.1 months [70-72]. In the Checkmate 032 trial, 28% of all patients with metastatic GC were determined to be MSI-H, and ORR to nivolumab was the highest (29%) in MSI-H patients. In addition, the MSI-H patients reached longer median OS (14.75 months) compared with the other subgroups [67,73]. These studies suggest that the MSI status should be determined to avoid enrolling MSI-GC patients into unnecessary chemotherapy regimens, and MSI can be used as predictive biomarker of response to immunotherapy and of prognosis.

Diagnosis of MSI: Currently, there are several well-validated methods to evaluate the functionality of the MR system, including amplification of microsatellite sequences of representative genomic loci by polymerase chain reaction (PCR), evaluation of nuclear expression of MMR proteins by immunohistochemistry (IHC), and detection of MSI by next-generation sequencing (NGS) [44,53,54].

Deficiency of MMR proteins usually result in insertions or deletions of nucleotides in the microsatellite regions during DNA replication [37,40,74]. The variation of microsatellite regions can be detected by PCR, using specific primers to amplify certain specific microsatellite loci in both tumor and normal tissue [75-77]. MSI can be determined by the presence of shifts in the size of the amplicons from the microsatellite loci in tumor and normal tissue. To increase the specificity and sensitivity and ensure the reproducibility and standardization between different laboratories, five microsatellite markers, including two mononucleotide loci (BAT-25 and BAT-26) and three dinucleotide loci (*D2S123, D5S346,* and *D17S250*) were recommended by Bethesda Panel as references for diagnostic testing [36,54,77,78]. MSI-H status is defined by a shift in size in at least 2/5 microsatellite loci, MSI-L by a shift in size in 1/5 loci, and microsatellite stable (MSS) by no shift in cancer tissue compared to the reference [36,44,54,76-78].

Alternatively, MMR deficiency can be determined by IHC to show the loss of nuclear expression of one or more MMR proteins. Monomeric MLH1 and MSH2 are stable, while PMS2 and MSH6 are unstable as monomers and quickly degrade [36,40,44]. Thus, tumors with MLH1 or MSH2 mutations usually show loss of not only the affected protein, but also their functional partners, PMS2 or MSH6, respectively. On the other hand, PMS2 or MSH6 mutations would show loss of only the affected protein [36,40,44,78]. Although as an indirect method to assess MSI, the performance of the IHC method is comparable with that of the PCR method with >90% concordance rate [38]. However, IHC cannot detect the missense mutations of the MLH1 or MSH6 genes that render the protein unfunctional but still retain the antigenic epitope for the IHC antibodies [36,38,42]. In these cases, the MSI status can only be evaluated by either PCR-based testing or NGS.

The microsatellite status of a tumor can also be determined by NGS with coverage of a broader range of microsatellite loci. The disadvantages of this method are higher initial costs, longer turnaround time needed to perform the sequencing, and the requirement of bioinformatics analysis to interpret the data [79-81].

Immune check point factors and gastric cancer

Frequency of PD-L1/2 expression in GC: With the success of immune-checkpoint inhibitors in the treatment of many types of tumors including advanced gastric carcinoma, immunotherapy has gained considerable attention [82-85]. Recent molecular characterizations of the GC have shown elevated PD-L1/2 expression in both EBV positive (EBV+) and MSI-GC subtypes which may more likely respond to immunotherapies [9,10,86].

PD-L1 (B7-H1), a member of the immunoglobulin superfamily B7, is a 290aa transmembrane glycoprotein encoded by the *CD274* gene located on chromosome 9 and is the ligand of programmed cell death 1 (PD-1) [87-90]. *PD-L1* is normally expressed in antigen-presenting cells including dendritic cells, macrophages, and monocytes, but also aberrantly on the cell surface of a wide variety of solid tumors [88,89,91,92]. PD-1 (*PD-L1* receptor) is usually expressed by activated T-cells, tumor-infiltrating lymphocytes (TILs), and other immune cells [4]. Another ligand for PD1 is *PD-L2* (also known as B7-DC and CD273) which is thought to be a *PD-L1* homologue arising through gene duplication within 100 kb of each other in chromosomal region 9p24.1 [93-95]. Unlike PD-L1, *PD-L2* is usually inducibly expressed on dendritic cells, macrophages, bone marrow–derived mast cells, and certain tumor cells [96]. The PD-1/PD-L1/2 interactions are considered as important immune check point, which leads to the suppression of T-cell receptor signaling and the down regulation of the immune response to maintain the tolerance of self-antigens in normal host [87,97]. The inhibitory effects on the immune response by these checkpoint regulators would also allow the tumor cells to escape host immune destruction [88,89,97,98]. Targeting the PD-1/*PD-L1* immune checkpoint by therapeutic PD-1/*PD-L1* inhibitors could restore the

cancer cell-directed immune response by improving the immune functions of tumor-specific T cells [99-101].

