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Genetic Instability in Gastric Cancer

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1. Introduction

Gastric cancer remains a worldwide burden as a second leading cause of cancer death in both sexes (Globocan, 2011; Nobili et al., 2011). Although its incidence is in decline in developed countries, it is still the fourth most common malignancy in the world, behind cancers of the lung, breast, colon, and rectum (Globocan, 2011). The fall in its incidence is attributed mainly to the decline of the intestinal type of stomach cancer, whereas the incidence of the diffuse type has remained constant over time (Yamashita et al., 2011). On the other hand, there has been a progressive increase in the cardia and gastroesophageal junction adenocarcinoma (Milne et al., 2009; Yamashita et al., 2011). The exact cause of this shift in location is not known. The general decrease of gastric cancer frequency in developed countries is attributed to the changes in dietary habits and food preservation methods (Crew & Neugut, 2006; Kufe et al., 2003). The prevalence of gastric cancer varies throughout the world, with the highest rates reported in Korea, Japan, Central and South America, and Eastern Europe, whereas Western Europe, North America, Africa, Australia, and New Zealand are low incidence areas (Crew & Neugut, 2006; Tahara, 2008; Yamashita et al., 2011). Despite the decrease in its incidence and improvements in diagnosis, curative surgery, and treatment, gastric cancer remains major health burden due to its poor prognosis (Smith et al., 2006; Yamashita et al., 2011).

Adenocarcinoma is the major histological type of gastric cancer; accounting for 90% to 95% of all gastric malignancies, and this chapter will focus only on this type of gastric tumours (Hamilton & Meltzer, 2006). Adenocarcinoma develops from the glandular cells of stomach mucosa, while other rare stomach cancers develop in lymph tissue (lymphoma), hormone – producing cells (carcinoid tumours), muscle cells (soft tissue sarcomas) or certain nerve cells (gastrointestinal stromal tumours or GIST) (Smith et al., 2006). Based on the widely used Lauren classification, adenocarcinomas are divided into two distinct pathological entities, intestinal and diffuse types, which have different clinicopathological and prognostic features (Yamashita et al., 2011). Intestinal type is associated with *Helicobacter pylori* infection, obesity and certain dietary factors, such as high intake of salt, smoked meats and food preserved with nitrites or nitrates, and is believed to arise through a long-term multistep progression from chronic gastritis to chronic atrophy to intestinal metaplasia to dysplasia (Crew & Neugut, 2006; Hamilton & Meltzer, 2006; Yamashita et al., 2011). Histologically it is well differentiated and occurs more commonly in older patients, males and blacks (Crew & Neugut, 2006). Diffuse type is poorly differentiated with infiltrating,

non-cohesive cells and is more frequent in younger patients (Crew & Neugut, 2006; Panani, 2008). Studies showed that *Helicobacter pylori* infection also plays a role in the development of diffuse gastric cancer, through chronic inflammation, but without occurrence of intermediate steps, such as gastric atrophy and intestinal metaplasia (Milne et al., 2009).
It is believed that the pathogenesis of gastric cancer represents a classic example of gene-environment interactions (Panani, 2008). Epidemiologic studies have shown a reduction of its incidence in migrant populations, when they move from high-risk areas to low-incidence ones. Subsequent populations acquire risk levels similar to those in the host country, indicating the importance of environmental influences on its development (Crew & Neugut, 2006; Matysiak-Budnik & Megraud, 2006). Therefore, it is generally acknowledged that both, environmental and genetic factors are implicated in the pathogenesis of gastric cancer development (Milne et al., 2009). Furthermore, several researchers believe that environmental factors have a greater influence on the development of intestinal type, whereas diffuse type might have a stronger genetic background (Matysiak-Budnik & Megraud, 2006; Milne et al., 2009). Nevertheless, despite tremendous efforts in the past few decades, there is still no clear agreement on the genetic and epigenetic changes underlying the initiation and progression of both types of gastric adenocarcinoma (Milne et al., 2009; Panani, 2008).
This review is intended to focus on different molecular hypotheses of gastric carcinogenesis. New advances in the fields of high-throughput methodologies, functional genomics and molecular profiling will be discussed.

2. Molecular mechanisms of gastric carcinogenesis

Numerous cytogenetic and molecular genetic studies tested common cancer hypotheses, such as oncogene overexpression, suppressor, mutator, and methylator pathway hypotheses, but exact molecular mechanisms of gastric cancer development remain elusive. In nearly two decades of research a vast amount of articles referring to overexpression and silencing of genes was published (Resende et al., 2010). Several studies reported amplification and overexpression of growth factors, growth factor receptors, tyrosine kinases, nuclear factors, matrix metalloproteinases, cell cycle regulators cytokines and other genes (Panani, 2008; Tahara, 2008; Wu et al., 2010). Furthermore, other studies have shown that loss of heterozigosity (LOH) and inactivation of tumour suppressor genes seem to be involved in the development of gastric adenocarcinomas (Gazvoda et al., 2007; Juvan et al., 2007; Panani, 2008; Resende et al., 2010). The presence of spontaneous DNA replication errors in simple repetitive microsatellite sequences indicated a novel pathway of carcinogenesis, microsatellite instability (MSI) (Loeb, 2001; Panani, 2008; Simpson et al., 2001). It was found that it could be the consequence of defective DNA mismatch repair mechanism (MMR), caused by genetic alterations in *MLH1*, *MSH2*, *PMS1*, and *PMS2* genes (Hudler et al., 2004; Loeb, 2001; Panani, 2008; Simpson et al., 2001). In recent years, epigenetic changes, such as promoter methylation, hypomethylation and histone acetylation have been also recognized in gastric carcinogenesis (Hudler et al., 2004; Mitani et al., 2005; Schneider et al., 2010; Suzuki et al., 2006; Yamamoto et al., 2011).
In the 90's a model, describing genetic events of colorectal carcinogenesis, was suggested by Fearon and Vogelstein, which has shaped our understanding of the evolution of most types of malignancies today (Fearon & Vogelstein, 1990). The so-called 'Vogelgram' predicts that alterations in at least four to five cancer-related genes (oncogenes and tumour suppressor

genes) are needed for malignancy to occur, and that the total accumulation of changes rather than the order of their appearance is responsible for progression of the cancer (Fearon & Vogelstein, 1990).

