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Pathogenesis of gastric cancer: genetics and molecular classification

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Abstract

Gastric cancer is the fifth most incident and the third most common cause of cancer-related death in the world. Infection with *Helicobacter pylori* is the major risk factor for this disease. Gastric cancer is the final outcome of a cascade of events that takes decades to occur and results from the accumulation of multiple genetic and epigenetic alterations. These changes are crucial for tumor cells to expedite and sustain the array of pathways involved in cancer development, such as cell cycle, DNA repair, metabolism, cell-to-cell and cell-to-matrix interactions, apoptosis, angiogenesis and immune surveillance. Comprehensive molecular analyses of gastric cancer have disclosed the complex heterogeneity of this disease. In particular, these analyses have confirmed that Epstein Barr virus (EBV)-positive gastric cancer is a distinct entity. The identification of gastric cancer subtypes characterized by recognizable molecular profiles may pave the way for a more personalized clinical management and to the identification of novel therapeutic targets and biomarkers for screening, prognosis, prediction of response to treatment, and monitoring of gastric cancer progression.

Keywords

Gastric cancer; *Helicobacter pylori*; Epstein Barr virus; chromosomal instability; microsatellite instability; hereditary gastric cancer

1. Gastric cancer epidemiology

Gastric cancer is the fifth most incident malignancy in the world, with 952,000 new cases (6.8% of the total number of cancers) estimated to have occurred in 2012 (Ferlay et al. 2013). Of these, 823,000 cases were at non-cardia anatomic subsites. The incidence greatly varies between countries and the majority of cases are registered in developing countries, half of them occurring in Eastern Asia. Gastric cancer incidence is twice as high in men than in women, suggesting that environmental, hormonal, and/or genetic exposures may affect risk.

Globally, gastric cancer is the third most common cause of death by cancer, representing 723,000 deaths (8.8% of all cancer deaths) in 2012 (Ferlay et al. 2013). The mortality rate is quite similar to the incidence rate with the highest estimated mortality rates observed in Eastern Asia. The high mortality rates of gastric cancer are due to the paucity of clinical symptoms, consequent late diagnosis, absence of screening, and ineffective treatments. Thus, the majority of patients present advanced-stage tumors at the point of diagnosis. The five-year survival of gastric cancer patients of all stages is about 25% and the median overall survival is less than one year (Gastric Group et al. 2013).

Gastric cancer incidence and mortality have been declining over the past decades, and this has been related to a decrease in the prevalence of *Helicobacter pylori*, probably as a result of improved living conditions, hygiene and sanitation, as well as to the widespread use of antimicrobials (Ferro et al. 2014). Nevertheless, and despite the declining rates, the global burden of gastric cancer is expected to increase in the coming years due to the demographic effects of growth and ageing of the population worldwide (Ferlay et al. 2013).

2. Histological phenotypes of gastric cancer

From the morphologic standpoint, gastric cancer is very heterogeneous, which is reflected by the diversity of histopathological classifications (Carneiro et al. 1995; Lauren 1965; Ming 1977; Lauwers et al. 2010). The most widely used classification was proposed by Lauren, in which gastric cancer is divided in two main types - intestinal and diffuse - which also differ from each other clinically and epidemiologically (Lauren 1965). Intestinal-type tumors are usually exophytic, often ulcerating, and are associated with intestinal metaplasia of the stomach. Diffuse-type tumors are poorly differentiated infiltrating lesions, which lead to thickening of the stomach (*linitis plastica*). Patients with diffuse-type tumors appear to have a worse prognosis than those with intestinal-type tumors. Intestinal-type cancers are more common in proximal (fundus) location while diffuse-type tumors predominate in younger patients (Lo et al. 1996). Interestingly, diffuse-type tumors demonstrate a nearly equal sex ratio, compared with the male preponderance in the intestinal-type.

Correa proposed a model of gastric carcinogenesis according to which the intestinal-type carcinoma represents the end-product of a cascade of sequential changes in gastric mucosa including superficial gastritis, chronic atrophic gastritis, small intestinal metaplasia, colonic metaplasia and adenomatous (flat or polypoid) dysplasia (Correa 1988, 1992). The sequence of histogenetic changes leading to diffuse-type cancer is less well-defined. A further

complication for these models of precancerous stages is that a substantial fraction of gastric cancers are characterized by the co-existence of both intestinal-type and diffuse-type morphologic features (Carneiro et al. 1995).

3. *Helicobacter pylori* infection and gastric cancer

Helicobacter pylori infection is the major risk factor for gastric cancer and has been considered as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC 1994). A reevaluation incorporating a substantial amount of new available data provided support for an association between *H. pylori* infection and non-cardia gastric cancer (IARC 2011). Recent estimates show that 89% of all non-cardia tumors (about 780,000 cases) is attributable to *H. pylori* infection, making this bacterium responsible for at least 6.2% of all cancer cases worldwide (Plummer et al. 2015). It must be noted, however, that the detection methods may not be sensitive enough and that the true fraction of attributable cases may actually be higher than 89%.

Adding to the geographic overlap between the prevalence of *H. pylori* infection and the incidence of gastric cancer, epidemiological data from cohort studies, case-control studies, and meta-analyses also evidenced the carcinogenicity of chronic *H. pylori* infection in humans (IARC 2011). Risk estimates from a combined analysis of 12 independent prospective cohort studies showed an association between *H. pylori* seropositivity and non-cardia tumors with an overall odds ratio of 2.97 (95% CI 2.34–3.77), but not with cardia tumors (Helicobacter and Cancer Collaborative Group 2001). The magnitude of the risk is higher, reaching 21-fold, when more sensitive assays are used for determining the *H. pylori* status (Gonzalez et al. 2012; Ekstrom et al. 2001). The risk associated with infection with CagA-positive *H. pylori* strains is also higher than that of infection with CagA-negative strains (Huang et al. 2003; Kamangar et al. 2006; Palli et al. 2007). Moreover, *H. pylori* eradication reduces the incidence of gastric cancer. In a meta-analysis of six randomized eradication trials, including 6497 participants, *H. pylori* eradication was superior to placebo or no treatment in preventing gastric cancer, with a risk ratio of 0.66 (95% CI 0.46–0.95) (Ford et al. 2015).