The EBV+ GC represents roughly 7-10% of all GC with no difference between intestinal and diffuse histological types and among geographical regions but is more prevalent in younger patients and in males, with a male/female ratio of 2:1 [9,10,102,103]. In EBV+ GC, *PD-L1* expression was observed both in tumors (50% of cases) and in tumor-surrounding immune cells (94% of cases) [57, 105]. In addition, 15% of EBV+ GC showed amplification in the chromosomal region 9p24.1 which contains the *PD-L1* and *PD-L2* loci [104,105]. The frequency of *PD-L2* positivity in EBV + GC is much lower and was observed in tumor cells of 22% of cases and in stromal immune cells of 38.8% of cases [106]. In contrast, a lower *PD-L1* expression was observed in tumor cells of 10% of EBV- GC cases and in tumor-surrounding immune cells of 39% of EBV- GC cases [100]. Similar findings were reported in other retrospective studies in which PIK3CA and *PD-L2* were more highly expressed in EBV+ GC than in EBV- GC [104,105]. As previously mentioned, MSI-GC is characterized by increased lymphocytic infiltrate with significantly higher rates of *PD-L1* expression (37-87%) and *PD-L2* expression compared with MSS -GCs. *PD-L1* expression was more frequently observed in 61% for EBV+ GC and 37% of MSI GCs [105].

PDL-L1/2 expression as a prognostic biomarker for GC: The correlation of *PD-L1* expression with the prognosis in patients with GC is still considered controversial due to the conflicting reports. Some studies claimed that *PD-L1* expression was independently associated with improved longer survival [107,108]. Others reported that *PD-L1* expression was not associated with a poor prognosis in patients with gastric cancer [109,110]. However, most studies showed that *PD-L1* expression is an independent negative prognostic predictor for GC. Expression of *PD-L1* was significantly associated with tumor size, invasion, lymph node metastasis, advanced clinical and pathological stage, and shorter survival time of patients [107,108,111-113]. In addition, several meta-analyses have demonstrated that *PD-L1* overexpression is a worse prognostic factor in GC [100,114,115]. It has been hypothesized that the poor prognosis of GC with positive *PD-L1* expression are related to its inhibitory effects on tumor-specific T lymphocytes [116]. The *PD-L2*-positivity in the stromal immune cells correlates with lower T stage, negative lymph node metastasis, and perineural invasion, but with no prognostic impact on DFS of EBV + GC [106].

***PD-L1* expression as a predictive biomarker for response to immunotherapy:** Several PD-1/PD-LI inhibitors have already been approved by the FDA for cancers like non-small cell lung carcinoma (NSCLC), Merkel cell carcinoma, and melanoma. The commonly known inhibitors include PD-1 inhibitors (pembrolizumab, nivolumab) and *PD-L1* inhibitors (avelumab, durvalumab, atezolizumab) [83,117-120]. All these inhibitors are also being tested for efficacy in treating GC in various phases of several ongoing clinical trials. Based on the encouraging early phase results obtained by KEYNOTE-012 (NCT01848834), KEYNOTE-028 (NCT02054806) FDA accelerated the approval of pembrolizumab for the treatment of patients with PD-L1-positive recurrent or advanced GC who have received 2 or more lines of chemotherapy [69,73,121]. Results from ONO-4538-12 (NCT02267343) phase III clinical study showed that nivolumab, significantly improved OS, PFS and ORR compared to placebo as a rescue treatment after failure of standard chemotherapy for GC [122]. Preliminary results from phase Ib/II studies (NCT02572687) demonstrated that durvalumab in combination with ramucirumab, an anti-VEGFR-2 inhibitor, induces synergic antitumor effects in GC [123].