Although molecular mechanisms and alterations contributing to initiation and progression of gastric tumorigenesis are still not completely understood, it is now widely accepted that it is initiated by several genetic and epigenetic alterations that result in overexpression of oncogenes and growth factors, as well as impaired expression of tumour suppressor genes. It has also become evident that alterations in genome stability genes can initiate and accelerate these neoplastic processes (Nobili et al., 2011; Oda et al., 2005; Zheng et al., 2004). It is also important to note that the prevalence of these abnormalities varies between intestinal and diffuse types of gastric cancer (Hamilton & Meltzer, 2006).

Recently, another type of genetic instability has been recognized as the most common feature of gastric cancers, namely chromosomal instability (CIN), leading to aneuploidy (Buffart et al., 2011; Nobili et al., 2011). New advances in high-throughput methodologies have shown that majority of solid tumours are characterized by gross chromosomal abnormalities, such as gain and/or loss of whole chromosomes or chromosomal segments (Duesberg & Rasnick, 2000; Gollin, 2005).

2.1 Oncogenes

Cell proliferation is tightly regulated through signal transduction pathways, which are regulated by growth factors and their receptors. Alterations in growth factors and other oncogenes result in constantly active genes or active under conditions in which the wild-type genes are not. Oncogenes are mainly activated due to gene amplifications, intragenic mutations that regulate the activity of gene product or chromosomal translocations, all leading to overexpression of the oncoproteins. The occurrence and development of gastric cancer was found closely related to a variety of oncogenes, few of which are briefly discussed below.

Ras family oncogenes play an important role in the pathogenesis of colon and pancreatic cancers and were reported, though less frequently, in gastric carcinomas (Pellegata et al., 1992; Soh et al., 1993). The prevalence of alterations in *HRAS* (*K-ras*), which encodes a protein involved in cellular signal transduction pathways, appeared to be dependent of geographic and ethnic origins of gastric cancer cases. While *HRAS* mutations were rare in Western Europe and Japan, the prevalence in China was up to 30% (Deng et al., 1994; Hiyama et al., 2002; van Rees et al., 1999). Genetic changes in *HRAS* have been observed in gastric intestinal metaplasia and gastric adenomas, and could be an early event in the development of gastric cancer (Hirohashi & Sugimura, 1991; Osaki et al., 1996). Despite many studies focused on *HRAS* mutations, there is still some controversial data on the functional role of these mutations that needs to be elucidated.

Overexpression or activation, due to either amplification or mutation of genes of some tyrosine kinases (hepatocyte growth factor receptor (*MET* or *c-met*), fibroblast growth factor receptor 2 (*FGFR2* or K-sam), human epithelial growth factor receptor 2 (*HER2*), and epithelial growth factor receptor (*EGFR*)) could be associated with human gastric cancer. Both, *HER2* and *EGFR*, were found overexpressed in gastric cancer, with prevalence in the intestinal type cancers (Garcia et al., 2003). Receptors have an intracellular domain with tyrosine kinase activity and *EGFR* can bind ligands with its extracellular domain, which induces homodimerization of the receptor and generates autophosphorylation, initiating

several signalling cascades that lead to DNA synthesis and cell proliferation. Overexpression of *EGFR* promotes cell migration, angiogenesis and inhibits apoptosis and has been observed in up to 47% of gastric cancers. Moreover, it was found to correlate with disease progression and poor clinical outcome (Malden et al., 1989; Yonemura et al., 1992; Yoshida et al., 1990). *HER2* does not bind to any known ligand, but it is known to heterodimerize with other members of the family, especially when it is overexpressed. The protein has been reported to be overexpressed or activated in 19% of gastric cancer cases. Studies suggest that overexpression of *HER2* might be prognostic factor for intestinal-type gastric cancer associated with shorter relapse-free survival and overall survival (Vizoso et al., 2004; Zhang et al., 2009).

Abnormalities in genes, such as *FGFR2* (*K-sam*), belonging to fibroblast growth factor receptor family, are associated with diffuse-type gastric cancer. Activation of *FGFR2* has been found in approximately 50% of diffuse type gastric cancers, and was associated with neoplastic progression and metastasis (Hara et al., 1998; Hattori et al., 1996; Werner et al., 2001).

The oncogene *MET* (*c-met*) encodes a receptor with tyrosine-kinase activity that binds hepatocyte growth factor. Aberrantly active receptor was preferentially found in intestinal-type gastric cancer tumours and was correlated with poor prognosis (Nakajima et al., 1999; Tsugawa et al., 1998). Employing a simple method of fluorescent multiplex RT-PCR assay and capillary electrophoresis separation we found overexpression of *MET* in 56% of Slovenian patients with gastric cancer (Rajcevic et al., 2007; Rajcevic et al., 2001). *MET* amplification could constitute an important biomarker for selecting patients for a targeted therapy, because it has been observed that a fraction of gastric cancer cell lines appeared to be exquisitely sensitive to a specific *MET* inhibitor (Smolen et al., 2006).

Vascular endothelial growth factor (*VEGF*), a pro-angiogenic molecule, was found frequently overexpressed in poorly differentiated gastric cancer (Brown et al., 1993; Scartozzi et al., 2004; Tian et al., 2001; Yamamoto et al., 1998). Recently, a *VEGF* +1612G/A gene polymorphism was found to be associated with gastric cancer in Chinese Han patients and was previously shown to affect *VEGF* plasma levels (Zhou et al., 2011). Several other oncogenes have been found overexpressed in gastric carcinomas (Nobili et al., 2011; Rajcevic et al., 2007; Tahara, 2004). Nevertheless, years of research have shown that overexpression of oncogenes is not the sole mechanism implicated in gastric cancer pathogenesis.

2.2 Suppressor phenotype

Tumour suppressor genes are targeted in the opposite way than oncogenes. Molecular abnormalities that result in a truncation of the proteins, deletions or insertions or epigenetic silencing, reduce the activity of the gene product. Generally, alterations in both alleles are required to confer impairment of the gene product, except in the case of haplo-insufficient genes (Dang et al., 2008; Vogelstein & Kinzler, 2004). Inactivation of the wild-type allele arises due to allelic loss, termed also loss of heterozygosity (LOH) or mutations (Knudson, 1993). The suppressor phenotype in gastric cancer is characterized by inactivation of suppressor genes, such as *TP53 (p53)*, *APC*, *MCC*, *DCC*, *CDH1*, *Rb1*, *FHIT,* and other (Hamilton & Meltzer, 2006; Nobili et al., 2011).