Noteworthy, not all of the *H. pylori*-infected individuals will develop gastric cancer, which points to a multifactorial etiology of this disease. Indeed, host genetic susceptibility factors that influence the inflammatory response to the infection and lifestyle factors such as smoking and diet with a high-salt content are associated with increased gastric cancer risk (Figueiredo et al. 2002; Gonzalez et al. 2003; Persson et al. 2011; Peleteiro et al. 2011).

The mechanisms of malignant transformation mediated by *H. pylori* infection are discussed in chapters 2, 8–11 and 13.

4. Epstein-Barr virus infection and gastric cancer

Epstein Barr virus (EBV) is almost ubiquitous in the human population, primarily maintained as a latent infection in a subset of B-lymphocytes comprising roughly 0.001% of peripheral blood mononuclear cells. *In vitro*, EBV infection of B cells is highly efficient with most B cells becoming immortalized after infection. In B cells, the viral envelope

glycoprotein gp350 binding to the B-cell surface coreceptor CD21, tethers the virus to the host cell surface and subsequent binding to human leukocyte antigen (HLA) class II facilitates fusion of viral and cell membranes, releasing the viral capsid into the cytoplasm (Fingerroth et al. 1984; Li et al. 1997; Nemerow et al. 1987; Tanner et al. 1987). HLA binding and fusion are mediated by a different set of envelope glycoproteins: gH, gL, gp42 and gB (the fusion core complex) (Hutt-Fletcher 2007). gp42 is the main HLA class II-binding protein bridging between a gH/gL heterodimer and HLA. gH/gL binds gB, the main effector of viral fusion (Backovic et al. 2007a; Backovic et al. 2007b).

On the other hand, epithelial cells are nearly refractory to EBV and susceptibility to infection is facilitated only under special experimental conditions, e.g. after ectopic expression of CD21 or after B cell-mediated transference of viral particles (Borza et al. 2004; Shannon-Lowe et al. 2006; Shannon-Lowe and Rowe 2011). In epithelial cells EBV entry is thought to be mediated by integrins. Several interactions have been described, including gH/gL with the integrin receptors $\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha v\beta 8$, and the viral envelope glycoprotein BMRF2 with the cellular transmembrane receptor integrins αv , $\alpha 3$, $\alpha 5$, and $\beta 1$ (Chesnokova et al. 2009; Hutt-Fletcher and Chesnokova 2010; Chesnokova and Hutt-Fletcher 2011; Tugizov et al. 2003; Xiao et al. 2008). Viral fusion also requires binding to the cellular transmembrane glycoprotein neuropilin 1 (Wang et al. 2015).

EBV released by B cells and by epithelial cells bear distinctive envelopes displaying different tropisms. gH/gL/gp42 trimers are enriched in epithelial cell-derived viruses, while gp42 is lacking on virus released by B cells (Chen et al. 2012; Wang et al. 1998). Since gp42 mediates binding to HLA class II, B cell-originated virus has an enhanced tropism for epithelial cells, and epithelial cell-originated virus for B cells. This switching tropism has important implications at an individual level. Integrins are exclusively expressed at the basolateral face of polarized epithelia, the side contacting circulating B cells (Lee and Streuli 2014; Manninen 2015). On the contrary, virus present in saliva contains gp42 exhibiting an enhanced tropism for B cells (Jiang et al. 2006). Thus, persistent infection of epithelia most probably follows virus released by reactivated B cells in chronically infected hosts. Also, epithelial infection is more permissive for IgA- and CD21-bound virus and after formation of B cell-epithelial cell conjugates (Shannon-Lowe et al. 2006; Shannon-Lowe and Rowe 2011; Sixbey and Yao 1992), all mechanisms favored at the basolateral face. All these data support a model of viral transmission in which EBV-infected B cells circulating close to the basolateral side would be reactivated by stimuli that are still not clear, producing viral particles with an increased capacity to infect epithelial cells.

In B cells, viral expression starts with the EBV nuclear antigens (EBNAs), EBNA-LP (leader protein), EBNA-2, EBNA-1, the EBNA-3 family ($-3a$, $-3b$ and $3c$) and the latent membrane proteins (LMP), LMP1, LMP-2A and LMP-2B (the latency III program). In vivo, EBV switches between different gene expression programs (latencies), presumably to counteract immune cell responses. In immune-competent hosts the germinal center model argues that infection guides the B cell into a series of stages that mimic antigen-activation and formation of long-lived memory B cells (Thorley-Lawson et al. 2013). While passing through the germinal center, viral gene expression is reduced to EBNA-1, LMP1 and LMP2A (latency II), EBNA-1 only (latency I) and no protein expression (latency 0). Latency

0 is found in memory cells and is considered the ultimate state of EBV persistence (Babcock et al. 2000). During these latency programs, EBV also expresses several untranslated RNAs: the EBER-1 and EBER-2 (EBV encoded small RNAs), and about 25 pre-microRNAs (pre-miRNAs). Expression of EBNA-1, LMP2A and non-coding RNAs with or without LMP1 has been demonstrated in epithelial cells *in vitro* and *in vivo*.

