Review Article

INK4/ARF and gastric carcinogenesis

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Abstract: Gastric cancer (GC) is an aggressive disease, with a huge impact on global health, as it still remains the fourth most common type of cancer and the second leading cause of cancer-related death worldwide. Genetic abnormalities involved in the pathogenesis and progression of gastric carcinoma were identified, some of them also correlated with prognosis. Among these genetic abnormalities, the loss-of-function of genes encoded by *INK4-ARF* locus frequently occurs in cancers. This locus is located on the human chromosome 9p21, spans approximately 35 kilobases and encodes five types of tumor suppressor genes, being the most studied $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$. Among the several types of INK4/ARF molecular inactivation, one can highlight genetic abnormalities, such as genomic/chromossomic instabilities and microsatellites, mutations and polymorphisms and DNA methylation. In this review we will discuss the main types of molecular alterations on INK4/ARF described on GC.

Keywords: Gastric cancer (GC); genetic; methylation; molecular markers

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Gastric cancer (GC): general aspects

GC remains an aggressive disease that has a huge impact on global health. In the last few decades, the overall incidence of GC has been decreasing, but it still remains the fourth most common type of cancer and is the second leading cause of cancer-related death worldwide. The decline in its incidence is due to the use of antibiotics, changes in the consumption of chilled foods, increased use of preventive examinations and the eradication of *H. pylori* (1,2).

The incidence rates can vary by up to ten times around the world. Although Japan and Korea have the highest GC rates in the world, nearly two-thirds of stomach cancers occur in developing countries. High incidence areas include East Asia, Eastern Europe and South and Central America. Low incidence areas are found in South Asia, North America, East Africa, Australia and New Zealand (3).

One reason for this variation is the complex etiology dependent on a combination of environmental, host and genetic factors (4,5). One should emphasize the production of reactive oxygen species that leads to oxidative damage to DNA, mutations and aberrant DNA methylation (mainly hypermethylation of CpG islands in the promoter regions of certain genes), which results in the silencing of the tumor suppressor gene. In general, the prognosis for patients with gastric adenocarcinoma remains poor, and little progress has been made in improving the long-term survival rate (6,7).

Lauren (8) proposed the most used and widespread GC classification. He distinguishes between two main types of gastric carcinoma based on histological features: intestinal (well differentiated) and diffuse (poorly differentiated). Depending on the classification criteria and population studied, the proportion of intestinal and diffuse cancer varies (9-12). This classification is widely and routinely used by pathologists, epidemiologists and clinicians for the evaluation of gastric adenocarcinoma, particularly with respect to their incidence and etiology, although this is with limited value in relation to therapeutic decisions (13).

The intestinal form is composed of malignant cells that are bound to form structures similar to functional glands

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of the gastrointestinal tract. Its development has been characterized by a series of sequential steps that begin with gastritis, which ultimately leads to mucosal atrophy (atrophic gastritis) followed by intestinal metaplasia, dysplasia, carcinoma, and subsequent metastatic spread (14). This pattern reflects a more differentiated cancer, and is the most common type in high-risk populations (15,16).

The diffuse subtype of GC is considered more aggressive than the intestinal type, is originated from native cells of the gastric mucosa and tends to be poorly differentiated (17). Despite being associated with an *H. pylori* infection, it is more associated with the loss of E-cadherin expression and, unlike the intestinal type, is not associated with any pre-neoplastic lesions. Due to its aggressive characteristics, the diffuse type can invade surrounding tissues and organs, for example, the duodenum and esophagus (2).

Genetic abnormalities involved in the pathogenesis and progression of gastric carcinoma were identified, and several alterations were correlated with the prognosis. Molecular mechanisms, such as changes in oncogenes, tumor suppressor genes, and cell adhesion molecules, are involved in gastric carcinogenesis. Moreover, genetic instability and changes in cytokines and growth factors contribute to the complexity of pathways involved in gastric carcinogenesis (14,18).

Among these genetic abnormalities, the loss of the functions of genes encoded by the *INK4-ARF* locus occurs frequently in cancers, which raises questions about the alterations in biochemical pathways of these proteins when transformed into cancer cells (19,20).