In our study we evaluated LOH on loci associated with the following tumour suppressors: *TPp53*, *APC*, *nm23*, and *RB*) and found that 52% of all cases exhibited LOH in at least one locus (Gazvoda et al., 2007). The highest frequency of LOH was at APC locus (36%),

followed by *TP53-1* (33%), *nm23* (33%), *TP53-2* (24%) and *RB* (24%). Interestingly, 5% of the samples exhibited MSI on all the evaluated loci (in LOH as well as in MSI evaluation). These samples were associated with clinicopathological features that differed from the rest. All tumours belonged to intestinal type, displayed expansive growth and were mostly tubular. Furthermore, we found that LOH on loci *TP53-1* and *TP53-2* was associated with more expansive growth and LOH on *TP53-1* locus tended to be associated with intestinal type tumours. In contrast, tumours without LOH on *TP53-1* locus were associated with ulcerating, infiltrating type of gastric adenocarcinoma (Gazvoda et al., 2007).

The *TP53* gene encodes a main regulator of cell growth and division, and its function in intestinal type of gastric cancer is mainly altered due to LOH and mutations. When protein p53 is impaired, the cells may not be able to induce apoptosis and control tumour growth (Vousden & Prives, 2005). Studies showed that mutations in *TP53* are present in a range of 40%-70% of early and advanced gastric cancers, and inactivation of *TP53* resulting from LOH is found in 60%-70% of intestinal-type gastric cancers, thus making this gene among the most frequently mutated genes in cancers (Hamilton & Meltzer, 2006; Werner et al., 2001). It was suggested that accumulation of mutations in *TP53* is involved in initiating carcinogenic processes, though not all studies are in agreement with this hypothesis (Liu et al., 2001; Zwick et al., 1997). The expression of p53 protein can be easily detected by immunohistochemical staining, because mutations in *TP53* gene increase the half-life of its product, and it was postulated that it could be used as a biomarker in a clinical setting (Zheng et al., 2004). However, there are conflicting results regarding the prevalence and of *TP53* mutations and its expression and their relationship to clinicopathological features of gastric cancer (Panani, 2008). We and some other researchers found that the *TP53* mutational status was not in association with p53 expression (Bataille et al., 2003; Juvan et al., 2007; Panani, 2008). Furthermore, we found that positive *TP53* expression was associated with poorer survival, which was accordance with some other studies (Bani-Hani et al., 2005; Lazar et al., 2010). On the other hand, other studies did not reveal this association, therefore, the prognostic value of *TP53* remains controversial (Panani, 2008).

Loss of *APC* gene function was first identified in 60%-80% of patients with familial adenomatous polyposis-associated colorectal cancers (Kinzler et al., 1991; Lynch & Lynch, 1998). Mutations and LOH of the gene were also reported in more than 50% of gastric cancers of intestinal type (Tahara, 1995; Wright & Williams, 1993). Functional product of *APC* gene targets β-catenin for ubiquitination, and thus prevents β-catenin associated induction of genes involved in growth control (Caca et al., 1999; Park et al., 1999).

E-cadherin, encoded by *CDH1* gene, is an adhesion molecule expressed from epithelial cells, which plays a crucial role in epithelial structural integrity and was found to be implicated in carcinogenesis. Germline mutations in *CDH1* were first described in patients with hereditary diffuse type gastric cancer, however the rate of *CDH1* mutations in sporadic gastric cancer was found to be as high as 50%, and reduced expression of E-cadherin protein was found in 51% of diffuse type gastric cancers (Becker et al., 1994; Guilford et al., 1998; Xiangming et al., 1999). Susceptible individuals with a germline mutation in tumour suppressor gene *CDH1* require the inactivation of the second allele due to somatic mutation or DNA methylation, rendering E-cadherin completely inactive (Becker et al., 2000). Abnormal expression of E-cadherin is thought to promote metastatic ability of gastric cancer cells (Kanai & Hirohashi, 1997).

2.3 Alterations in other genes

Genetic and epigenetic abnormalities have been found in numerous other genes that participate in proliferation, invasion and metastasis, such as cell cycle regulators, cell-adhesion molecules, growth factors, cytokines, nuclear factors, matrix metalloproteinases, DNA repair genes, and apoptosis regulators (Nobili et al., 2011; Tahara, 2004; Yokozaki et al., 2001). For example, cyclin E1 together with cyclin-dependent kinase, *CDK2*, promotes the entry into the S-phase of the cell cycle, and it was found overexpressed in one third of gastric cancer cases. Amplification of this gene was found to correlate with tumour aggressiveness (Jiaqing et al., 1998; Nobili et al., 2011; Xiangming et al., 2000). In our study we observed overexpression of cyclin E1 in 42% of patients with gastric cancer and in 57% of patients with precancerous lesions, indicating that abnormalities in this gene could be early event in gastric carcinogenesis (Rajcevic et al., 2007). Moreover, we also found overexpression of epidermal growth factor family members, such as *TDGF1* and *EGF*, and *NRG1*, signalling protein, that mediates cell-cell interactions and plays critical roles in the growth and development of multiple organ systems (Rajcevic et al., 2007). Several other genes have been reviewed extensively elsewhere (Nobili et al., 2011; Resende et al., 2010; Tahara, 2004; Wu et al., 2010; Yokozaki et al., 2001).

2.4 MSI and mutator phenotype

Molecular abnormalities in oncogenes and tumour suppressor genes drive the neoplastic process by increasing tumour growth. The increase is achieved by activating of genes that drive the cell cycle or by inhibiting normal apoptotic pathways (Vogelstein & Kinzler, 2004). The third class of genes that contribute to cancer development are the stability genes, which, when mutated, promote tumorigenesis in a completely different way. They keep genetic alterations to a minimum, and thus, when they are inactivated, mutations in oncogenes and tumour suppressor genes occur at a higher rate (Freiberg, 2003). As with tumour suppressor genes, both alleles must be inactivated for physiologic effect to result.

Mismatch repair (MMR) genes are an example of genome stability genes and molecular inactivation of these genes is a hallmark of so-called mutator pathway, which results in microsatellite instability (MSI) or mutator phenotype. Microsatellites are short tandem repeats abundant throughout the genome. They are polymorphic among individuals, but their length is stable in every noncancerous tissue within a given individual. Patients with MSI phenotype exhibit a high frequency of changes in length of microsatellites within a tumour tissue compared to normal tissue, due to slippage of DNA polymerase during DNA replication on repetitive sequences, which leads to insertion or deletion of nucleotides. In short, MSI phenotype is characterized by appearance of new alleles not present in the normal genotype. These postreplicational DNA errors are detected and repaired by a complex of MMR proteins, rather than proofreading activity of the polymerase. Inactivation or deficiency of one or more MMR genes, particularly *MLH1* or *MSH2*, leads to manifestation of MSI phenotype in gastric cancer. As shown in Figure 1, MSI often leads to additional genetic changes and allelic losses, due to frameshift mutations in coding repetitive sequences of genes involved in cell growth regulation, apoptosis and DNA repair (Buermeyer et al., 1999; Ottini et al., 2004). Remarkably, every human MMR gene except *MLH1* includes a mononucleotide repeats, suggesting that the MMR process becomes increasingly defective with subsequent losses of involved proteins (Perucho, 1996).