EBV is considered to be a human carcinogen linked to development of several types of lymphoma and carcinoma, accounting for about 200,000 new cancer cases annually worldwide. In particular, this virus was first detected (via polymerase chain reaction) in a gastric tumor in 1990 (Burke et al. 1990). Approximately 9% of gastric cancer cases have latent EBV infection in every tumor cell (Murphy et al. 2009). This fraction is relatively constant in both high- and low-gastric cancer incidence populations worldwide. Consequently, EBV-related gastric cancer is the most common malignancy associated with EBV infection, exceeding even EBV-related nasopharyngeal carcinoma in number of cases annually (Cohen et al. 2011).

Since EBV infection is so common in humans, presence of the virus in tumor tissue is needed to implicate the infection in gastric carcinogenesis. However, the tissue inflammation often present in gastric cancer may lead to infiltration of incidental EBV-infected leukocytes as a non-specific source. Conventionally, EBV is localized to tumor cells by *in situ* hybridization for the abundant EBER-1 and -2 transcripts of unknown function. Next-generation sequencing provides a versatile alternative for identification of EBV-positive tumors (Camargo et al. 2016a). In viral-positive tumors, the nucleic acids are present as circularized episomes and chromosomal integration does not appear to occur. The episomes are monoclonal with uniform terminal repeats in a given tumor, indicating infection was present at the time of transformation in the clonal progenitor cell.

EBV-infected lymphocytes in inflamed gastric mucosa may represent the route of introduction into the gastric epithelium (Ryan et al. 2012). Nevertheless, studies are inconsistent about the presence of EBV in epithelial cells of premalignant lesions, including intestinal metaplasia and dysplasia (Gulley et al. 1996). In prospective follow-up of a high-risk population, serologic markers of viral reactivation were associated with gastric pre-neoplastic lesions and risk of progression (Schetter et al. 2008).

EBV genome sequences vary among isolates, with two recognized major types based primarily on variation of EBNA-2 and EBNA-3 genes (Palser et al. 2015). Furthermore, some strains have greater propensity for infecting epithelial cells, which may be indicative of carcinogenic competence (Tsai et al. 2013). However, there is no known association to date of particular viral genotypes with EBV-positive gastric cancer.

EBV reactivation from latency is a postulated mechanism for the development of EBV-associated malignancies. Supporting this hypothesis, serologic studies have found significantly higher immunoglobulin G antibody titers against the viral capsid and early antigens (although not against EBNA) in patients with EBV-positive tumors as compared to EBV-negative cases (Levine et al. 1995; Shinkura et al. 2000).

There is limited evidence on the possible interaction or antagonism between EBV and *H. pylori* in gastric carcinogenesis. In a study comparing *H. pylori* serologic profiles of patients with EBV-positive and -negative non-cardia tumors, antibody levels to 15 *H. pylori* proteins did not vary by tumor EBV status (Camargo et al. 2016b). This similarity of host-response indicates that *H. pylori* plays an essential role in the etiology of EBV-positive tumors as well.

Taken together, much has been learned about EBV-positive gastric cancer since it was first documented, but important knowledge gaps remain. It is still unclear when and how the virus appears in the gastric epithelium prior to tumor emergence. Elucidating the viral contribution to gastric cancer pathophysiology could lead to novel strategies for prevention and treatment, with possible extension to other EBV-related malignancies.

5. Molecular phenotypes of sporadic gastric cancer

It has long been recognized that gastric cancer, like other cancers, harbors genetic and epigenetic alterations that contribute to cancer development, progression, and response to therapy (Peltomaki 2012). Previous to the genome-wide characterization of gastric cancer, several molecular phenotypes, chromosome instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and stable or diploid tumors, were already described and widely accepted (Ottini et al. 2006). The advent of high-throughput genome-, transcriptome- and proteome-wide approaches, confirmed the remarkable genetic and epigenetic intertumoral heterogeneity of gastric cancer and highlighted the molecular complexity of this disease.

Profiling of gastric tumors also led to the emergence of several molecular classification schemes. Gene expression profiling has been used for the discovery of subtypes that may predict patient survival and response to therapeutic modalities. Unsupervised hierarchical clustering analysis of data obtained with cell lines identified two major gastric cancer subtypes with distinct genomic signatures: one associated with intestinal tumors and enriched in biological functions related to carbohydrate and protein metabolism and cell adhesion, designated G-INT; and one associated with diffuse tumors and enriched in cell proliferation and fatty acid metabolism functions, designated G-DIF, (Tan et al. 2011). Although related with Lauren's histotypes, the overall concordance was only 64%, and thus G-INT and G-DIF molecular subtypes were considered distinct. In fact, and unlike Lauren's classification, the G-INT and G-DIF gene expression patterns were prognostic of survival in various patient cohorts, and patients with G-DIF gastric cancer had worse survival outcomes. Furthermore, distinct *in vitro* sensitivities of G-INT cell lines to chemotherapeutic agents 5-fluorouracil and oxaliplatin and of G-DIF cell lines to cisplatin were observed, and patients with G-INT tumors benefited from adjuvant 5-fluorouracil based therapy.

Following this early discovery study, gene expression profiling of gastric tumors led to the identification of proliferative, metabolic, and mesenchymal gastric cancer subtypes (Lei et al. 2013). Proliferative subtype tumors are mostly of the intestinal-type, have high levels of genomic instability with recurrent *ERBB2*, *KRAS*, *CCNE1*, and *MYC* gene amplification, DNA hypomethylation and high levels of tumor suppressor gene *TP53*

mutations. Mesenchymal subtype tumors are mostly of the diffuse-type, contain cells with cancer stem cells features, and are particularly sensitive to inhibitors of the PI3K-AKT-mTOR kinase pathway. Metabolic subtype tumors include both diffuse and intestinal histological types and show high expression of genes associated with metabolic pathways as well as features of spasmolytic polypeptide-expressing metaplasia (SPEM). Metabolic subtype tumor cells were sensitive to 5-fluorouracil *in vitro*, which concurred with the findings that metabolic subtype gastric cancer patients from two independent cohorts had benefit from 5-fluorouracil treatment in terms of cancer-specific and disease-free survival. However, there were no major differences in survival among these three gastric cancer subtypes.

