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Ph.D in Medical Genetics

Expression analysis of key trigger genes in gastric cancer: from etiology and subtyping to early prognostic assessment

Dr. Paraskevi Vogiatzi

Supervisor: Prof. Antonio Giordano

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Ph.D. dissertation board

Prof. Gianpaolo Papaccio

Department of Experimental Medicine, Section of Histology and Embryology, Second University of Naples, Naples, Italy

Prof. Sergio Ferrari

Department of Biomedical Sciences, Section of Biochemistry, University of Modena and Reggio Emilia, Modena, Italy

Prof. Salvatore Oliviero

Department of Molecular Biology, University of Siena, Siena, Italy

Prof. Mario De Marchi

Department of Clinical and Biological Sciences, Division of Medical Genetics, University of Turin, Orbassano, Italy

Prof. Antonio Giordano

Department of Human Pathology and Oncology, University of Siena, Siena, Italy
Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, PA, USA

Prof. Alessandra Renieri

Department of Molecular Biology, Medical Genetics, University of Siena, Italy

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1. Introduction

1. Introduction

Gastric cancer (GC) is a common malignancy and a leading cause of cancer-related death in the world. Development of gastric cancer is a multifactorial phenomenon involving many etiologic factors, both endogenous and exogenous. It is characterized by gene-environment interactions, and is correlated to peculiar pathological, occupational and social conditions and, as indicated in recent studies, with epigenetic events. Over the past 2 decades, many exciting discoveries regarding the genomics of gastric cancer have been made, but this health problem was not radically resolved. This fact emphasizes the importance of identification of diagnostic and prognostic markers useful in the earliest stage of the disease. The study of expression the many genes involved in gastric cancer may foster early diagnosis and individual therapeutic strategies.

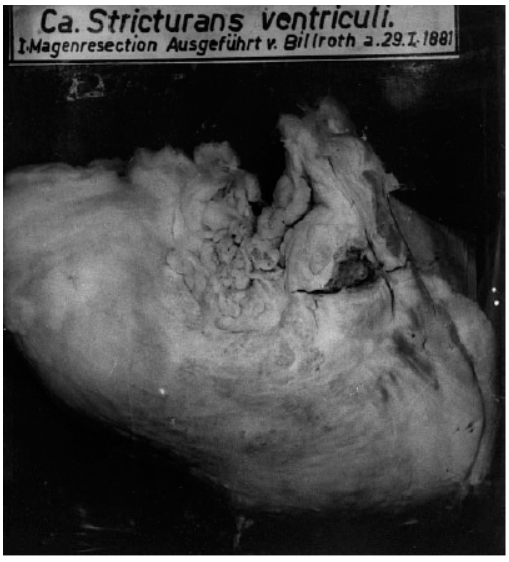

Figure 1.
Specimen of the tumor of Therese Heller, in whom the first gastrectomy was performed by Billroth on January 29, 1881 (reproduced with the kind permission of Proff. Manfred Scopec and Walter Mauritz, Museum of the Institute for History of Medicine, Vienna).

2. Pathology and molecular biology of gastric cancer (GC)

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2.1. Epidemiology

2.1.1. Incidence and geographic variability

Gastric adenocarcinoma is one of the most common cancers in Asia. Although its incidence in other regions is lower, it is still a major health problem worldwide. From 1998 to 2001, gastric cancer was the second most frequent cause of cancer death, being second only to lung cancer, and the fourth most common cancer in the world; most cases were in developing countries. In Japan, the age-standardized incidence of gastric cancer (per 100,000 people) ranged from 60 to 92 in men and from 24 to 39 in women; these rates were comparable to those in Korea. Among the white population in the U.S.A., the incidence was one-tenth of that observed in Japan: 6.6 in men and 2.6 in women. Marked geographic variability is also observed in Europe. The annual age-standardized incidence rate (per 100,000 people) is higher in Eastern (34.1 in men) and Southern Europe (19.5 in men) than in Northern (6.1 in women) and Western Europe (7 in women) (1-5).

The incidence of stomach cancer has substantially declined over the last few decades especially in the developed countries. The cancer death rates for gastric cancer in US population (both sexes from 1930 to 2002) are compared with those due to other cancers in figures 2 and 3, in which a remarkable decline for stomach cancer is evident. The causes of this decline may include improvements in diet, food storage and lower rates of *Helicobacter pylori* infection, which means better overall sanitary conditions and methods of intervention such as eradication or immunization (5, 6).

But the problem is still important. In a very recent report, in the United States alone among 22,280 patients diagnosed with gastric cancer in 2006, 11,430 are expected to die (7). This tumor also remains a serious problem in the elderly from low socioeconomic

classes. The geographical areas with more cancer deaths (per 100,000) in 2002 were Japan, China, Latin America (Chile, Colombia), parts of Eastern Europe (Russian Federation, Azerbaijan, Estonia, Lithuania, Croatia, Hungary, Romania), and Portugal (7). Gastric cancer incidence in Italy was found to be medium (7), with particularly "hit" regions, such as Emilia-Romagna, Tuscany, Umbria and The Marches (8, 9). The peak incidence is estimated to occur at 50-70 years, as this tumor is rare before 30 years of age (10, 11). Males are affected more often than females (2, 3) and African Americans, Hispanics and Native Americans more than Caucasians (7, 12).

Although this disease is better understood now, low survival rates persist due to the lack of suitable and specific biomarkers for early detection, with most cases being diagnosed in the late stages. Continued intensive studies are necessary to improve diagnostic and treatment plans.

2.1.2. Overall survival

The 5-year relative survival rate for all cases in US from 1995 to 2001 is only 23% (7). In Europe, the relative survival from stomach cancer in 1990-1994 was poor in both sexes: 42% at 1 year and 23% at 5 years (13). The younger patients (under 45 years) had the higher 5-year survival (35%), while in patients over 74 years it was only 17%. As logically expected, in Finnish Cancer Registry with survival data up to the year 1995, the 5-year survival is closely dependent on the stage of the tumor, which means only 3% survival for lesions with distant metastasis and 61% for still localized malignancies (14, 15). Finally, as relatively good news, 5-year survival for stomach cancer in Europe slightly increased from 18% in 1983-1985 to 21% in 1991-1994 (16). Anyway, as the corresponding survival rate in Japan is reported to be approximately 60% (17), the crucial question is whether and how survival in countries other than Japan could be improved.