Impairment of MMR, eventually leading to cancer development, can occur: 1) by mutational inactivation of one or two MMR genes, or 2) by epigenetic inactivation of MMR genes. In gastric cancer, functional inactivation of MMR is mainly caused by latter. Epigenetic hypermethylation of *MLH1*promoter has been found to be responsible for the development of the majority, more than 50%, of MSI-H positive gastric cancers, whereas mutations in *MLH1* and *MSH2* are being reported in 12-15% of gastric cancer exhibiting MSI-H phenotype (Bacani et al., 2005; Wu et al., 2000; Yamamoto et al., 1999) (Figure 1). Silencing of multiple genes, including known tumour-related genes such as *CDKN2A (p16)*, *hMLH1*, *THBS1,* and *CDH1,* due to promoter hypermethylation, is an important epigenetic event in stomach carcinogenesis and was shown to occur in early stages of gastric cancer development. This pathway of methylation of CpG islands characterizes alternative molecular phenotype of gastric cancer, referred to as the CpG island methylator phenotype (CIMP) (Nobili et al., 2011; Oue et al., 2001; Resende et al., 2010).

2.4.1 MSI analysis

MSI can be detected with polymerase chain reaction (PCR), where each microsatellite under investigation is amplified using specific primers. Lengths of PCR obtained products are usually assessed and compared between normal and tumour tissues from each individual using a simple and cost effective fluorescent multiplex PCR, followed by capillary electrophoresis separation (Gazvoda et al., 2007; Suraweera et al., 2002). Because of a huge number and diversity of microsatellite regions in the human genome, it is difficult to determine the prevalence of MSI in human cancers and its incidence varies depending on which loci are investigated (Lawes et al., 2003). To overcome this confusion, a standard panel of microsatellite markers, including mononucleotide repeats (BAT25 and BAT26) and dinucleotide repeats (D2S123, D5S346 and D17S250) has been recommended to identify MSI phenotype (Nobili et al., 2011). Cancers were subdivided in three groups based on the number of markers displaying instability: those demonstrating instability in > 30-40% of the loci investigated were classified as high-level MSI (MSI-H); those demonstrating instability in <30-40% of the loci investigated were classified as low level MSI (MSI-L); and stable cancers (MSS) showing no instability (Boland et al., 1998). Although these criteria were initially aimed at identifying MSI positive colorectal cancer, they were also successfully used for detecting MSI-H gastric cancers. Incidence of MSI-H has been observed in range 2-18% of gastric cancer cases, depending on the ethnic background. In Japan the incidence of MSI-H phenotype in patients with gastric cancer was reported in 5% of cases, whereas in Western populations it was ranging from 2 to 15% (Gu et al., 2009; Hudler et al., 2004; Leung et al., 1999; Pedrazzani et al., 2009; Schneider et al., 2000; Zhou et al., 1998). Moreover, studies reported 3-fold higher prevalence of MSI-H status in intestinal rather than diffuse-type gastric cancers (Leite et al., 2011). As reviewed by Lawes et al., patients with gastric cancer that exhibit MSI-H phenotype were associated with a better survival (64-88%) when compared to MSS counterparts (39-53%) (Lawes et al., 2003). Furthermore, we and other researchers have found that MSI-H phenotype was not associated with LOH-H phenotype, which is in agreement with other studies proposing that the mutator and suppressor pathways are independent of each other at least in the early stages of gastric carcinogenesis (Gazvoda et al., 2007; Kim et al., 2001). Likewise, patients with LOH-H were associated with MSI-L or did not show MSI (microsatellite stable, MSS) on evaluated loci. In our study we

evaluated MSI on loci BAT25, BAT26, BAT40, D2S123, D3S1277, and D10S107, and as mentioned before, LOH on loci, associated with tumour suppressors. Interestingly, the highest frequency of MSI was found at RB locus (21%), which was initially tested for LOH, followed by BAT25 (15%), D3S1277 (14%), D2S123 (13%), D10S107 (13%), BAT40 (12%) and BAT26 (10%) (Gazvoda et al., 2007). We observed that in our study BAT26 was the most informative locus. We also correlated MSI with clinicopathological features and found that MSI-L phenotype was associated with diffuse or mixed types of gastric cancers.