A more comprehensive analysis of gastric cancer by The Cancer Genome Atlas (TCGA) network which studied a series of 295 untreated, surgically-resected tumors with array-based somatic copy number analysis, whole-exome sequencing, array-based DNA methylation profiling, mRNA sequencing, microRNA sequencing, and reverse-phase protein array, led to the proposal of four molecular subtypes (Figure 12.1 A): tumors with CIN, tumors with MSI, genomically stable (GS) tumors, and EBV-positive tumors (Cancer Genome Atlas Research 2014). Using this classification, no significant differences in patient survival and tumor recurrence rates were observed between subtypes, but the follow-up time of the cohort was limited at the time of analysis.

The Asian Cancer Research Group (ACRG) used gene expression data to define four gastric cancer molecular subtypes that were linked to distinct patterns of molecular alterations, disease progression and prognosis (Figure 12.1 B): the epithelial to mesenchymal transition (EMT)/microsatellite stable (MSS) subtype, the MSI subtype, the MSS/*TP53*⁺ and the MSS/*TP53*⁻, the last two based on the status of *TP53* activation (Cristescu et al. 2015). EMT/MSS tumors are mainly of the diffuse type, occur at an earlier age, and patients have the worst prognosis and the highest recurrence rate of the four subtypes. MSI tumors are hypermutated, occur frequently in the antrum, are mostly of the intestinal histotype, and patients have the best overall prognosis and the lowest recurrence rate. Patients with MSS/*TP53*⁺ and MSS/*TP53*⁻ tumors have intermediate prognosis and recurrence rates, the former showing better prognosis. EBV infection occurred more frequently in the MSS/*TP53*⁺ subtype. For the sake of an easier correspondence with the classical gastric cancer phenotypes, we will now refer to a more thorough discussion of each of the phenotypes: gastric cancer with CIN, gastric cancer with MSI, genomically stable gastric cancer and EBV-positive gastric cancer.

5.1 Tumors with chromosomal instability

CIN is one of the major genomic instability pathways involved in gastric carcinogenesis, characterized by losses or gains of whole chromosomes resulting in altered chromosome copy number (aneuploidy). CIN may also involve changes in portions of chromosomes, which include allelic losses (LOH), gene deletions and/or amplifications (Lengauer et al. 1998; Ottini et al. 2006).

In the TCGA series, CIN subtype tumors represent about 50% of the gastric cancers and are localized mainly in the gastroesophageal junction/cardia (Cancer Genome Atlas Research

2014). These tumors have marked aneuploidy and harbor a very high frequency of *TP53* mutations (71%). CIN subtype tumors frequently harbor gene amplifications.

One of the major functional groups showing gene amplifications in gastric cancer are receptor tyrosine kinases (RTKs). While *ERBB2* (HER2), *EGFR*, and *MET* amplifications have long been described in gastric cancer, *FGFR2* has only been detected quite recently (Lemoine et al. 1991; Tsugawa et al. 1993; Hara et al. 1998; Sakakura et al. 1999; Graziano et al. 2011; Zang et al. 2011; Deng et al. 2012; Dulak et al. 2012; Wang et al. 2014; Wong et al. 2014; Cancer Genome Atlas Research 2014).

FGFR2 has been recently described as amplified in about 4% to 10% of tumors, with no association with histological type (Deng et al. 2012; Jung et al. 2012; Su et al. 2014). Dysregulation of the FGFR signaling pathway is associated with cancer development and progression, and in preclinical models of gastric cancer amplification of *FGFR2* is associated with increased tumor cell proliferation and survival (Xie et al. 2013). A large international multicentric study showed that *FGFR2* amplification is related to poor prognosis in patients with resectable tumors, irrespective of ethnic origin and underlying significant differences in clinicopathological parameters, survival and treatment (Su et al. 2014). Importantly, gastric cancer cell lines and patient-derived xenograft models carrying *FGFR2* amplification exhibit sensitivity to *FGFR* inhibitors, thus supporting this type of therapeutic intervention (Deng et al. 2012; Xie et al. 2013).

Interestingly, amplifications in RTK genes appear to be mutually exclusive (Deng et al. 2012; Dulak et al. 2012; Das et al. 2014; Wong et al. 2014). RTK amplification (either of *FGFR2*, *ERBB2*, *EGFR*, or *MET*) was a predictor of poor prognosis, but considering each of the RTKs as independent factors, patients with *MET*-amplified tumors and *ERBB2*-amplified tumors were the ones with the worst prognosis (Deng et al. 2012). After adjustment for tumor stage and grade, *ERBB2* amplification had the strongest prognostic impact. *ERBB2* amplification and high HER2 immunohistochemical overexpression have already been used as stratification biomarkers for anti-HER2 therapies in gastric cancer (Bang et al. 2010; Satoh et al. 2014).

KRAS amplification has only been recently detected in about 10% of gastric tumors and appears to be mutually exclusive to RTK gene amplification (Deng et al. 2012; Dulak et al. 2012). Patients with tumors exhibiting *KRAS* amplification have significantly worse prognosis compared with patients with tumors lacking either *KRAS* or RTK amplification (Deng et al. 2012).

Overall, amplification in this group of genes is present in around 30% to 40% of the gastric cancer cases (Deng et al. 2012; Dulak et al. 2012). Considering that several inhibitors that target components of the RTK/RAS signaling pathways are currently in clinical trials, this subgroup of patients may benefit from stratification based on the presence of these genetic alterations.