INK4/ARF locus

The *INK4/ARF* locus is located on the human chromosome 9p21, spans approximately 35 kilobases and encodes five types of tumor suppressor genes: $p16^{INK4A}$, $p15^{INK4B}$, $p14^{ARF}$ ($P19^{ARF}$ in the mouse), $p16^{INK4A\gamma}$ and *P12* (21-25): the physiological functions of some of these remain unknown (26). Additionally, it is known that this locus has the *CDKN2BAS* gene (also known as *CDKN2b* anti-sense), which is responsible for producing a noncoding antisense RNA named *ANRIL*, which plays a regulatory function on *CDKN2A* and *CDKN2B* expression (27,28).

The most studied proteins produced by this locus ($p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$) interact with other proteins that activate two critical anti-proliferative pathways (Rb and p53 pathways) which play important roles in cell cycle inhibition, senescence, and stress-induced apoptosis (29). Functionally, both $p16^{INK4A}$ and $p15^{INK4B}$ bind specifically to CDK4 and CDK6, inhibiting the activity of cyclin D-dependent kinases, consequently blocking the cell proliferation by preventing phosphorylation of Rb, resulting in a G1 arrest (30,31). $p14^{ARF}$, encoded in part by an alternative reading frame within the second of three exons that comprise the *INK4A* gene, acts primarily by inhibiting murine double minute 2 (MDM 2)-mediated ubiquitination and degradation of p53, triggering p53-dependent cell cycle arrest and apoptosis (30).

Given the central role in tumor suppression of INK4-ARF in modulating activities of Rb and p53 pathways, it is not surprising that genetic and epigenetic changes in this locus are frequently detected in the majority of tumor types (29,32).

Some studies have shown that the *INK4/ARF* locus is tightly controlled, and polycomb group complexes are required to initiate and maintain the silenced state (29,33,34). The polycomb repressive complex 1 (PRC1) proteins (BMI1, PCGF1, PCGF2/MEL18, CBX2, CBX7, CBX8, and RING1B), and the PRC2 proteins (EED, SUZ12, and EZH2) have been shown to directly bind to and repress the locus (33,35-37).

Genetic alterations in *INK4/ARF* in gastric carcinoma

Genomic instability

Genomic instability is considered a hallmark of human cancers (38), which results in several genetic aberrations, from gene (changes in a single nucleotide) to structural levels (structural changes, losses and gains of entire chromosomes) (38,39). Therefore, these aberrations can affect the expression of tumor suppressor genes, oncogenes, DNA repair genes (genome stability genes), growth regulators, and cell cycle checkpoint control genes (40). Currently, the genomic instability can be classified into two types: chromosomal instability (CIN) and microsatellite instability (MSI) (39).

CIN and MSI

CIN is characteristic of various tumors, including GCs, which commonly are associated with chromosomal aberrations responsible for major modifications of DNA content, i.e., changes in chromosome copy numbers, high-level loss of heterozygosity (LOH), and gene deletions and/or amplifications (41). Most GCs exhibit significant CIN at several chromosome arms, including 9p (41-44); these instabilities have been associated with a shorter survival in

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GC patients (45).

Array-based comparative genomic hybridization (aCGH) is a powerful method used to identify alterations of DNA copy number changes on a genome-wide scale (46). This method has been applied to a number of solid tumors, including GCs (47,48), revealing several regions of consensus change in DNA copy numbers, indicating the possible location of candidate oncogenes or tumor suppressor genes involved in gastric tumorigenesis (49). In particular, the chromosome 9p21.3, which has the tumor suppressor genes $p16^{INK4A}$ and $p15^{INK4B}$, is known to be deleted frequently in GCs, particularly in undifferentiated GC (50-52).

Fan *et al.* (52) explored aCGH profiles of 64 human GC samples and eight GC cell lines using bacterial artificial chromosomes (BAC). They found out that the most frequent homozygous deleted region was 9p21 (8/72), and that homozygous deleted regions were higher in the cell lines than in the primary GC tumor samples. Weiss *et al.* (48) similarly observed that the loss of 9p21.3 was present in 29% of gastric adenocarcinoma.