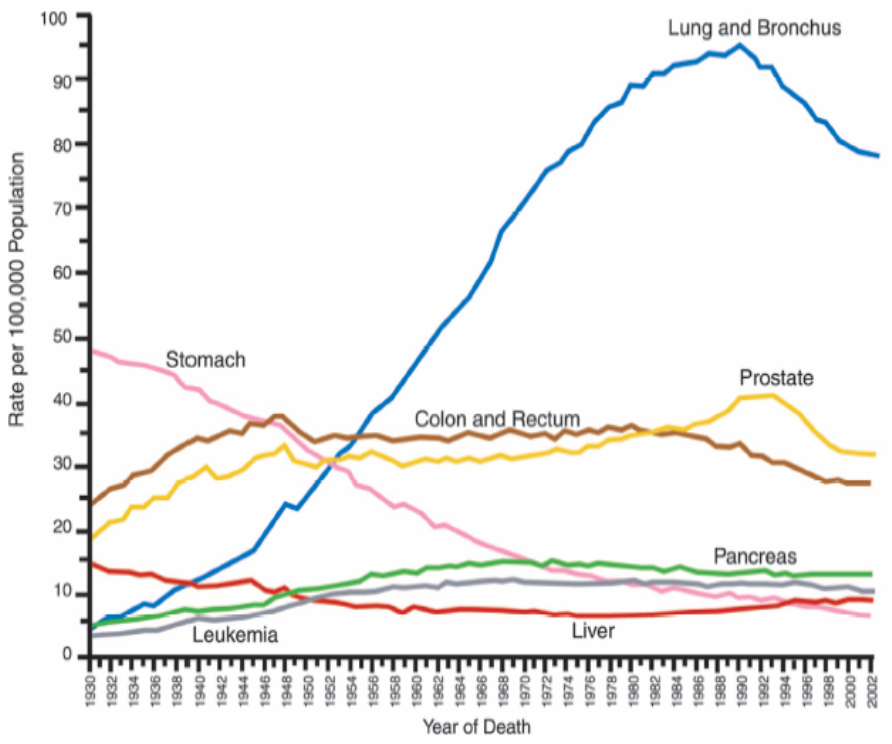

Figure 2.
Annual age-adjusted cancer death rates among males for selected cancers, among these stomach cancer, in US population from 1930 to 2002.
Source: Jemal *et al.*, 2006 (ref. 7).

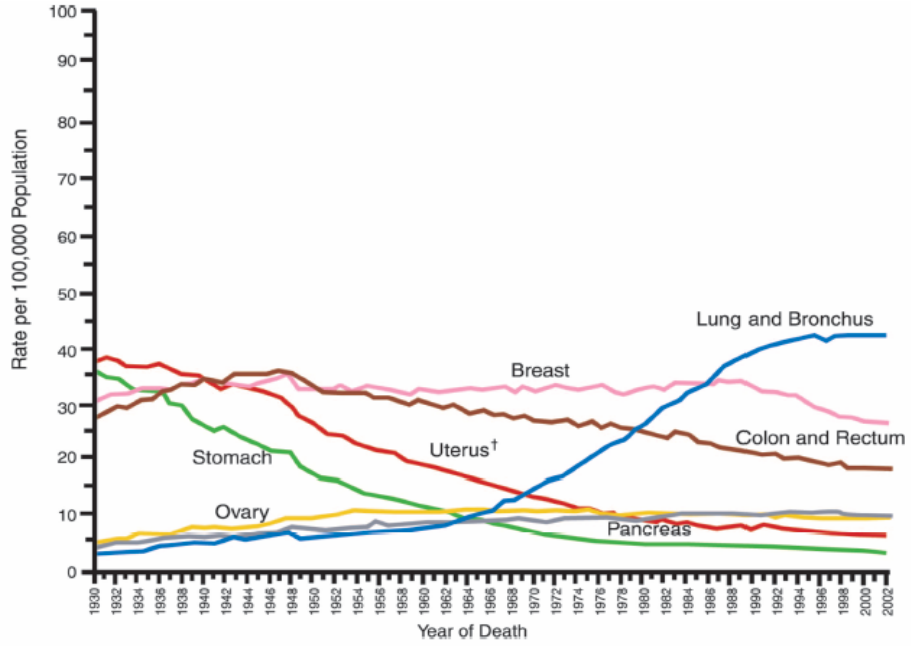

Figure 3.
Annual age-adjusted cancer death rates among females for selected cancers, among these stomach cancer, in US population from 1930 to 2002.
Source: Jemal *et al.*, 2006 (ref. 7).

2.2. Etiology of gastric cancer

Due to demographic variability and recent changes in disease incidence, much emphasis has been placed on studying etiologic and risk factors in gastric cancer. Environmental factors, occupational factors, associated pathological conditions, genetic and/or epigenetic factors all play role in the development of this disease. Current view of etiologic and risk factors are summarized in table 1.

Table 1. Etiologic and Risk Factors of Gastric Cancer

Environmental Factors	Exposure to aluminum, arsenic, chromium, chlorophenols, lead, zinc, cadmium, nichel, talcum powder, some polyaromatic hydrocarbons, azo dye compounds (ref. 18) Cigarette smoking (refs. 19-23) Exposure to radiation (uranium, radon, other natural radionuclides) (ref. 24) Diet rich in salted, smoked, grilled, and fried food (refs. 6, 25) Frequent consumption of red meat (ref. 26) Low vegetable intake (ref. 25) Nitrate compounds (ref. 27) Alcohol abuse (possibly) (refs. 19, 28, 29)
Occupational and Social Factors	*High risk*: carpenters, steelworkers, tin miners (ref. 18) *Increased risk*: chemical industry workers, coal miners, coke plant workers, oil refinery workers, rubber manufacturing (ref. 18) *Possible risk*: agricultural workers, gold miners, lorry and coach drivers, jewelery workers, metal (and components) manufacturers (ref. 18) Living in developing countries (ref. 3) Lower socioeconomic groups for distal GC (ref. 30) Higher socioeconomic groups for proximal GC (ref. 30)
Associated Pathological Conditions	Pernicious anemia (ref. 31-33) Chronic atrophic gastritis (ref. 31) Intestinal metaplasia (ref. 31) Partial gastrectomy (ref. 31) Low level of gastric acid (ref. 31) Infection by *Helicobacter pylori* (refs. 5, 6, 34-40) Infection by *Epstein-Barr Virus* (refs. 41-45)
Genetic Factors	ABO blood groups (A and in some cases B) (refs. 32, 33, 46, 47) Familiar cases and Hereditary syndromes (refs. 48-50 and OMIM databases) "Racial" or "ethnic" factors (refs. 51, 52) Microsatellite and chromosomal instability (refs. 53-59) Oncogenes (refs. 60-69) Tumor suppressors genes (refs. 69-85) Cell-adhesion and metastasis-related molecules (refs. 86-99) Cell-cycle regulators (refs. 100-102) Growth factors and cytokines (refs. 61, 103-106)
Epigenetic Factors	Aberrant promoter methylation of genes (refs. 60, 61, 107-116) Histone hypoacetylation (H3, H4) (refs. 61, 117, 118) DNA hypomethylation (ref. 119)