Genes that encode transcription factors also exhibit amplification in gastric tumors. *MYC* amplification and overexpression in gastric cancer are predictors of poor clinical prognosis, especially in patients that have tumors of the intestinal-type (Kozma et al. 2001; Burbano et

al. 2006). In addition to *MYC* and *GATA4* that were previously shown to be amplified in gastric cancer, more recent studies detected amplifications in *GATA6* and *KLF5* transcription factors (Weiss et al. 2004; Deng et al. 2012; Dulak et al. 2012; Cancer Genome Atlas Research 2014; Cristescu et al. 2015). *KLF5*, *GATA4* and *GATA6* are each amplified in about 10% of tumors and, although their amplification occurs in an independent pattern, they are co-expressed in gastric cancer, especially of the intestinal-type (Cancer Genome Atlas Research 2014; Chia et al. 2015). Recently, Chia *et al.* showed that *KLF5*, *GATA4* and *GATA6* functionally cooperate to regulate common downstream pathways and to promote gastric tumorigenesis (Chia et al. 2015). Interestingly, they identified hepatocyte nuclear factor-4 α (HNF4 α) as a direct target of *KLF5/GATA4/GATA6*. HNF4 α is present at high levels in intestinal-type tumors with *KLF5/GATA4/GATA6* amplifications, is required for gastric cancer proliferation, and can be targeted by the anti-diabetic drug metformin. These findings forecast a therapeutic opportunity for *KLF5/GATA4/GATA6*-amplified tumors, which together represent about 30% of gastric cancer cases.

The octamer transcription factor 1-encoding gene (*OCT1*) is also recurrently amplified and upregulated in gastric cancer and associated with poor prognosis of patients (Qian et al. 2015). Functionally, *OCT1* amplification contributes to ERK/MAPK activation leading to accelerated tumor growth. Interestingly, *OCT1* amplification displays mutual exclusivity with amplifications in *KRAS* and *FGFR2* (Qian et al. 2015).

Amplifications in genes encoding cell cycle regulators are also observed in gastric cancer. Amplifications of *CCNE1* and *CCND1*, which encode cyclin E1 and cyclin D1, are observed, respectively, in 15% and in 10% of tumors (Akama et al. 1995; Bizari et al. 2006). *CCNE1* amplification is associated with the presence of lymph node metastasis (Akama et al. 1995). *CDK6*, encoding cyclin dependent kinase 6, is also found amplified in about 15% of tumors (Isinger-Ekstrand et al. 2010). A recent high-resolution profiling of copy number alterations in a panel of 233 gastric cancers confirmed amplifications in *CCNE1*, *CCND1* and *CDK6* and, most interestingly, detected a pattern of co-occurrence of amplifications in the same tumor between *CCNE1* and *ERBB2* (Deng et al. 2012). This pattern of amplification co-occurrence has been previously associated with resistance to anti-HER2 therapies in several cancers (Kim et al. 2014b) and may partially explain the modest efficacy of HER2-directed therapies in gastric cancer (Bang et al. 2010; Satoh et al. 2014).

VEGFA is also reported to be amplified in gastric cancer (Dulak et al. 2012; Andreozzi et al. 2014). *VEGFA* encodes vascular endothelial growth factor A, a growth factor that contributes to tumor-induced angiogenesis that is a critical step in tumor growth and metastasis (Ellis and Hicklin 2008). This may represent an interesting biomarker candidate for predicting clinical outcome in patients with advanced gastric cancer treated with bevacizumab (Van Cutsem et al. 2012).

In the context of CIN, translocations, amplifications and rearrangements may result in gene fusions. For example, an oncogenic fusion of *CD44* and the glutamate transporter *SLC1A2* gene was identified in a small subset (1% to 2%) of gastric tumors (Tao et al. 2011). Interestingly, silencing of *CD44-SLC1A2* sensitized cells to cisplatin, a chemotherapeutic

agent commonly used in gastric cancer, suggesting that this gene fusion represents a potential drug target.

A novel fusion transcript between dihydrouridine synthase 4-like and B-cell receptor-associated protein 29 (*DUS4L-BCAP29*) that shows functional tumorigenic potential was also described both in gastric cancer cell lines and tumors (Kim et al. 2014a). Additional rare gene fusions involving *AGTRAP-BRAF*, *ERBB2-CDK12*, *ERBB2-NEUROD2*, *SLC34A2-ROSI*, *SNX2-PRDM6*, and *MLL3-PRKAG2* have been detected in gastric cancer (Palanisamy et al. 2010; Zang et al. 2011; Lee et al. 2013; Yao et al. 2015). Whether they are functionally relevant or can identify novel subclasses of patients that may clinically benefit from targeted therapies remains to be determined.

5.2 Tumors with microsatellite instability

The MSI phenotype results from the accumulation of numerous mutations across the genome in short repetitive sequences called microsatellites, due to a defective DNA mismatch repair (MMR) system (Velho et al. 2014). The MMR system corrects base-base mismatches and insertion/deletion mispairs generated during DNA replication and recombination, to prevent mutations from becoming permanent in dividing cells and thus preserve genomic stability (Li 2008). Epigenetic silencing of *MLH1* gene by promoter hypermethylation is the main mechanism leading to MMR deficiency in sporadic gastric cancer, accounting for 75% to 100% of the cases (Leung et al. 1999; Pinto et al. 2000; Leite et al. 2011).

Tumors can be classified as MSI with high instability (MSI-H), MSI with low instability (MSI-L), or microsatellite stable (MSS) (Umar et al. 2004). It is still not clear in gastric cancer whether MSI-L and MSS represent separate groups, as both appear to have similar phenotypes (Pinto et al. 2000). For the sake of simplicity, hereafter MSI stands for MSI-H.