Although many studies have investigated mutations or deletions of genes in 9p21, there has been no precise characterization of the break-points. Lee *et al.* (50) analyzed the entire set of large homozygous deletions in six human GC cell lines (SNU-1, SNU-5, SNU-16, SNU-520, SNU-638, and SNU-668) through genome and transcriptome approaches, and defined 9p21.3 homozygous deletions precisely. Moreover, they investigated the effect of the 9p21.3 deletions on gene expression by transcriptome sequencing, finding that no genes within the 9p21.3 deletion region were expressed in SNU-16 and SNU-668 compared to other GC cell lines analyzed.

Some studies recognize that the loss of chromosome 9p has been reported to occur more frequently in cases of GCs that are relapsed or histologically malignant (53,54).

In the literature, there are few studies reporting alterations in the $p14^{ARF}$ gene. Tang *et al.* (55) showed that homozygous deletion of $p16^{INK4A}$ and $p14^{ARF}$ was present in 35.4% of the GC cases analyzed, and the loss rate of $p14^{ARF}$ mRNA was 45.8% (22/48) in GC. When analyzing 11 cell lines for mRNA expression, homozygous deletion, mutation, and promoter methylation, Iida *et al.* (56) did not find any mutation in the whole coding region, but demonstrated that the $p14^{ARF}$ gene was more frequently inactivated by homozygous deletion or methylation in diffuse-type GC cell lines (5/7, 71.4%) than in intestinal ones; thus suggesting that $p14^{ARF}$ may be involved in the tumorigenic process in diffuse-type GCs.

Another type of genomic instability, commonly recognized in GC, is MSI. In GC tissues, MSI was a frequent event, with an average frequency varying from 25% to 33.9% (57-59). Zhang *et al.* (60) showed that the LOH frequencies of D9S171 and D9S1604 microsatellite loci in GC, located upstream of the $p16^{INK4A}$ gene, were 15% and 50%, respectively. Moreover, the LOH frequency in well-differentiated GC tissue was lower than in the moderately and poorly differentiated GC tissue without any significant difference ($P>0.05$).

Mutations and polymorphisms

The number of studies linking genetic polymorphisms and GC has increased exponentially over the past decades, in parallel with major advances in sequencing and genotyping, resulting in the identification of polymorphisms that may be useful indicators for assessing the risk of GC (39,61). However, it is worth noting that the results derived from polymorphism studies still need to be carefully interpreted, as these biomarkers are generally population dependent, with a strong ethnic influence (39).

Li *et al.* (62), studying 9p21 single nucleotide polymorphisms (SNPs) from eight genome-wide association studies (GWAS), including studies of esophageal squamous cell carcinoma (ESCC), GC, pancreatic cancer, renal cell carcinoma (RCC), lung cancer (LC), breast cancer (BrC), bladder cancer (BC) and prostate cancer (PrC), identified that the SNP on rs3731239 ($p16^{INK4A}$ intronic) was significantly associated with an increased risk of GC, and that it overlapped with weak but potential enhancers, leading them to suggest that SNPs in the CDKN2A/2B-AS1/2B cluster may modulate disease susceptibility, primarily through regulating expression levels of genes in the cluster.

Kim *et al.* (63) investigated the importance of $p16^{INK4A}$ and $p15^{INK4B}$ mutations in four stomach cancer cell lines and 14 stomach adenocarcinomas. They detected mutations in both genes only in stomach cancer cell lines; however, in the SNU5 cell line, they discovered a nonsense mutation (CGA→TGA/Arg→Stop) at codon 72 of the *CDKN2* gene. The absence of mutations in the $p16^{INK4A}$ and $p15^{INK4B}$ genes in stomach cancer tissues suggests that mutations in both genes may not be a critical genetic change in GC pathways.

INK4/ARF methylation and GC

Methylation is an important characteristic of DNA and is responsible for events such as gene regulation, X chromosome inactivation, aging and cancer. Several studies

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have identified methylation contribution to both oncogene activation and silencing of tumor suppressor genes in the development of cancers (64).

The inactivation of the $p16^{INK4A}$ gene by hypermethylation has been regarded as an important mechanism for initiation and development of GC (65). It is widely accepted that gene promoter methylation can repress gene expression at the transcriptional level, promoting the inactivation of tumor suppressor genes, leading to the development of malignant tumors (66).