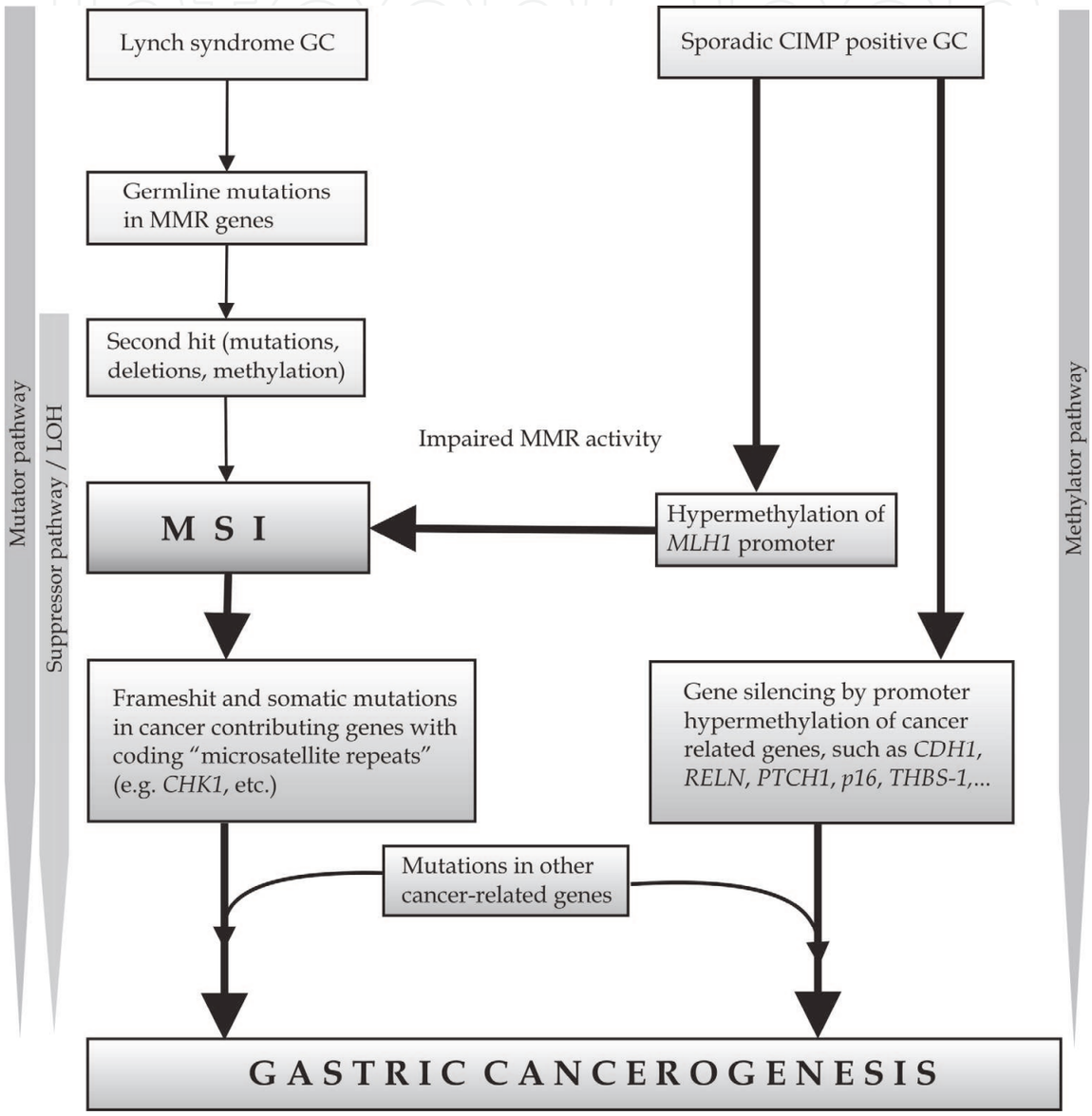

Fig. 1. Mutator pathway overlapping with suppressor and methylator pathways in gastric tumorigenesis. These changes should not be considered a specific sequence of alterations, but rather an overall collection of abnormalities that contribute to the pathogenesis of gastric cancer (Adopted from Boland & Goel, 2010).

It has recently become evident that dinucleotide repeats are less sensitive than mononucleotide repeats for detection of MSI-H, therefore revised criteria proposes the use of a mononucleotide markers in order to define MSI-H instability (Umar et al., 2004). A panel of five mononucleotide repeats (BAT25, BAT26, NR-21, NR-22 and NR-24) that may be more instrumental for detecting MSI-H status in humans has been suggested (Buhard et al., 2004). It has been further demonstrated that these markers are quasimonomorphic in 1206 studied individuals from 55 different populations worldwide, and can therefore be used for MSI-H determination without the requirement for matching normal DNA (Buhard et al., 2006). By adopting the panel, MSI-H phenotype was reported in a range from 5% to 50% of all gastric carcinomas with significant differences in various population groups (Leite et al., 2011; Ottini et al., 2004; Simpson et al., 2001).

2.4.2 Mutational impairment of MMR activity and pathogenic significance of observed alterations

The most common inherited condition that gives rise to MSI positive cancers is Lynch syndrome, an autosomal dominant disease, also referred to as Hereditary Non-polyposis Colorectal Cancer (HNPCC), where gastric cancer is a common neoplasia, occurring in 6% of Lynch syndrome cases (Percesepe et al., 2001; Samowitz et al., 2001). Predisposed individuals carry a recessive, first-hit germline mutation in the MMR genes, including large genomic rearrangement, which account for 5-20% of all mutations. In reference of Knudson's hypothesis, the MSI-H phenotype requires the "second hit" inactivation of the responsible MMR gene for development of malignant phenotype.

In Lynch syndromes, somatic inactivation of the remaining wild-type allele can occur due to different mechanisms: loss of heterozygosity (LOH), somatic mutation and promoter methylation (Imai & Yamamoto, 2008). The relative risk of gastric cancer development in Lynch syndrome individuals has been reported to be 4-19-fold higher, compared to general population, suggesting that screening for MMR mutations in predisposed carriers could be of importance for the detection of predisposed individuals (Gylling et al., 2007). Particularly, patients with MSI-positive gastric carcinomas, but lacking *MLH1* promoter hypermethylation are regarded as potential germline MMR-related mutation carriers.

Majority of MMR alterations, found in patients with Lynch syndrome are known to be pathogenic as they result in premature termination of protein synthesis and thus loss of MMR activity. However, hundreds of MMR variants that do not lead to truncation of the respective MMR protein have been identified in Lynch cancer cases and their pathogenic significance is often difficult to establish on clinical samples alone.

Information on functional nature of MMR alterations is essential for accurate early diagnosis and prognosis as well as for proper genetic counselling for members from affected families. Therefore in the past decade, many functional assays have been developed to ease the interpretation of pathogenicity of unclassified variants (UVs). Recent and some of the most recognized *in vivo* and *in vitro* assays together with available *in silico* algorithms are summarised in Table 1.

While many *in vitro* assays characterize specific biological functions of MMR proteins, *in vivo* tests strive to assess the MMR repair capacity as a complex cellular process (Ou et al., 2007). Since efficiency of MMR repair relies on several successfully completed biochemical events of involved proteins (e.g. protein expression levels and stability, localization of MMR protein to the nucleus, heterodimerization ability and effective recognition and repair of the DNA lesions, etc.), *in vivo* approaches are preferable and are either cell line- or yeast-based. However, all assays have their limitations and problems, mostly concerning toxic episomal overexpression of MMR proteins and lack of evolutionary conserved regions between yeast

and human MMR proteins at the regions of interest. Moreover, since variety of strategies have been used, it is difficult to establish and compare clinical significance of analysed variants. Finally, it is also not easy to determine sensitivity and specificity of these tests, therefore results should still be utilized with caution and interpreted alongside clinical data of the affected carriers.