Tumors with MSI phenotype represent up to 37% of all stomach cancer cases and constitute a well-defined subset of tumors (Zhu et al. 2015; Cancer Genome Atlas Research 2014). MSI gastric cancer is characterized by antral tumor location, intestinal histological type, expanding growth pattern, absent or minimal desmoplastic response, abundant lymphoid infiltration, diploid DNA content, female gender, old age at presentation, and better prognosis (dos Santos et al. 1996; Yamamoto et al. 1999; Falchetti et al. 2008). No significant associations were observed between the MSI phenotype and anatomical location, Lauren's classification, and lymphocytic infiltration in the TCGA series (Cancer Genome Atlas Research 2014). Divergent studies regarding the prognostic value of MSI in gastric cancer exist, but two recent meta-analyses confirmed that patients with MSI tumors have better prognosis than those with MSS tumors (Choi et al. 2014; Zhu et al. 2015).

Next-generation sequencing (NGS)-based studies provided a comprehensive view of the mutations present in MSI tumors. MSI gastric cancers are characteristically hypermutated tumors with an estimated mutation rate of 31.6 mutations/megabase (Mb) in comparison to 3.3 mutation/Mb in MSS tumors (Wang et al. 2011). Moreover, MSI tumors also portray a distinctively high C-to-T transition signature compared to other gastric cancer molecular subtypes that have enrichment of the C-to-A transversion signature (Yamamoto et al. 1999;

Wang et al. 2011; Nagarajan et al. 2012; Liu et al. 2014; Wang et al. 2014; Cancer Genome Atlas Research 2014).

Exome sequencing studies that characterized the mutational repertoire of the MSI gastric cancer subset identified *ARID1A*, *PIK3CA*, *PTEN*, *ERBB2*, *ERBB3*, *KRAS*, *RNF43*, and *TP53*, among other genes as significantly mutated (Wang et al. 2011; Zang et al. 2012; Wang et al. 2014; Cancer Genome Atlas Research 2014; Liu et al. 2014; Cristescu et al. 2015).

Somatic inactivating mutations in *ARID1A*, encoding the AT-rich interactive domain-containing protein 1A, are consistently identified more frequently mutated in MSI than in MSS tumors (Wang et al. 2011; Zang et al. 2012; Cancer Genome Atlas Research 2014). The *ARID1A* mutation spectrum also differs between gastric cancer subtypes and while the great majority of mutations in MSI are frameshift due to insertions or deletions (indels) involving short mononucleotide repeats, in MSS tumors mutations are both indels and single nucleotide variations. The high rate of *ARID1A* mutations is consistent with a driver gene being targeted by the MSI mechanism, since it was 12- to 61-fold higher than the global background mutation rate of somatic indels at mononucleotide tracts of similar length in MSI tumors (Wang et al. 2011). Reduced or absent *ARID1A* protein expression is observed in the majority of *ARID1A*-mutated samples and loss of *ARID1A* expression is more frequent in MSI than in MSS tumors (Wang et al. 2011; Zang et al. 2012; Abe et al. 2012). While no significant differences in disease-free or overall survival are observed in the MSI subtype with mutated or wildtype *ARID1A*, in MSS tumors loss of *ARID1A* expression significantly correlates with worse disease-free and overall survival (Abe et al. 2012). In the MSI subset, mutations in *ARID1A* are significantly associated with activating *PIK3CA* mutations, but not with *TP53* mutations, suggesting that *ARID1A* and *TP53* mutations drive alternative subsets of gastric cancer (Wang et al. 2011).

In MSI tumors, *PIK3CA* activating mutations are frequent and are mainly localized in the kinase and helical domains (Cancer Genome Atlas Research 2014) and this may represent a target for PI3K inhibitors.

RNF43, encoding an E3 ubiquitin ligase, was identified as frequently mutated in the MSI gastric cancer setting and was also found frequently mutated in MSI colorectal and endometrial cancers (Giannakis et al. 2014). *RNF43* is expressed in Lgr5-positive stem cells and negatively regulates Wnt/ β -catenin signaling. In the pancreatic tumor model, *RNF43* inactivating mutations lead to aberrant Wnt activation. Moreover, in preclinical models, blocking of Wnt inhibited the growth of *RNF43*-mutant pancreatic tumors *in vivo*, suggesting the use of *RNF43* mutations as a biomarker for patient selection for treatment with Wnt inhibitors (Jiang et al. 2013).

Remarkably, in the TCGA series, analysis of genes mutated within MSI tumors, revealed frequent alterations in major histocompatibility complex class I genes, including *HLA-B* and *B2M* (Cancer Genome Atlas Research 2014). Mutations affecting *B2M* and *HLA* inactivation have been previously associated with the MSI phenotype in gastric cancer (Hirata et al. 2007). In colorectal cancer, β 2-microglobulin deficiency reduces antigen

presentation and generates tumor phenotypes that are able to escape immune surveillance (Bernal et al. 2012).

In gastric cancer, and in addition to *MLH1*, other genes can be silenced by promoter hypermethylation, including for example genes involved in DNA repair, such as *MGMT*, cell-cell and cell-matrix interactions, such as *CDH1* and *TIMP3*, cell cycle regulation, like *CDKN2A/P16INK4A*, and transcriptional regulation, such as *RUNX3* (Qu et al. 2013).

Frequently, MSI tumors shows concurrent hypermethylation in multiple *loci* and this has been termed as CpG island methylator phenotype (CIMP). Controversy exists as to the full overlap between the MSI and the CIMP phenotype in gastric cancer (Lee et al. 2004; Zouridis et al. 2012; Moarii et al. 2015). A genome-wide characterization of the DNA methylation events associated with gastric cancer disclosed a subgroup of tumors characterized by a CIMP phenotype, which was associated with widespread hypermethylation, young patient age, and adverse outcome in a disease stage independent manner (Zouridis et al. 2012). Interestingly, it was also observed that cell lines with the CIMP phenotype were sensitive to treatment with 5-Aza-2'-deoxycytidine, a DNA methylation inhibitor. This suggests that pharmaceutical interventions with demethylating drugs towards CIMP gastric cancer may be a possibility.