2.2.1. Environmental factors

Dietary intake data support the role of certain foods as risk factors for the development of gastric cancer. Lack of, or infrequent consumption of vegetables and fruits, is a risk factor for gastric cancer, with the prevalence ranging from 15% to 40% (25). Fried, very salted, cured and smoked foods are diet components contributing to the development of gastric carcinomas. Carcinogens in cooking fumes, possibly heterocyclic amines (HCA) formed during high temperature cooking certain foods such as red meat and meat sauce may play role in the development of gastric cancer (26). Several N-nitroso compounds, present in foods and beverages or formed in the stomach from their precursors, act as alkylating agents. Mean dietary nitrate intake was significantly higher in gastric cancer patients supporting that N-nitroso compounds from dietary sources may play a role in the etiology of gastric cancer (27).

Cigarette smoking and alcohol may promote gastric carcinogenesis, and preventive measures addressing these factors could considerably reduce the incidence of gastric cancer. In a recent population-based, prospective cohort study in Norway, no statistically significant associations between various degrees of exposure to alcohol and risk of gastric cancer was revealed, but combined high use of cigarettes and alcohol increased the risk of noncardial gastric cancer nearly 5-fold, compared to nonusers (19). It has been shown that smoking elevates the levels of pepsinogen I and pepsinogen I/II ratio; therefore, they have been used as markers for gastric cancer (20). More recently, it has been suggested that polymorphisms, such as the cytochrome P-450 2E1 (CYP2E1) and N-acetyltransferase 1 (NAT1) polymorphism, and smoking may alter the susceptibility to cancer development in the stomach (21). Prospective studies on cigarette smoking and stomach cancer have suggested that gastric cancer is a tobacco-related disease (22, 23). Some authors have described a possible effect of ethanol in promoting gastric cancer at the distal segment in

patients abusing alcohol (28). Other researchers have suggested that concomitant alcohol abuse and the hOGG1 Ser (326) Cys polymorphism may alter the susceptibility of Chinese individuals to develop gastric cancer; Cys/Cys carriers seem to be particularly predisposed (29).

2.2.2. Associated pathological conditions

Particular medical conditions, such as infection by *Helicobacter pylori* (Hp) and/or Epstein-Barr Virus (EBV), adenomatous gastric polyps, pernicious anemia (31-33), intestinal metaplasia (31), partial gastrectomy (31), which decreases gastric acid (e.g., low acid production in Menetrie's disease) or achlorhydria (31) and chronic atrophic gastritis, could promote stomach cancer. In particular, epidemiological and biological evidence indicates that atrophic gastritis represents an important risk factor for gastric adenocarcinoma of the intestinal-type (31).

Helicobacter pylori

Helicobacter pylori (Hp) is a spirally-shaped, microaerophilic, Gram-negative bacterium with urease, catalase and oxydase activities and with a tuft of sheated unipolar flagella (34-36). These features, together with an unusual resistance to acidic pH conditions, allow the bacterium to survive in the stomach lumen. The gastric antrum is its most favorite site, but other parts of the stomach may be colonized.

H. pylori transmission is primarily "person-to-person" via oral-oral, or possibly fecal-oral routes. Infection rates are strongly related to poor sanitary conditions, low socioeconomical level and overcrowding during childhood. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages, while in industrialized countries its prevalence remains under 40% of the population (34).

H. pylori is the first formally recognized bacterium as a category 1 carcinogen by the World Health Organization International Agency for Research on Cancer (37) and is

also one of the most successful human pathogens, as over half of the world's population is colonized with this bacterium (34). It represents the main cause of gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (34-36) (figure 4). The strong association of the MALT lymphoma with *H. pylori* infection is confirmed by observations of complete regression after elimination of the bacteria. Expression of Hp genes, such as *cag*A and probably *vac*A, increase the risk for intestinal gastric cancer (38). It was reported that the absence of the HLA DQA1*0102 allele may confer susceptibility to intestinal gastric cancer in Hp-seropositive patients. The presence of the *1601 allele in Hp-negative patients, on the other way can also significantly increase the risk for diffuse-type gastric cancer (39). Polymorphisms in the human interleukin 1 beta (IL-1a) gene and IL-1 receptor antagonist gene may be an important tool in defining which *H. pylori*-infected individuals are at increased risk for developing gastric cancer (40).