Assay type	Biochemical feature analysed	Assay	References
In vitro	Protein-protein interaction	GST pull-down	Raevaara, 2005; Guerette, 1999; Belvederesi, 2006; Perera, 2008
		Expression of MMR genes in human cell lines	Trojan, 2002
	Protein expression	Western blotting	Takahashi, 2007
	mRNA splicing	pCAS minigene	Tournier, 2008
	MMR activity	Cell-free assay w/ protein extracts	Takahashi, 2007; Raevaara, 2005;
In vivo	Protein-protein interaction	Human-yeast hybrid MLH1 in yeast	Kondo, 2003;
	Protein expression	Immunohistochemical staining	Leite, 2011
	Intracellular localization	Fluorescence microscopy	Raevaara, 2005
	mRNA splicing	*In vivo* splicing assay in human cells	Auclair, 2006; Sharp, 2004; Arnold, 2009
	MMR activity	Yeast-based chromosome-integrated hMMR gene	Vogelsang, 2009; Vogelsang, 2010
		Dominant mutator effect	Raevara, 2005;Takahashi, 2007; Shimodaira, 1998
		Functional assay using yeast	Ellison, 2001;Wanat, 2007
		Utility of *MLH1*-deficient cells	Blasi, 2006
In silico	Effect of amino acid substitution on protein functions	SIFT	Kumar, 2009; Ng, 2003
		PolyPhen	Ramensky, 2002
		MAPP-MMR	Chao, 2008
		Align GVDV	Tavgtigian, 2006; Mathe, 2006
	mRNA splicing	NNSPLICE	Sharp, 2004

Table 1. Compilation of functional assays used in characterizing pathogenic significance of MMR variants found in Lynch syndrome patients.

We have recently described an *in vivo* yeast-based functional approach, expressing human MMR genes in yeast, enabling all variants found within the coding region of the MMR gene to be analysed. With chromosomal integration of relevant human MMR genes we obtained their stable expression throughout the experiment (Vogelsang et al., 2009). With our

approach we have functionally characterized four missense *MLH1* variants, which we previously identified in MSI-H positive gastric cancers with limited *MLH1* hypermethylation. We also assessed two of the variants, which were described for the first time in our study (Hudler et al., 2004). We have shown that identified missense mutations were not causally associated with MSI-H phenotype in analysed gastric cancer tissues (Vogelsang & Komel, 2010).

2.5 Chromosomal instability (CIN) and aneuploidy

In contrast to MSI, CIN is characterized by gross chromosomal abnormalities, such as gain or loss of whole chromosomes and/or fractions of chromosomes (LOH, amplifications, translocations) (Martin et al., 2010). Aneuploidy is the state of altered chromosome number in malignant cells (Pino & Chung, 2010). Studies showed that MSI phenotype is characteristic for hereditary type of gastric cancer, developed in the context of Lynch syndrome, and a smaller subset of sporadic cancers ranging from 15% to 35% (Panani, 2008). CIN, however, has been recently recognized as the most common feature of sporadic gastric cancers, and has been reported in up to 84% of gastrointestinal tumours (Grabsch et al., 2004; Ottini et al., 2006).

Several techniques, such as karyotyping, cytometry, detection of LOH, and fluorescent *in situ* hybridization (FISH) have been developed to measure CIN and some of them have already been successfully transferred to clinical practice. New methods, such as CGH arrays and copy number variation analysis (CNV), have advanced the field, due to their ability to detect chromosomal abnormalities with higher resolution and accuracy (Pino & Chung, 2010).

CIN has been recognized as valuable prognostic factor and tumour stage indicator in gastric cancers, although in the study of Birkbak et al. it has been found that intermediate CIN had more impact on poor prognosis than extreme CIN phenotype (Birkbak et al., 2011; Suzuki et al., 2003). Furthermore, it has been found that DNA copy number changes are not uniform in gastric cancers and subgroups with different patterns of DNA copy number alterations have been recognized, which have been associated with prognosis, lymph node status and metastasis (Buffart et al., 2007b; Kang et al., 2006; Morohara et al., 2005; Panani, 2008; Weiss et al., 2004; Wu et al., 2002).

Buffart et al. explored the differences in DNA copy number by CGH arrays and reported that the mean number of chromosomal events was lower in adenomas compared to gastric carcinomas, suggesting that distinct losses and gains on chromosomes likely represent early events in carcinogenesis (Buffart et al., 2007b). In another study they compared CGH profiles of gastric cancers in young and old patients (Buffart et al., 2007a). They found out that chromosome regions 11q23.3 and 19p13.3 contributed most to age-related differences in tumour profiles and that tumours of younger patients showed gains in chromosomal regions 6p21, 9p34, 11p15, 11q23, 17p13, 19p13, and 22q13, whereas in the majority of older patients normal copy status was observed. They concluded that these differences in genomic profiles likely reflect different pathogenic mechanisms of the disease.

Varis et al., similarly observed that the most frequent cytogenetic aberrations were gains seen at 17q, 19q, and 20q in younger patients (Varis et al., 2003). They also found that DNA copy number changes were mostly detected in intestinal or mixed types of tumours.

Tsukamoto et al. observed higher frequencies of DNA copy number aberrations, especially in the case of 20q13 chromosome gain, which was detected in 97% of cases, compared to other studies (Tsukamoto et al., 2008). They used laser microdissection method to isolate tumour cells, therefore their samples contained fewer cells from tumour microenvironment. They also identified 114 upregulated candidate genes located in regions of amplification and 11 down-regulated genes located in regions of deletion.

Several other studies reported different DNA copy number changes in patients with gastric cancer (Buffart et al., 2007b; Hou et al., 2008; Junnila et al., 2010; Kimura et al., 2004). Hou et al., for example, used an integrated approach using CGH and 100K SNP arrays, FISH, reverse transcription PCR, Western immunoblotting, and siRNA-mediated gene knockdown to determine and identify potential overexpressed genes in region 6p11p12, which they found to be amplified in their study (Hou et al., 2008). They identified *RAB23*, which could be implicated in invasion.

Despite the remarkable effort made by researchers to identify significant chromosomal aberrations in gastric cancers and to correlate them with clinicopathological features, the results are still inconclusive and not consistent with each other (reviewed in Nobili et al., 2011; Panani, 2008).

2.5.1 LOH

As stated before, LOH studies have already revealed several chromosomal loci with significant allelic losses, facilitating the identification of tumour suppressor genes, which could be important in gastric tumorigenesis (Gazvoda et al., 2007; Juvan et al., 2007; Kim et al., 1991; Kondo et al., 2005; Panani, 2008; Tamura, 2006). LOH is also a marker of chromosomal instability and might indicate a second inactivational hit of a cancer suppressor gene. Allelic losses are typically detected by using highly polymorphic microsatellite sequences that are dispersed throughout the human genome. Several LOH studies demonstrated that the extent of chromosomal loss appeared to be of prognostic significance (French et al., 2004; Gazvoda et al., 2007; Koo et al., 2004). It was established that there was a trend of two distinct subtypes, high-level LOH (named LOH-H) and low-level LOH (named LOH-L), being correlated with intestinal or mixed and diffuse growth patterns, respectively (Hong et al., 2010). In our study we also found out that LOH-H was associated with intestinal type of gastric cancer (Gazvoda et al., 2007). LOH has been shown to relate to cancer progression, where a transition from LOH-L to LOH-H is thought to reflect an increase in chromosomal instability during tumour advancement. These findings on LOH events suggest that the degrees of allelic loss may have an influence on the clinical course of gastric cancer.