5.3 Genomically stable tumors

According to the TCGA study, GS tumors have an enrichment of the diffuse histological type and are diagnosed at an earlier age. Compared to CIN subtype, GS tumors have a low degree of aneuploidy and lower frequency of *TP53* mutations. These tumors frequently harbor alterations in the gene encoding the cell-cell adhesion molecule E-Cadherin (*CDH1*) and in the gene encoding the small GTPase that regulates cell motility Ras homolog gene family, member A (*RHOA*) (Corso et al. 2013; Cancer Genome Atlas Research 2014). Mutations in *RHOA* are almost exclusively found in GS tumors. Accordingly, others have identified *RHOA* mutations in 14% to 25% of diffuse-type but not in intestinal-type tumors (Wang et al. 2014; Kakiuchi et al. 2014). *RHOA* mutations occur at hotspots that cluster at functional domains that are important to the interaction of RhoA with its effectors. Functional analyses revealed that hotspot mutants had defects in RhoA-mediated signaling which can contribute to the lack of cell adhesion and to the invasive growth pattern of diffuse-type tumors (Wang et al. 2014; Cancer Genome Atlas Research 2014).

Interestingly, recurrent structural genomic alterations that are implicated in dysregulated Rho signaling were also found enriched in the GS subtype. *CLDN18-ARHGAP26* and *CLDN18-ARHGAP6* gene fusions which juxtapose the tight junction protein claudin 18 with GTPase-activating proteins (GAPs) that are RhoA regulators have been identified (Cancer Genome Atlas Research 2014). Functional analyses showed that the *CLDN18-ARHGAP26* fusion impairs epithelial integrity by reducing cell-cell and cell-extracellular matrix adhesion and by increasing cell invasion (Yao et al. 2015). *CLDN18-ARHGAP* fusions were mutually exclusive with *RHOA* mutations and, within the TCGA GS subtype, *RHOA* and *CLDN18-ARHGAP* alterations represent about 30% of cases.

In accordance with the aforementioned genetic alterations, integrated pathway analysis revealed that cell adhesion was the most enriched biological pathway in GS subtype tumors (Zang et al. 2012; Cancer Genome Atlas Research 2014).

5.4 EBV-positive tumors

EBV-positive tumors differ from other gastric cancer types, exhibiting distinct histopathological and epidemiological features. EBV-positive tumors tend to occur in non-antral anatomic subsites and more frequently exhibit diffuse-type histology; tumor EBV-positivity is 2-fold higher in males and 4-fold higher in post-surgical remnant tumors (Murphy et al. 2009; Lee et al. 2009). Since gastric cancer overall is twice as frequent in men than in women, the 2-fold sex difference in EBV positivity implies that the incidence of EBV-positive tumors is approximately 4 times higher in males than females. This relative protection of females is, as yet, unexplained, but similar sex disparities are apparent in other EBV-associated cancers, including Hodgkin's lymphoma, Burkitt lymphoma and nasopharyngeal carcinoma. Regarding behavioral risk factors, tumor EBV positivity has been associated with cigarette smoking but not with alcohol drinking (Camargo et al. 2014b).

Apart from an etiologic contribution in EBV-positive gastric cancer, viral presence may influence clinical progression. In a large, multi-center series, EBV positivity was associated with 30% lower mortality adjusted for stage and other confounders (Camargo et al. 2014a). Thus, EBV-positive tumors appears to have a more favorable clinical course than gastric cancer overall.

Based on the comprehensive molecular analysis performed by the TCGA, EBV-positive tumors were characterized by extreme CIMP. Notably, EBV-positive tumors had a higher prevalence of DNA hypermethylation than any other subtypes, showing *CDKN2A/p16INK4A* promoter hypermethylation, while lacking *MLH1* hypermethylation that is otherwise characteristic of MSI-associated CIMP. Additionally, EBV-positive tumors are characterized by frequent *PIK3CA* and *ARID1A* mutations, absence of *TP53* mutations, and recurrent amplifications of the chromosome 9 locus containing *JAK2*, and the immunomodulatory *PD-L1*, and *PD-L2* genes (Cancer Genome Atlas Research 2014; Wang et al. 2011).

PIK3CA mutations were present in 80% EBV-positive tumors in comparison with 42% of the MSI tumors, and in the former, mutations were dispersed throughout the gene, contrasting with the preference for the kinase and helical domains in EBV-negative tumors (Cancer Genome Atlas Research 2014).

The viral genes with the highest levels of expression in EBV-positive tumors exhibit a uniform transcription pattern, primarily of sequences encoded in the *BamH1A* gene region of the genome (Cancer Genome Atlas Research 2014). However, other genes are more variable, with about half of EBV-positive gastric cancers exhibiting moderate expression of BNLF2a, a virally-encoded inhibitor of antigen peptide transport (Strong et al. 2015). Viral transcripts and their protein products represent candidate targets for functional studies to explore mechanisms of viral carcinogenesis.

6. Hereditary gastric cancer

While the great majority of gastric cancers are sporadic, familial aggregation occurs in about 10% of the cases, and of these, only 1–3% clearly constitute hereditary forms. Hereditary gastric cancers include syndromes such as hereditary diffuse gastric cancer (HDGC), gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), and familial intestinal gastric cancer (FIGC). gastric cancer has also been identified as part of the tumor spectrum of other hereditary cancer syndromes like hereditary non-polyposis colorectal cancer (HNPCC), Li-Fraumeni syndrome (LFS), familial adenomatous polyposis (FAP) and Peutz-Jeghers syndrome (PJS). The clinical features, genetics, and pathogenesis of the hereditary gastric cancer syndromes have been reviewed recently (Oliveira et al. 2015).