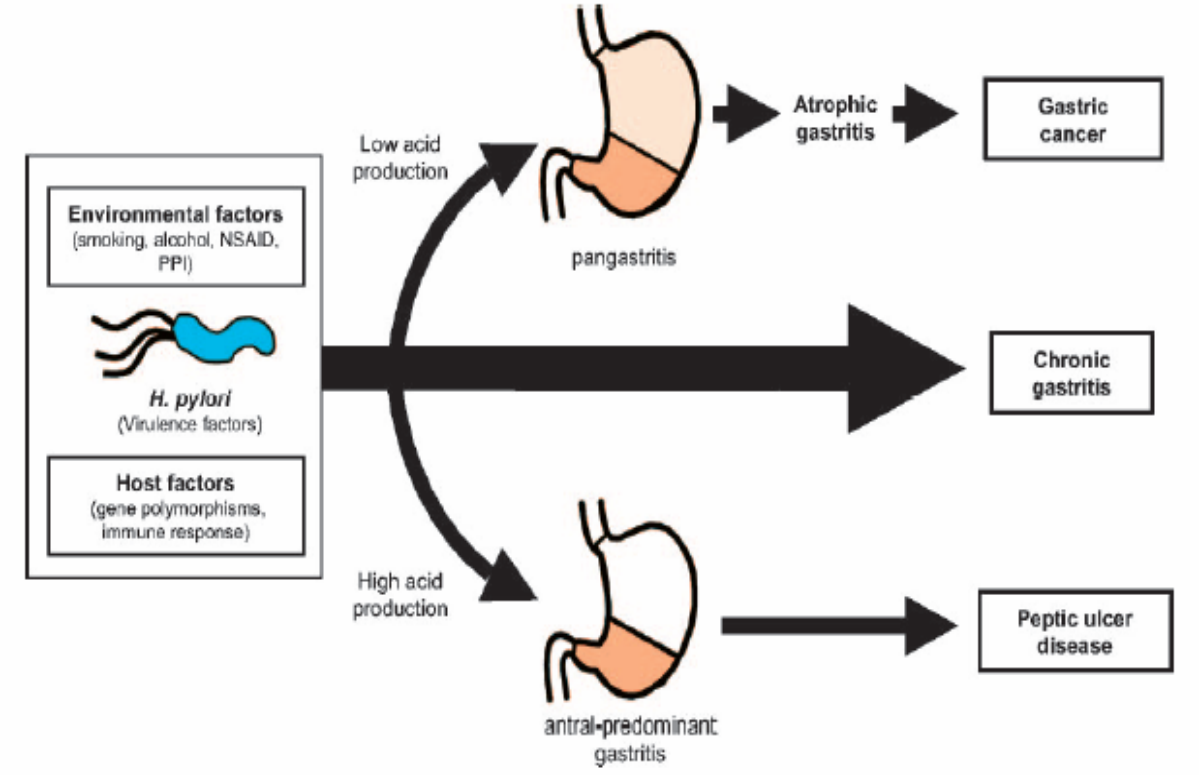

Figure 4. Schematic representation of the factors contributing to gastric pathology and disease outcome in *H. pylori* infection. *Source*: Kusters *et al.*, 2006 (review) (ref. 34).

Epstein–Barr Virus

Epstein-Barr Virus (EBV) was first described in 1964, is a gamma-herpes-virus, genus lymphocryptovirus, transmitted by saliva or, rarely, by transfusion of fresh blood to seronegative recipients. The majority of the population is infected early in life and even in developed countries the incidence is high (41). The presence of EBV has been demonstrated in a subset of stomach cancer cells, namely lymphoepithelioma-like carcinomas and its presence is associated with a late event in gastric carcinogenesis (42, 43). Promoter hypermethylation of *E-cadherin* gene and its abnormal expression has been found in Epstein-Barr virus-associated gastric carcinoma. The frequency of this aberrant methylation is significantly higher than that in EBV-negative gastric carcinoma (44). In a recent report, the authors investigated the methylation profile and clinicopathologic features including overall survival in four subgroups defined by EBV infection and CpG island methylator phenotype status (CIMP) status. EBV-associated gastric carcinoma showed global CpG island methylation, comprising a pathogenetically distinct subgroup in high (CIMP-H) gastric carcinoma (45).

Pernicious anemia

Megaloblastic or pernicious anemia is a rare disorder in which the body does not absorb enough vitamin B12 from the digestive tract, resulting in an inadequate amount of red blood cells produced. The association of gastric cancer with blood group A and pernicious anemia has been known for a long time (31-33).

The distribution of ABO blood groups varies in different geographical areas and ethnic groups (46, 47). In a study in China blood group B was found more frequently associated with cardia gastric cancer in males and carcinoma in the upper third of the esophagus (47).

2.2.3. Relevance of genetic events in gastric oncogenesis

Genetic predisposition to gastric cancer has been suggested. The first striking example of dominantly familial predisposition to gastric cancer has been described for Napoleon Bonaparte's family (OMIM *192090). Napoleon, his father, Charles Bonaparte, his grandfather, Joseph Bonaparte, his brother and his three sisters, all died of stomach cancer, most of them at an early age (48-50) (figure 5). At present, in OMIM database, 90% of gastric cancers are considered sporadic, 10% hereditary, which only 2% of total gastric cancers present an autosomal dominant pattern of inheritance (OMIM # 137215).

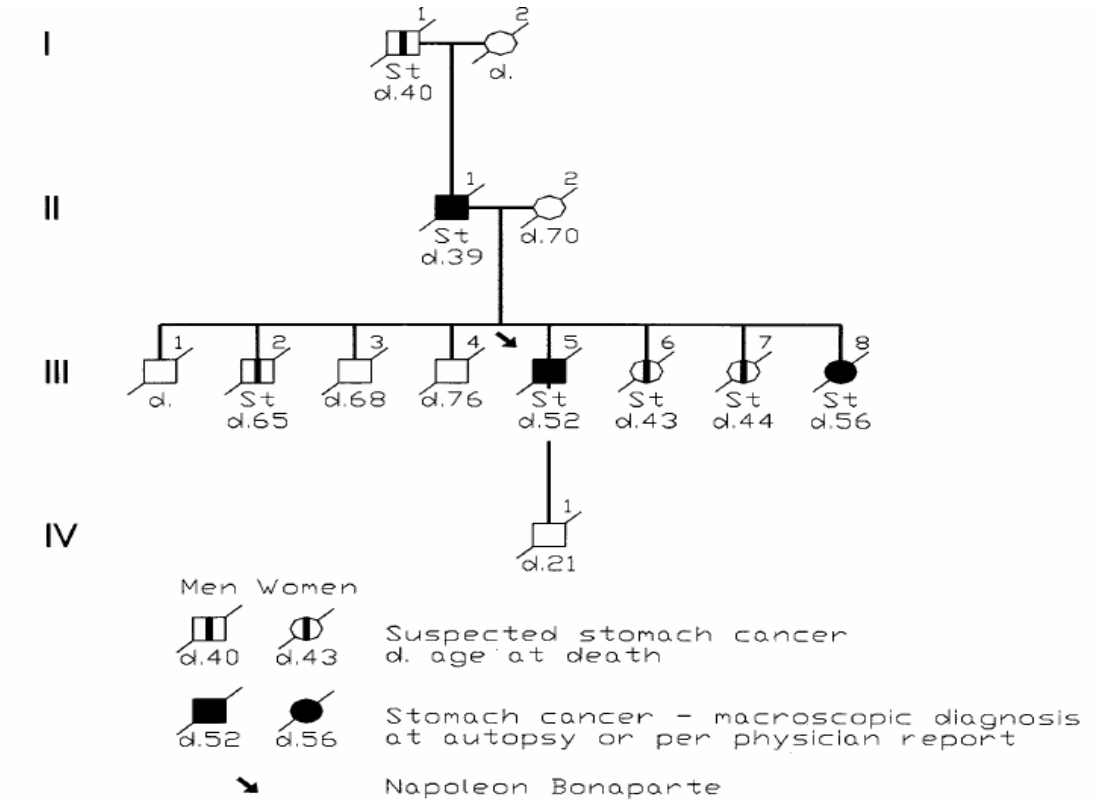

Figure 5. Pedigree of Napoleon Bonaparte's family showing susceptibility to gastric cancer, adapted from Sokoloff, 1938 (ref. 48). *Source*: Lynch *et al.*, 2005 (ref. 50).