2.5.2 Aneuploidy

Although some opinions still diverge regarding the clinical impact of aneuploidy alone (mostly measured by FISH, flow cytometry or image cytometry), recently there are reports pointing out that it could be of importance as a predictive marker in gastric cancer, and its potential clinical practicability in pre-malignant disease to stratify patients by their cancer risk. It is important to note recent evidence supporting the hypothesis of stepwise ploidy progression: from diploid or minor aneuploid in most early cancers to aneuploid in most advanced cancers (Duesberg et al., 2005). As a progressive increase in the severity of aneuploidy with neoplastic progression has been observed, it has thus been shown to be a

useful prognostic indicator for patient classification as low or high-risk cases for cancer development (Russo et al., 2000; Yasa et al., 2005).
Interestingly, aneuploidy was found in human tumours more than 100 years ago by von Hansenmann and Boveri (Duesberg & Rasnick, 2000; Ricke et al., 2008). However, in the last decades, the research was oriented towards oncogenes and tumour suppressors' hunt, and in identifying mutator and methylator pathways of gastric carcinogenesis. Yet to date, not one subtype of gastric adenocarcinomas has been completely described and no cancer-causing genes or combination of genes have been found to be specific for gastric cancers, although a number of mutations and other genetic changes have been described (Duesberg & Rasnick, 2000; Nobili et al., 2011; Panani, 2008; Weber, 2002).
Recently, it has been found that aneuploidy, either in the form of LOH or gross chromosomal copy number changes, stands out as the most consistent marker of neoplastic cells in solid tumours (Duesberg & Li, 2003; Ottini et al., 2006). Indeed, several studies confirmed a high frequency of aneuploidy in sporadic gastric cancers, even up to 84% (Belien et al., 2009; Buffart et al., 2007b; Buffart et al., 2011; Grabsch et al., 2004; Russo et al., 2000).

2.5.3 Mechanisms leading to chromosomal instability

The mechanisms leading to abnormal chromosome content and other chromosomal abnormalities are poorly understood, although it is now believed that CIN might, through stepwise clonal progression, lead to oncogene activation, tumour suppressor inactivation and alterations in other crucial genes, implicated in establishing the malignant phenotype of cells. Several different mechanisms have been proposed by researchers, such as telomere dysfunction, defective DNA damage response, impaired chromosomal segregation, and aberrations in cell cycle regulators (Castro et al., 2007; Gollin, 2005; Grabsch et al., 2004; Yasui et al., 1999).
Lately, the attention of researchers in the field of epithelial tumours, including gastric adenocarcinomas, has focused on genetic changes in mitotic genes, with emphasis on chromosome segregation. Segregation is one of the fundamental processes in cells, which are rapidly dividing, such as gastric epithelial cells. Therefore, if regulation mechanisms, governing this process are damaged, the cells might proceed through cytokinesis with DNA or spindle errors and thus could inherit unrepaired mutations or gain an abnormal number of chromosomes (aneuploidy) (Schmit & Ahmad, 2007). However, the molecular defects underlying CIN and aneuploidy and weather it is a cause or consequence of tumour phenotype are not completely clear. At least two possible mechanisms for CIN development have been suggested: mutations and/or polymorphisms in mitotic genes, implicated in chromosome segregation, or the activity of carcinogens on susceptible genetic background of individuals. (Duesberg et al., 2005; Iovino et al., 2006).
Studies on several animal species and humans showed that certain genetic mutations and polymorphisms in genes involved in segregation of chromosomes might cause an increased incidence of a particular tumour type (Shepard et al., 2007; Tomonaga & Nomura, 2007). Kim et al. analysed expression of *MAD2L1*, a component of the mitotic spindle assembly checkpoint, and kinase gene *BUB1*, involved in activating the spindle checkpoint. They found mutations in *MAD2L*, whereas they did not detect any mutations in *BUB1*.
Grabsch et al., on other hand, observed overexpression of BUB1 protein in gastric cancers, which was significantly higher in tissues of patients with diffuse type adenocarcinomas

(Grabsch et al., 2004). However, their study did not reveal any association between BUB1 protein expression level and DNA ploidy status of examined tumour types.

Aurora kinase A (*AURKA* or *STK15*) located at 20q13, a region that is frequently amplified in gastric cancer, has been found overexpressed in stomach adenocarcinomas (Dar et al., 2008). Functional analysis of upregulated *AURKA* gene, done by the same researchers, revealed a possible novel oncogenic pathway, involved in gastric carcinogenesis. *AURKA* overexpression led to a significant increase in mRNA levels of several direct targets of the β-catenin/TCF transcription complex (cyclin D1, *MYC*, *MYC*-binding protein, *CLDN1*, *FGF18*, and *VEGF*).

However, these and several other studies, explored overexpression and/or mutations of these genes, which could already be the consequence of CIN. Therefore, it has been proposed that minor alterations in mitotic genes could contribute to the onset of cancer (Frank, 2004). The mounting evidence is suggesting that subtle variations, such as single-nucleotide polymorphisms (SNPs) or non-lethal mutations, might induce CIN and aneuploidy. This hypothesis of low-penetrance allelic variants or risk alleles is further supported by the fact that non-heritable cancers usually develop in elderly, whereas dominant mutations in oncogenes and tumour suppressors usually induce the disease early in life (Duesberg & Rasnick, 2000; Frank, 2004). Minor genetic variants in mitotic genes could in combination with environmental factors modulate mitotic pathways, and could thus exert minor changes in the DNA of replicating epithelial cells. The search for these changes has begun only recently, and further investigations are needed to clarify these aberrations and their involvement in carcinogenesis.