Currently, *PD-L1* expression is the only available biomarker in predicting the tumor response and survival prognosis as the results from various clinical trial showed that increased response rates and longer overall survival (OS) were observed in patients with higher *PD-L1* expression [124,125]. However, many studies showed that *PD-L1* expression was not sufficient to fully differentiate responders and non-responders: consistent responses were only observed in a fraction of patients with high PDL-1 expression, while durable responses were also observed in patients with negative *PD-L1* expression [126-129]. Even long-term favorable clinical outcomes were seen be achieved in patients that are *PD-L1* negative [130-132]. A variety of causes may contribute to the misclassification of the *PD-L1* expression status including dynamic and heterogeneous *PD-L1* expression in different tumor histology, tumor sampling, use of archived material for testing, existence of various antibody clones, positivity or negativity cut-offs, and sometimes the scoring system [128,133-137]. Although some studies tried to identify other predictive markers for PD-1/*PD-L1* inhibitors such as tumor mutation burden, EBV status, or MSI status, none of them has been routinely used in clinical practice [110,125,138,139]. In fact, according to the FDA, pembrolizumab must be used in conjunction with its companion *PD-L1* test in GC, while testing *PD-L1* expression for nivolumab and atezolizumab are considered complementary [12,14].

Testing for *PD-L1* expression in GC for pembrolizumab: Evaluating the *PD-L1* expression for treatment of GC with pembrolizumab is different from that in NSCLC or other cancer types [84,133]. *PD-L1* expression was assayed using FDA-approved *PD-L1* IHC 22C3 pharmDx on sections from formalin fixed paraffin embedded tissue blocks. The number of PD-L1-stained cells (clear membranous stain with or without cytoplasmic stain at any intensity) including tumor cells, lymphocytes, and macrophages are counted in the areas that contains at least 100 viable tumor cells. A combined positive score (CPS) is calculated by dividing the total number of all *PD-L1* positive cells (tumors cells, lymphocytes, and macrophages) by the total number of viable tumor cells. A CPS ≥ 1% is considered positive for *PD-L1* expression [133,140]. In the KEYNOTE-059 trial, the CPS has been shown to be a robust, reproducible *PD-L1* scoring method that predicts response to pembrolizumab in patients with G/GEJ cancer better than the tumor proportion score (TPS) [133,141].

Diffuse-type GC and E-cadherin (CDH1): The diffuse type GC represents 32% of all GC and is more frequently associated with female and young patients [6,142,143]. It is enriched in the GS-subtype by the TCGA classification scheme and in the EMT subtype by the ACRG classification scheme [9,10]. Unlike the complicated genetic variations that underlie carcinogenesis in intestinal-type tumors, diffuse-type GC most frequently harbors molecular defects in the E-cadherin gene (CDH1), resulting the loss of expression of the cell adhesion molecule E-cadherin [144]. Somatic mutations of CDH1 have been detected in approximately 30% of sporadic diffuse gastric carcinoma (SDGC) and are associated with poor prognosis [9,145,146]. Germline mutations of CDH1 were found in about 40% of patients with hereditary diffuse gastric cancer (HDGC) and are inherited in an autosomal-dominant pattern [143,147]. In addition, the epigenetic inactivation by promoter hypermethylation of the CDH1 gene may also contribute to GC development [148,149].

CDH1 and HDGC: HDGC accounts for about 1-3% of gastric cancers and is characterized by early-onset of the disease with familial clustering [150,151]. By genetic linkage and sequencing analysis, mutations in CDH1 gene were first identified as the genetic cause for

early-onset, diffuse gastric cancer in large kindred from New Zealand [152]. Since then, more than 150 different CDH1 germline mutations have been identified in HDGC families of multiple ethnical origins [153,154]. These pathogenic mutations spread across the entire CDH1 gene including small insertions and deletions, large exon deletions, missense mutations, nonsense mutations, and splice site mutations [153,155]. The location or type of germline CDH1 mutation does not correlate with phenotype, particularly in regard to the presence of lobular breast cancer in HDGC families [153,155].