Gastric cancer is a known manifestation of inherited cancer predisposition syndromes, including hereditary nonpolyposis colon cancer (HNPCC1; OMIM #120435), Li-Fraumeni syndrome (LFS1; OMIM #151623), familial adenomatous polyposis (FAP;

OMIM +175100), Peutz-Jeghers syndrome (PJS; OMIM #175200) and Cowden disease (CD; OMIM #158350), suggesting the presence of predisposing genes with pleiotropic effects.

Genetic predisposition to gastric cancer has also been correlated with ethnic differences. New Zealand Maori families, Hawaiians and African Americans have shown a high frequency of gastric cancer (51). The genetic differentiation between the races is basely associated with Alu polymorphisms and Short Tandem-Repeat (STR) sequences; most of the variations were already present in our shared African ancestors, not more than ~100,000 years ago (52).

The catalogue of gene alterations in gastric cancer is growing rapidly, adding further complexity in this disease. Multifactorial models fit significantly better than single major gene models in the genesis of gastric cancer.

Multiple genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules and growth factor/receptor systems are involved over the course of the multi-step conversion of normal epithelial cells to gastric cancer (60, 61). Identification of specific genetic pathways in gastric cancer may have an impact on prognosis and selection of treatment strategies.

Two genetic pathways are present in gastric carcinoma. The first is *microsatellite instability,* which targets mononucleotide tracts with coding regions of cancer-related genes and is more associated with the intestinal-type. The second pathway is *chromosomal deletion* involving tumor suppressor genes and correlated with the growth pattern of diffuse-type carcinoma.

Microsatellite and chromosomal instability

Widespread tumor-associated microsatellite instability (MSI) is believed to be caused by altered repair of spontaneous DNA replication errors after mutational inactivation or epigenetic silencing of at least one of various mismatch repair genes (MMRs), including *hMLH1*, *hPMS1*, *hPMS2*, *hMSH2*, and *hMSH6/GTBP*. The term "microsatellite" refers to short repetitive nucleotide sequences whose detection is proof of an enhanced mutation rate. Yeast and mammalian cell studies suggest that MMR genes act not only on base/base mismatches or small insertion/deletion loops, which escape proofreading by the replicating DNA polymerase, but also on chemically altered nucleotide base pairs. MMR gene modifications underlie the development of several types of cancer, including gastric cancer.

Tumors with a MSI (+) phenotype have diploid DNA and follow a distinctive pathway of molecular progression, including frame-shift mutations at mononucleotide runs within key cancer-related genes. Ottini *et al.* have demonstrated that high frequency of MSI in gastric cancer is associated with female sex, antral location, intestinal-type histology, advanced tumor stage, vascular invasion, and positive family history (53). Leung *et al.* suggested that high-frequency MSI in sporadic gastric cancer is mostly due to epigenetic inactivation of *hMLH1* gene, and the loss of HMLH1 protein is a significant event in the development of invasive tumor (54). Frameshift mutations of human gastrin receptor gene (hGARE) are observed in gastrointestinal tumors with MSI, although the exact role of *hGARE* mutations in tumorigenesis remains to be elucidated (55). Mutations in *TGF-beta RII*, *IGFII R* and *BAX* genes in sporadic gastric tumors with MSI show a decreased tendency for nodal metastasis and wall invasiveness (56).

In a high proportion of gastric cancer cases is observed loss of heterozygosity (LOH) at chromosomes 1p, 5q, 7q, 11p, 13q, 17p, and 18p, which are possible sites of tumor suppressor genes (57, 58). Usually loss of heterozygosity is required to inactivate a mismatch repair gene (MMR) in stomach tumor.

Semba *et al.*, 1998 suggested that in young patients with diffuse gastric cancer, the presence of high MSI, which might be due to defect of DNA repair system rather than *hMLH1* and *hMSH2*, was frequently associated with LOH on chromosome 17q21 including the *BRCA1* gene (59).

Oncogenes

Many proto-oncogenes are activated in gastric malignancy. The *c-met* gene, a transmembrane tyrosine kinase receptor of hepatocyte growth factor (HGF), is found amplified in 19% of intestinal-type and 39% of diffuse-type gastric cancers (62). The majority of gastric carcinomas express two forms of the transcript, sized 7.0 kb and 6.0 kb. The 6.0-kb transcript of the *c-met* gene was expressed at considerable levels in 52% of the gastric carcinoma tissues and was closely correlated with tumor staging, lymph-node metastasis and depth of tumor invasion (63).

Amplification of *K-sam* gene is restricted to poorly differentiated types of gastric cancer (61, 64). *K-sam* was first gene found amplified in the gastric cancer cell line KATO-III. It encodes a receptor tyrosine kinase that belongs to the heparin-binding growth factor receptor, or fibroblast growth factor receptor, gene family. *K-sam* has at least four transrciptional variants (65). One of these, type II, encodes a receptor for keratinocyte growth factor. Amplification of *K-sam* was found preferentially in advanced diffuse or scirrhous-type gastric cancers (33% of all) but not in intestinal-type carcinomas (64). Over-expression of this oncogene is associated with worse prognosis in gastric malignancy.

The *c-erbB-2* gene is another potential cell surface receptor of the tyrosine kinase gene family. Indeed, the *c-erbB-2* gene is a v-erbB-related proto-oncogene which encodes a protein similar to but distinct from the epidermal growth factor (EGF) receptor. It is commonly amplified in the intestinal-type of gastric adenocarcinoma (66). c-erbB-2

protein expression is enhanced in advanced stages during the progression of gastric carcinoma and is an indicator of poor short-term prognosis (67).

Mutations of *K-ras* oncogene can be found in intestinal-type cancer and the precursor lesions, intestinal metaplasia and adenoma. However, *K-ras* point mutations are uncommon in stomach cancer and are not present in diffuse gastric tumor histology (68, 69).