The hypermethylation of $p16^{INK4A}$ may also be an important biomarker for identifying the possible malignant potential of precancerous lesions. In a study by Sun *et al*. (65), for the first time, a positive correlation between the aberrant methylation of the $p16^{INK4A}$ gene with the malignant transformation of gastric dysplasia was observed. This methylation pattern was also present in all of the GC samples that progressed from methylated gastric dysplasia lesions.

Iida *et al*. (56), using cell lines of the Human Science Research bank (Osaka, Japan), 7 (21.7%) of 32 samples of diffuse type, and 6 (21.4%) of 28 samples of intestinal type GC showed methylation of the *p16INK4A* gene, showing the lack of correlation between the methylation of this gene and the histological type.

In their experiment, Song *et al*. (64) analyzed the methylation profile of the $p16^{INK4A}$ gene in 322 samples from patients diagnosed with GC in Huizhou Municipal Central Hospital of Guangdong (China) and reported that 75% (242/322) were hypermethylated. Unfortunately, no comparison with the histological type was performed; however, it is important to highlight the association between DNA hypermethylation with MTHRF polymorphisms C677T and C1298A evidenced in this study.

Mino *et al*. (67) studied 35 patients with GC at Osaka City University Hospital, and observed the hypermethylation of the $p16^{INK4A}$ gene in 12/35 (34.3%) cancerous tissues: a frequency significantly higher than that of non-cancerous tissues. Although no relationship was observed between $p16^{INK4A}$ promoter hypermethylation and clinicopathologic characteristics, it seems that this event must be considered a biomarker of gastric carcinogenesis. This was also observed by do Nascimento Borges *et al*. (18) in a Brazilian population.

Finally, in a meta-analysis performed by Peng *et al*. (68), nine clinical trials with 487 GC patients and 271 healthy controls showed that the methylation rate of the $p16^{INK4A}$ gene in GC was significantly higher than in the healthy control, showing that the $p16^{INK4A}$ gene promoter array is a useful method for the diagnosis of GC.

The loss of function of p16 protein results in increasing activity in the corresponding kinase, leading to the phosphorylation of the pRb protein and resulting in the loss of cell cycle control. The transcriptional inactivation due to methylation of the promoter may also be linked to tumor cell proliferation and neovascularization (69).

Some evidence points to a close relationship between MSI and the aberrant promoter methylation of the $p16^{INK4A}$ gene (70,71). Shim *et al*. (70) found that 13/21 (61.9%) tumors that were MSI positive had the $p16^{INK4A}$ promoter methylation, as opposed to 24/67 (35.8%) MSI negative tumors. These results suggest that MSI was associated not with a p16 protein loss, but rather with the methylation status of the gene.

There are few reports in the literature regarding the $p15^{INK4B}$ gene methylation profile in GC. do Nascimento Borges *et al*. (18) analyzed 35 tumoral and 37 non-tumoral samples from a Brazilian population and found a high methylation frequency in the promoter region (25.9% and 27.3%, respectively). Interestingly, a correlation was noticed between patients under 60 years old and $p15^{INK4B}$ hypermethylation, suggesting a relationship between $p15^{INK4B}$ hypermethylation and the aging process.

Lee *et al*. (72) analyzed the *p15* methylation pattern in 54 patients with gastric adenocarcinoma and observed 68.5% with promoter methylation, which is a frequency similar to that obtained from a patient's serum (55.65%).

Promoter methylation of the $p14^{ARF}$ gene has been found in a great number of cancers including GCs (56,73-75).

Tsujimoto *et al*. (76) found a positive correlation between $p14^{ARF}$ promoter methylation and GC. Regarding the difference between histological types, $p14^{ARF}$ methylation was more frequently found in the early stages of intestinal type cancers and in the advanced stages of cancers of the diffuse type. These results are in agreement with those found by Iida *et al*. (56), who investigated the methylation status of $p14^{ARF}$ in 11 GC cell lines and 62 primary GCs, and found hypermethylation patterns in 9% and 35%, respectively, leading to the hypothesis that $p14^{ARF}$ methylation might also occur as an early event in a fraction of intestinal type cancers.

Concluding remarks

INK4/ARF, especially 9p21 deletion and $p16^{INK4A}$ promoter hypermethylation, may be used as a marker of gastric carcinogenesis, whereas mutations and polymorphisms

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should be carefully used, as they have a strong ethnic influence.

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Footnote

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