Patients with CDH1 germline mutations carry a relatively high risk of developing advanced GC and breast carcinoma with incomplete penetrance [153,155,156]. Prior study showed that the mean age at diagnosis of advanced GC was 40 years (range, 14–85 years) [157]. The cumulative risk for gastric cancer is 67% for men and 83% for women by age 80 years, and the cumulative risk for breast cancer is 39% [156]. The combined risk of gastric cancer and breast cancer in women is 90% by age 80 years [153,155,158]. The prognosis of HDGC patients with germline CDH1 mutations are worse with an overall five-year survival rate of only 4% comparing to 13% in patients without CDH1 mutations [154] while germline CDH1 mutations accounts for about 40% of HDGC, the genetic causes of 60% of HDGC remains unknown [143,147,159]. Mutations in several candidate genes have been identified in HDGC patients without CDH1 mutations, including CTNNA1, MAP3K6, BRCA2, PALB2, INSR, FBXO24, and DOT1L. However, the significance of these mutations needs to be confirmed in more patients to determine their genetic linkage, prevalence, and penetrance [33,153,159-161]. As such, genetic testing for HDGC is restricted to CDH1 gene per current guideline defined by the International Gastric Cancer Linkage Consortium (IGCLC) [33].

Molecular pathogenesis of CDH1 mutation in GC development: E-cadherin is a transmembrane protein of the cadherin superfamily encoded by the CDH1gene located in human chromosome 16q22.1 [162]. The mature E-cadherin protein consists of a cytoplasmic domain, a single transmembrane domain, and an extracellular domain with five tandem repeat regions [163]. The cytoplasmic domain of E-cadherin interacts with α-, β-, and γ-catenins to form cadherin-catenin complexes, linking the cadherins to the actin cytoskeleton network and to many other transmembrane and cytoplasmic proteins [164]. The epithelial cell-cell adhesion is mediated through the homophilic interactions between the extracellular domains of the cadherin molecules in a calcium-dependent manner [165]. These structural functions of E-cadherin create an intricate transcellular network (adherens junction) and are essential in regulating a variety of cellular processes including cell migration, differentiation, tissue architectural homeostasis, endocytosis, exocytosis, autophagy, signal transduction, gene expression, and receptor/channel recycling [165-171]. Inactivation of E-cadherin leads to loss of cell adhesiveness and impaired cell-proliferation signaling pathways resulting in abnormal morphogenesis, unregulated growth, and invasion of adjacent tissues through epithelial-mesenchymal transition [168,169,171-175].

Several lines of evidence suggest that E-cadherin deficiency is likely the initiating event in the tumorigenesis of HDGC. Down-regulation of E-cadherin expression is first observed in the presumable gastric epithelium stem cells residing in the upper isthmus of the gastric gland [152,176]. E-cadherin expression is consistently reduced or completely lost in not only the multifocal microscopic foci of signet ring cells (SRC), the earliest apparent HDGC disease, but also in late stage HDGC in CDH1 germ line mutation carriers [74,177]. The downregulation of E-cadherin in both the *in situ* and invasive components of HDGC with germ line mutations suggests that inactivation of CDH1 requires a second hit for disease initiation. Potential molecular mechanism behind this secondary hit includes somatic mutation, loss of heterozygosity (LOH), and epigenetic silencing by promoter hypermethylation of the second of CDH1 allele [178-181]. Studies have shown that promoter hypermethylation accounts for 50% of cases, while mutation and LOH-mediated gene inactivation are less frequently [178-181]. However, inactivation of CDH1 alone is not sufficient for development of tumor invasion. Additional disease modifying genes including SMAD4, C-SRC, and TP53 might be involved in tumor invasion and metastasis [174,182,183].

Clinical management of patients with germline CDH1 mutations: HDGC is a highly invasive tumor that is usually identified at advanced stage with a poor prognosis. Individuals with germline CDH1 mutations carry a high lifetime risk for developing HDGC with the median age at diagnosis of age 40 [156,184]. Therefore, optimal clinical management of these high-risk individuals requires identification of asymptomatic mutation carriers followed by prophylactic gastrectomy at the appropriate age, or endoscopic surveillance followed by therapeutic gastrectomy if diffuse gastric carcinoma was detected [155,185,186].

Only selected patients who meet the following the 2015 IGCLC criteria are eligible for genetic testing to identify germline CDH1 mutation carriers: 1) two or more gastric cancer cases in one family at any age, with at least one confirmed diffuse gastric cancer; 2) diffuse gastric cancer under the age of 40 years without a family history; or 3) family history with the diagnoses of both diffuse gastric cancer and lobular breast carcinoma, at least one under the age of 50 years [34]. Additionally, families in whom genetic testing could be considered include: presence of bilateral lobular breast cancer or family history (first or second degree relative) of two or more cases of lobular breast cancer below age 50; a personal or family history (first or second degree relative) of cleft lip/palate in a patient with diffuse GC; or an individual with *in situ* signet ring cells and/or pagetoid spread of signet ring cells on a gastric biopsy [33]. The sensitivity, specificity, positive predictive value and negative predictive value of the 2015 IGCLC criteria were 0.79, 0.70, 0.19, and 0.97, respectively [187].