In our study, we genotyped two polymorphic sites, T91A (F31I) and G169A (V57I) in serine-threonine-kinase *STK15* (*AURKA*), which is involved in the regulation of several cell cycle events (Hudler et al., 2009). It is responsible for the functioning of centrosome, for microtubule formation and stabilization at the spindle pole throughout all phases of segregation, and for chromosome segregation during anaphase. We found a putative protective role of the genotype A/T (F31I) in examined population of gastric cancer patients. We also found a weak protective association between homozygotes A/A, heterozygotes A/G (V57I) and A/T (F31I) genotype and reduced risk for perineural invasion. In another study we performed the case-control study of selected polymorphisms rs151658 and rs239559, rs1031963 and rs1801376 in mitotic segregation genes, TTK and BUB1B, respectively (Hudler et al., 2010). We found a significant interaction between patients and control cases for genotype A/G in rs151658 polymorphism. We also observed a statistically important difference in genotype frequencies between female patients and control cases for polymorphism rs1801376. Our results showed that this difference was significant only for female population of patients. Polymorphisms rs151658, rs1031963 and rs1801376 showed significant associations with certain clinicopathological factors, such as differentiation of tumours, infiltration, and intestinal type of gastric cancers. This study provides new support for the role of mitotic genes in gastric cancer development, suggesting that smaller changes could be associated with genetically unstable gastric tumours. However, the biological basis for the role of risk alleles of mitotic genes in cancers of the upper gastrointestinal tract needs to be established to understand its consequences and role during carcinogenesis.

Carcinogens are a second probable cause of CIN and particular agents, such as *Helicobacter pylori* infection, tobacco, nitrates, and nitrites have an important impact on gastric tumorigenesis in genetically susceptible individuals (Matysiak-Budnik & Megraud, 2006). In

addition, a combination of SNPs within pro-inflammatory genes IL-1β, IL-1RA, TNFα, and IL-10 conferred even greater risk for gastric cancer development in combination with CIN causing *Helicobacter pylori* infection (El-Omar et al., 2003).

3. Future directions

Recent advances in high-throughput methods revealed the lack of consistency regarding the number and species of genes mutated in all subtypes of gastric adenocarcinomas, or even from one cell to another within the same tumour, which points to amazing genetic diversity of cancer cells. The idea that mutations in a few specific genes are necessary and sufficient to cause the disease in any of the most common human cancer forms was opposed by observation that random mutations accumulate much faster inside genetically unstable malignant cells and that genome instability might be a critical early event that leads to the mutation of oncogenes and suppressor genes. Furthermore, in contrast to gene mutation hypotheses neoplastic transformation of normal epithelial cells is a slow process, which explains the fact that majority of cancers appear at an advanced age. All these facts make relevant molecular cancer diagnosis and treatment extremely complex and difficult to fulfil. Therefore, in the future we suggest performing combined analyses of gene expression profiles, genetic polymorphisms in mitotic genes, and functional analyses of these polymorphisms. Studies should be expanded on candidate genes by employing genome-wide association studies in order to identify novel genetic variants associated with gastric cancer.

4. Conclusion

It is apparent that majority of gastric cancers are characterized by genetic instability, either MSI or CIN. Whereas MSI is characterized by changes in short repeat sequences, the hallmark of CIN are gross chromosomal rearrangements, such as the gain or loss of whole chromosomes (Martin et al., 2010). Accumulating evidence shows that CIN and aneuploidy are the most common characteristics of sporadic gastric adenocarcinomas, accounting for more than 60% of cases, whereas MSI is characteristic for hereditary type of gastric cancer, developed in the context of Lynch syndrome, and a smaller subset of sporadic cancers, ranging from 15% to 35% (Panani, 2008). The newly formed chromosomal/aneuploidy hypothesis (aneuploidy could be the consequence of carcinogens or genetic changes in certain mitotic genes) could answer several questions remaining from the currently established classic oncogene overexpression model, mutator and suppressor theories, which postulate that cancer is caused by clonal expansion of one single cell, which has accumulated 4-7 mutations during the lifetime of a patient. (Castro et al., 2007; Duesberg et al., 2005; Duesberg et al., 2000). However, these theories do not explain the long latent periods in cancer development and more importantly, despite more than two decades of effort, they have failed to identify a particular sets of gene mutations that occur in every instance of gastric tumour development.

It is evident that gastric cancer is the consequence of a multistep process involving different genetic and epigenetic changes in numerous genes. Host genetic background and environmental factors also play an important role in the pathogenesis of the disease. The majority of genetic alterations contributing to the malignant transformation were observed in growth regulatory genes, and in genes involved in cell cycle progression and arrest.

However, exact genetic steps involved in the stomach carcinogenesis still remain uncertain. Different histological forms, as well as different aetiologies point to different genetic pathways for intestinal and diffuse tumours. To date, no single genomic abnormality is known to be specific to sporadic gastric cancer, or to any of its histological subtypes. Some of the genetic changes occur commonly in both major types, intestinal and diffuse, but some differ depending on the histological type. Even more, recent studies supported the idea that there are subgroups where MSI, CIN, suppressor, and methylator pathways overlap during the development of malignant phenotype. In conclusion, further research is required, with emphasis on collecting as many genetic changes as possible, which could aid in deciphering the molecular mechanisms of gastric cancer and in the development of suitable methods for screening, risk assessment and prognostic evaluation.

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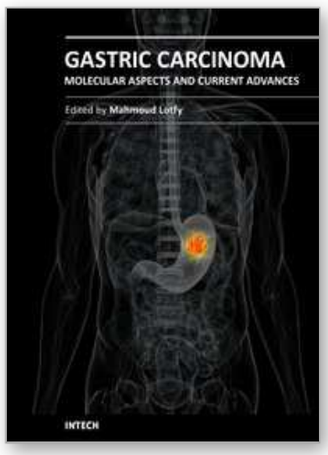

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Gastric cancer is one of the most common tumors worldwide. It has a heterogeneous milieu, where the genetic background, tumor immunology, oxidative stress, and microbial infections are key players in the multiple stages of tumorigenesis. These diverse factors are linked to the prognosis of the gastric cancer and the survival of gastric cancer patients. This book is appropriate for scientists and students in the field of oncology, gastroenterology, molecular biology, immunology, cell biology, biology, biochemistry, and pathology. This authoritative text carefully explains the fundamentals, providing a general overview of the principles followed by more detailed explanations of these recent topics efficiently. The topics presented herein contain the most recent knowledge in gastric cancer concerning the oncogenic signaling, genetic instability, the epigenetic aspect, molecular features and their clinical implications, miRNAs, integrin and E-cadherin, carbohydrate-associated-transferases, free radicals, immune cell responses, mucins, Helicobacter-pylori, neoadjuvant and adjuvant therapy, prophylactic strategy for peritoneal recurrence, and hepatic metastasis.

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