Tumor suppressor genes

The *p53* gene, probably the most famous tumor suppressor gene, couldn't be absent from the list of genes involved in gastric carcinogenesis. It is frequently inactivated in gastric carcinomas by loss of heterozygosity (LOH), missense mutations or frameshift deletions. Taken together, these genetic alterations are present in more than 60% of gastric carcinomas and are also found in intestinal metaplasia, dysplasia and adenomas (69-71). The *p53* gene (locus 17p13.1) frequently shows GC-AT transitions in diffuse-type gastric cancer, due to carcinogenic N-nitrosamines produced from dietary amines and nitrates in the acid gastric environment (72-74).

LOH and abnormal expression of the *p73* gene, another *p53* family member mapping at 1p36, a minimal region frequently mutated in gastric cancer, preferentially occur in the *de novo* pathway for well-differentiated adenocarcinomas of foveolar type expressing pS2 (TFF1), a gastric-specific trefoil factor (61, 75). The pS2 protein is normally expressed in gastric foveolar epithelial cells. Inactivation of the *pS2* gene is observed in dysplasia, adenoma and adenocarcinoma in mice (76), suggesting its role at early steps of gastric carcinogenesis (61).

Germline mutations in the adenomatous polyposis coli (APC) gene cause familial adenomatous polyposis (FAP), which is an autosomal-dominant colorectal cancer syndrome (77). Loss of heterozygosity of the two closely spaced *APC/MCC* genes, which are involved in colon tumorigenesis, has been also shown to be associated with the

development of gastric carcinomas (78). Inactivation of *APC* (locus 5q21-q22) as well as of *DCC* (locus 18q21.3) and *Rb1/p105* (locus 13q14.1-q14.2), found in gastric cancers, seems to be involved in the development and progression of some human gastric cancers, regardless of histologic type (78, 79). Notably, *APC* gene missense mutations are present in more than 50% of the intestinal-type gastric cancer, while they are not involved in diffuse-type cancers. Somatic mutations of the APC gene are observed in precursor lesions of the stomach, such as in 20-40% of gastric adenomas and in 6% of intestinal metaplasias, demonstrating its role in early steps of gastric carcinogenesis (80, 81).

Loss of heterozygosity on chromosome 10q23.31 of tumor suppressor gene *PTEN* appears in precancerous lesions. *PTEN* mutations are restricted to advanced gastric cancer. In fact, LOH and mutation of *PTEN* are closely related to infiltrating and metastatic gastric cancers (82). In a more recent paper based on immunohistochemical analysis in a large number of patients, it is shown that SURVIVIN (BIRC5), an inhibitor of apoptosis, is positively correlated with PTEN expression in gastric cancer and is a molecular marker of lymph node metastasis, while PTEN expression is reconfirmed as a molecular marker of advanced gastric cancer (83).

RUNX3 gene is a relatively recently discovered tumor suppressor, also involved in the complex process of gastric oncogenesis. Loss of *RUNX3* by hypermethylation of its promoter results in many tumors, including gastric malignancy. *RUNX3* methylation is observed in chronic gastritis, intestinal metaplasia and gastric adenomas, suggesting this gene as a target for epigenetic gene silencing in stomach cancer (84). Its role will be further discussed in separate chapter of the thesis.

Nuclear retinoic acid receptor β, *RARβ* is another tumor suppressor gene found hypermethylated in 64% of the intestinal-type gastric cancers, while alterations of this gene are not observed in the diffuse-type (85).

Cell-adhesion and metastasis-related molecules

Mutations in genes encoding for cell-adhesion molecules have been described in gastric cancer as well. Inactivation or down-regulation of E-cadherin protein, which belongs to the functionally related trans-membrane glycoprotein family, is found in gastric cancer and contributes to an increase in cell motility, the first step of cancer invasion and metastasis. This protein, a product of the *CDH1* gene (locus 16q22.1), is responsible for the Ca (2+)-dependent cell-cell adhesion mechanism; therefore, its inactivation has been suggested to play an important role in the growth and invasion either in hereditary gastric carcinoma (HGC) or in hereditary diffuse gastric cancer (HDGC) (86, 87). It has been shown that intragenic deletion or somatic mutations of the *CDH1* gene and promoter methylation synergistically induce CDH1 down-regulation in hereditary diffuse gastric cancer (HDGC) patients (88). In recent screenings, *CDH1* somatic mutations found in sporadic diffuse and in a diffuse component of mixed gastric cancer were especially in-frame deletions and missense, while the major germline mutations of *CDH1* gene found in familiar gastric cancer were missense (30% of all germline mutations reported to date), frameshift, and nonsense (86, 87). In particular the mutations of *CDH1* in exons 8 or 9 induced the scattered morphology, decreased cellular adhesion and increased cellular motility of diffuse-type gastric cancers (89). More papers also demonstrated that *E-cadherin* mutations together with those of β-*catenin* and γ-*catenin* are involved in the development and progression of diffuse and schirrhous-type cancers (90-92).

Rare genetic alterations of IQ Motif-Containing GTPase-Activating Protein 1 gene (IQGAP1), also called *p195* (locus 15q26), a negative regulator of cell-cell adhesion at adherens junctions, have been found especially in diffuse gastric cancers (93). Previous study demonstrated that mutant mice exhibited a significant increase in late-onset gastric hyperplasia relative to wild-type animals of the same genetic background (94).

Abnormal CD44 transcripts containing the intron 9 sequence are found in both types of gastric cancers and metastasis (95). This event is also found in 60% of intestinal metaplasias, and does not take place in normal gastric mucosa (96). Osteopontin (OPN), a protein ligand of CD44, is up-regulated in gastric cancers and together with abnormal CD44 result in lymphatic invasion and metastasis (97, 98). Galectin-3, a galactoside-binding protein is another molecule implicated in gastric tumor metastasis (99).

Cell-cycle regulators

Cyclin E overexpression is a common event in gastric cancer and is associated with increased aggressivity in the presence of aberrant p53. The combination of cyclin E overexpression with aberrant p53 expression in gastric cancer further distinguished a subgroup of patients with poor prognosis (100).

Moreover, reduced p27 expression is a negative prognostic factor for patients with cyclin E positive gastric tumors (101).

The E2F family of transcription factors plays a key role in the control of cell-cycle progression. Some family members may act as oncogenes, others as tumor-suppressor genes. Suzuki et al. reported increased expression of E2F-1 mRNA in 40% of the gastric carcinomas (102).