HDGC is one of the best genetically characterized forms of hereditary gastric cancer. Heterozygous germline *CDH1* (E-Cadherin) mutations, including frameshifts, splice-site, nonsense and missense mutations, as well as large rearrangements, were until recently the only known causative alterations of HDGC, representing up to 40% of patients belonging to families that fulfill the clinical criteria for HDGC (van der Post et al. 2015).

Efforts conducted to identify alternative causative genes for the remaining HDGC that do not harbor *CDH1* alterations enabled the identification of a germline truncating mutation in the α -1-Catenin (*CTNNA1*) gene (Majewski et al. 2013). This mutation owing to a two-base deletion in *CTNNA1* exon 2, was detected in ten individuals with invasive tumors or intramucosal signet ring cells that were part of a large non-*CDH1* HDGC pedigree. Loss of α -1-Catenin expression was observed in tumor cells, along with reduced levels of E-Cadherin, consistent with the prediction that the deletion removes key functional domains of the α -1-Catenin, compromising the binding with β -Catenin and the formation of the E-Cadherin complex. In another study, two novel germline truncating *CTNNA1* mutations, one frameshift and one nonsense, were identified in two individuals of unrelated families out of 144 non-*CDH1* HDGC probands (Hansford et al. 2015). Immunohistochemistry of the tumors from the two *CTNNA1* mutation-positive patients showed loss of α -1-Catenin expression, suggesting the occurrence of an as yet unidentified second hit event at the *CTNNA1* locus. Contrasting with previous findings, E-Cadherin expression was preserved in tumors of *CTNNA1* mutation-positive patients. These data support that germline *CTNNA1* mutations cause HDGC and strengthen the inclusion of *CTNNA1* testing in non-*CDH1* HDGC families. Screening of mutations in catenin family member genes in 22 non-*CDH1* HDGC families did not reveal mutations in *CTNNA1*, *CTNNB1*, or *CTNND1* and found two nonsynonymous variants in the γ -Catenin-encoding gene *JUP* (Schuetz et al. 2012).

Other pathogenic germline mutations in known gastric cancer-predisposing genes, such as *BRCA2* and *STK11*, and in other cancer-susceptibility genes, such as *PRSS1*, *PALB2*, *ATM*, *MSR1*, and *SDHB* have been identified (Hansford et al. 2015). In other HDGC families, screenings of *APC*, *BRCA1*, *BRCA2*, *STK11* and *TP53* did not identify novel variants that matched the expected inheritance pattern (Majewski et al. 2013). Thus, their role in HDGC needs further investigation.

Germline mutations in *MAP3K6* were also identified upon examination of a large family with a history of familial gastric cancer (FGC) without *CDH1* mutations, with many features of HDGC, but with diversity in clinical presentation and advanced age of onset (Gaston et al. 2014). Four individuals with gastric cancer and five of the 27 currently unaffected relatives harbored a germline truncating (p.P946L) *MAP3K6* mutation. A *de novo* second-hit somatic mutation in *MAP3K6* was further detected in tumor DNA from one of the p.P946L carriers. In an additional screening of 115 individuals from unrelated non-*CDH1* FGC families, four additional *MAP3K6* variants, one truncating and three missense, as well as the p.P946L variant, were identified. Within this cohort, DNA hypermethylation of the *MAP3K6* gene was observed as a second-hit in tumor DNA of one affected individual. Taking together these observations with the evidence that *MAP3K6* (also known as *ASK2* or *MEKK6*) functions as tumor suppressor gene *in vivo* and is mutated in primary tumors and gastric cancer cell lines, reinforce the notion that *MAP3K6* is a strong susceptibility gene candidate predisposing to FGC (Iriyama et al. 2009; Zang et al. 2011).

Germline mutations in *INSR* (Insulin receptor), *FBXO24* (F-box protein 24), and *DOT1L* (DOT1-like histone H3K79 methyltransferase) have been identified in three affected members of a family with six cases of diffuse-type gastric cancer in two generations (Donner et al. 2015). Analysis of tumor samples of the three patients did not show LOH in any *loci* of the variants. These variants and the adjacent regions were screened in an additional 26 gastric cancer patients with a confirmed or suspected family history of DGC, but no other nonsynonymous or additional mutations were identified, warranting further validation of these mutations in other HDGC families.

Regarding GAPPS, germline point mutations in the promoter 1B of the *APC* gene were recently identified in six families (Li et al. 2016). The three points mutations identified were located within a *Ying Yang* transcription factor (*YY1*) binding motif, which inhibits binding of *YY1* to the *APC* promoter, leading to reduced APC expression. LOH or somatic APC-truncating mutation second hits were found in the majority of the polyps of the fundic glands that are typical of GAPPS, although in only a small number of cells. This fact led authors to suggest that *APC* haploinsufficiency is responsible for the fundic gland polyposis in GAPPS, and the second *APC* hit might be the driver of dysplasia. No germline mutations have been identified so far in FIGC.

7. Concluding Remarks

The most recent results on the characterization and biology of gastric cancer lent further support to the prevailing concept that this disease is highly heterogeneous. While these findings may help explain the ineffectiveness of a one-size fits all approach to treating this disease, they certainly open the door for a more precise clinical management. Future clinical approaches will certainly take into consideration the specific features of the different subgroups. The prognostic significance of the various gastric cancer types may emerge as more data accumulate. Researchers and clinicians should take advantage of the information provided by these studies to both design and test new targeted therapeutic approaches.

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Fig. 12.1.

Current gastric cancer classifications based on comprehensive molecular analyses. The main features of each subtype are highlighted in the boxes below each subtype. (A) TCGA classification (Cancer Genome Atlas Research 2014). (B) ACRG classification (Cristescu et al. 2015).