Discussion

Genetic testing should begin at the age of consent (usually 16 - 18 years of age) for individuals from affected families [33,155]. Testing of younger unaffected family members can be considered on a case-by-case basis [33]. Factors such as the emotional and physical health of the individual and the earliest age of gastric cancer in the family should be considered [155]. Because there are no hot spot regions of CDH1 gene and no genotypic/phenotype correlations in regard to the risk of developing GC, genetic testing should be accomplished by direct sequencing of all coding regions of the gene, including intron-exon boundaries [104]. The missense mutations must be individually validated for their pathogenic relevance. Usually, this can be achieved using computational methods including frequency in normal controls, co-segregation within the pedigree, recurrence of the mutation, and in silico tools such as structural modelling and SIFT software in combination with databases containing CDH1 sequencing data [154,188]. For difficult cases, *in vitro* functional cell model or animal models may be needed to evaluate the impact of CDH1 missense alterations in protein structure, trafficking, and signaling [179,188,189].

The signet ring cell carcinoma in patients with CDH1 mutation initially locates beneath the foveolar epithelium and only become visible on endoscopy late in the disease process [190,191]. Therefore,

prophylactic gastrectomy, rather than endoscopic surveillance, is usually recommended for CDH1 pathogenic variant carriers after age 20. However, endoscopic surveillance is needed if gastrectomy is contraindicated owing to the comorbidity, younger than recommended age for surgery, CDH1 variants of undetermined significance, or the patient refuses surgery. Endoscopy should be performed annually using a white light high definition endoscope in high risk individuals and any endoscopically visible lesions should be biopsied [33]. Theoretically, 1768 biopsies are needed to assure a 90% rate of detecting at least 1 cancer focus [192]. A minimum of 30 biopsies is recommended, with 5 biopsies taken from each of the following anatomic zones: pre-pyloric area, antrum, transitional zone, body, fundus, and cardia, as described in the Cambridge protocol [158]. However, detection of early stage disease with direct endoscopic visualization is extremely difficult and only a small percentage of cancerous infiltrates were identified by endoscopic examination [184,193,194].

The recommended surgical management for carriers of a proven pathogenic germline CDH1 mutation is prophylactic total gastrectomy in their 20s or 30s [33]. However, the optimal time for gastrectomy should be individualized based on pathogenic significance of CDH1 mutation, the age of onset of disease in the pedigree, and the penetrance pattern of particular kindred [147,194,195]. Prophylactic gastrectomy carries a 3-6% mortality rate and a 100% morbidity rate due to post-surgery eating habit changes, dumping syndrome, diarrhea, and weight loss [194]. Current guidelines recommend a total gastrectomy with Roux-en-Y reconstruction [154]. Because the CDH1 pathogenic variant is present in all gastric tissues, all gastric mucosa should be removed, and the negative margins confirmed during prophylactic gastrectomy [33,195]. It is reasonable to performed D1 lymph node dissection as the majority of HDGC patients will have at least T1a disease and the presence of T1b lesions cannot be ruled out preoperatively [196,197].

Due to the increased risk of lobular breast cancer in women with pathogenic CDH1 mutations, current guidelines suggest that annual breast magnetic resonance imaging should start at age 30, and treatment decisions should be made on a case-by-case basis [33,198]. Finally, it is recommended that the gastrectomy and mastectomy specimens of CDH1 mutation carriers should be fully examined microscopically to determine the stage of cancer and better understand the phenotype and biology of this disease [33].

Conclusion

Gastric cancer is a highly heterogeneous disease as reviewed by recent molecular profiling and classification. Distinct molecular signatures including *HER2* overexpression, MSI status, PD1/*PD-L1* expression patterns, CDH1 mutations, have been shown to be associated with different GC subtypes. Identifying these molecular signatures in GC could provide instructive information for prognosis, stratifying GC patients with optimal treatments, and eventually improving clinical outcomes.

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