Growth factors and cytokines

A broad range of growth factors and cytokines are produced in the gastric tumor environment by different cells accounting for complex cell interactions and for regulation of differentiation, activation, and survival of multiple cell types. Besides the role of intratumoral cytokine network, we will discuss the role of growth factors and their effects in diverse histotypes. EGF, TGFα members of the EGF family are overexpressed in the intestinal-type of gastric carcinomas (61). TGFβ growth factor is more prevalent in diffuse-

type carcinomas with diffusely productive fibrosis (61, 103). IGF II and bFGF growth factors are overexpressed in both histotypes of gastric cancer (61).

Gastric cancer cells express neuropilin-1 (NRP-1), which is a membrane-bound coreceptor for both VEGF-165 and VEGF receptor 2 (VEGFR-2) in endothelial cells. It is known that NRP1 plays versatile roles in angiogenesis, axon guidance, cell survival, migration, and invasion. In the case of human gastric cancer, regulation of NRP-1 expression is intimately associated with the EGF/EGF-R system. It was shown that activation of EGF-R may contribute to gastric cancer angiogenesis by a mechanism that involves upregulation of VEGF and NRP-1 expression via multiple signalling pathways (104).

Angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and inteleukin-8 (IL-8) promote neovascularisation of gastric cancer. Moreover, VEGF promotes in particular the malignant progression of the intestinal-type and amphiregulin (AR), another member of EGF family, is overexpressed in both types of gastric cancer (61).

IL-8 is a member of the CXC family of chemokines, which plays a pivotal role in gastric oncogenesis; more than 80% of stomach tumors express both this cytokine and its receptor (105, 106). Among its activities, IL-8 enhances expression of EGF receptor, type IV collagenase (metalloproteinase (MMP)-9), VEGF and IL-8 mRNA itself by gastric cancer cells, while also reducing CDH1 mRNA expression.

2.2.4. Epigenetic events in gastric cancer development and progression

The field of cancer epigenetics is evolving rapidly on several fronts. Advances in our understanding of chromatin structure, histone modification, transcriptional activity and DNA methylation have resulted in an increasingly integrated view of epigenetics. In response to these insights, our knowledge in gastric carcinogenesis will be fragmentary, unless we consider the link between genetics and epigenetics (figure 6). Some examples of epigenetic alterations in gastric malignancy will follow.

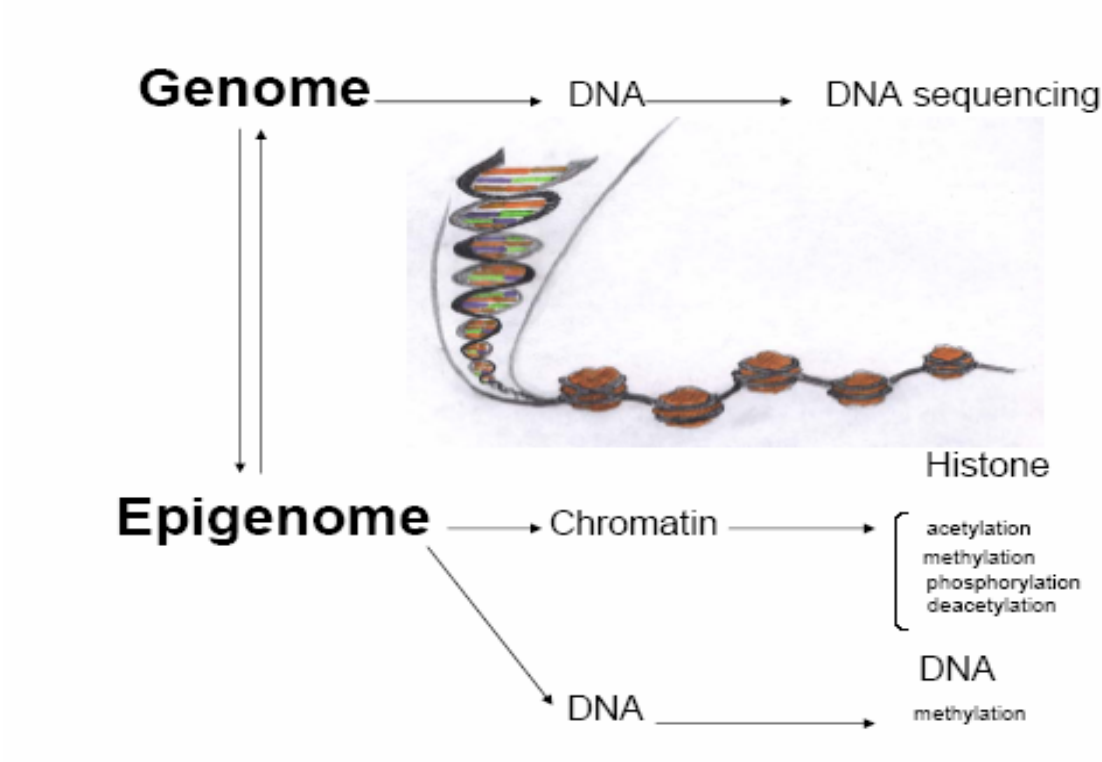

Figure 6. Current opinion on cancer mechanisms: the link between genome and epigenome. *Source*: Paraskevi Vogiatzi.

Transcriptional silencing of tumor suppressor genes by hypermethylation plays a crucial role in the progression of gastric cancer. It has been shown that there are at least two types of CpG islands in the intestinal-type and diffuse-type of gastric cancer with diverse methylation phenotypes; both are attractive for research (120).

In a study of methylation profiles of *p16*, *hMLH1* genes and four CpG islands (MINT1, MINT2, MINT25, and MINT31) in gastric carcinomas, the authors distinguished them in concordant methylation of multiple genes/loci (CIMP-high) (31% of tumors), CIMP-low (55% of tumors) and CIMP-negative (13% of all tumors). The CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) status in resected gastric cancers were compared with clinicopathologic features and overall survival (107). MSI status of the tumor was not a significant predictor of prognosis, while CpG island methylator phenotype (CIMP) status was a good but not independent prognostic factor of gastric cancer.

Diverse studies have been done to classify the methylation behavior of the genes in gastric carcinoma. In one study it has been suggested that genes could be divided in five different classes: 1) *GSTP1* and *RASSF1A* genes, which are methylated only in carcinoma, 2) *COX-2*, *hMLH1* and *p16* genes, presenting low methylation frequency in chronic gastritis, intestinal metaplasia and gastric adenoma but significantly higher methylation frequency in carcinoma, 3) *MGMT* gene, showing low and similar methylation frequency in all cancerous steps, 4) *APC* and *E-cadherin* genes, presenting high and similar methylation frequency in all cancerous lesions and 5) *DAPkinase*, *p14*, *THBS1* and *TIMP-3* genes, showing an increasing methylation frequency with the progression of the disease (108).

De novo methylations have been found in a large variety of neoplastic diseases and involve different pathways. In stomach cancer these are cell cycle regulation, apoptosis and cell signaling.

Among tumor suppressor cell cycle genes, hypermethylation of the $p16^{INK4A}$ (locus 9p21) and $p15^{INK4B}$ (locus 9p21) cell cycle inhibitors has been found in gastric cancer (109). In particular, aberrant methylation of $p16^{INK4A}$ promoter may predict the malignant potential of dysplasia, leading to early tumor identification (110, 111).

Other tumor suppressor genes related to an apoptotic response are also inactivated by *de novo* hypermethylation in gastric cancer. One of these is the tumor suppressor gene $p14^{ARF}$ (locus 9p21) which becomes unable to inhibit the MDM2 oncogenic protein, an inhibitor of p53 pro-apoptotic function (110).

Inactivation of APC/ E-cadherin pathways also seems to be influenced by epigenetic events in gastric cancer. Recently, it has been recognized that aberrant methylation of *APC* is common in many neoplasms of the aerodigestive tract and that *E-cadherin*, a member of the APC pathway, is also hypermethylated in gastric tumors (112). Another member of the cadherin family, *CDH4* (encoding for R-cadherin), containing CpG islands located at the 5' first exon, is methylated in 78% of colorectal and 95% of gastric carcinomas. *CDH4* methylation was also detected in histologically normal tissues located in proximity of the neoplasms, indicating that *CDH4* methylation is an early event in gastrointestinal tumor progression. It has been proven that *CDH4* methylation can be also revealed in the peripheral blood of cancer patients, thus suggesting that *CDH4* may act as a tumor suppressor gene in human gastrointestinal tumors and can potentially be used as an early diagnostic marker for gastrointestinal tumorigenesis (113).

Several studies have described the DNA methylation of many DNA mismatch repair genes such as *hMLH1*, whose promoter region was found to be methylated in 100% of MSI-H sporadic gastric cancers (114).

Transcriptional silencing of the nuclear retinoid acid receptor β (RARβ), a hormonal responsive gene, has been frequently associated with gastric carcinoma (115).

Another gene found to undergo aberrant methylation in gastric cancer is the RAS related gene, *RASSF1* (locus 3p21.3). At least two forms of RASSF1 are expressed in normal human cells; during carcinogenesis, however, the RASSF1A isoform is highly epigenetically inactivated in a variety of tumors such as lung, breast, ovarian, kidney, prostate, thyroid and gastric carcinomas. RASSF1A inactivation and K-ras activation are

mutually exclusive events in the development of certain carcinomas. This observation could further pinpoint the function of RASSF1A as a negative effector of K-ras in a pro-apoptotic signaling pathway. Re-expression of RASSF1A reduced the growth of human cancer cells, supporting a role for *RASSF1A* as a tumor suppressor gene. Loss or abnormal down-regulation of RASSF1A correlate with tumor stage and grade but not with histological types of gastric tumors. No somatic mutations have been detected in RASSF1 transcripts expressed in unmethylated tumors; therefore, *RASSF1A* methylation could serve as a useful marker for the early diagnosis and prognosis of cancer patients and could become an important target for the pharmacological therapy of cancer (116).

It has been reported that methylation of "Methylguanine-DNA Methyltransferase" (MGMT) gene (locus 10q26) is associated with advanced stages and poor prognosis in gastric carcinoma (110).

Histone acetylation appears to play an important role in transcriptional regulation. Inactivation of chromatin by histone deacetylation is involved in the transcriptional repression of several tumor suppressor genes, including $p21^{WAF1/CIP1}$. A study in gastric carcinoma investigating the status of histone acetylation found that histone H4 acetylation in both the promoter and coding regions of the $p21^{WAF1/CIP1}$ gene in cells expressing dominant-negative p53 was less than half compared to that observed in cells expressing wild-type p53, whereas histone H3 acetylation in both the promoter and coding regions was slightly reduced (by approximately 20%) in cells expressing the dominant-negative p53 (117).

"Pin2-interacting protein 1" gene (briefly *PINX1*) maps at 8p23 and is a potent telomerase inhibitor and a putative tumor suppressor gene (121). LOH of *PINX1* locus and hypoacetylation of histone H4 in the 5' UTR of *PINX1* are associated with reduced expression in this malignancy (118).

Although hypomethylation was the originally identified epigenetic change in cancer, it was overlooked for many years compared to hypermethylation. Recently, gene activation by cancer-linked hypomethylation has been rediscovered. Recent paper proved that demethylation of specific CpG sites within the first intron of R-RAS oncogene caused activation in more than half of gastric carcinomas. Silencing R-RAS-expressing cells resulted in the disappearance of the adhering cells, suggesting that functional blocking of the R-RAS-signaling pathway can be used in gastric cancer therapy (119).

2.3. Pathological classification

The stomach neoplasias are mainly composed of adenocarcinomas, but also mesenchymal tumors (i.e., stromal tumors, leiomyomas and leiomyosarcomas, and schwannomas), primary lymphomas, and carcinoid tumors can also be found; malignant tumors of these types occur much less often (122). Indeed, gastric adenocarcinomas account for more than 95% of gastric tumors, whereas gastrointestinal stromal tumors (GISTs) are the most common among the rare gastric mesenchymal tumors (123). However, our study on gastric cancer was focused on gastric adenocarcinomas.

Several attempts to classify gastric cancer (GC) have been made over the past decades. Most successful, and widely used, is the classification by Lauren, which distinguishes, by microscopical morphology alone, two main cancer histological types which appear clearly as dissimilar clinical and epidemiological entities. These are the intestinal-type, a well differentiated tumor characterized by cohesive neoplastic cells forming gland-like tubular structures and the diffuse-type, a poorly differentiated tumor resulting in individual cells infiltrating and thickening the stomach wall (so-called “linitis plastica” or “leather bottle appearance”) (124). There are, however, many gastric cancers

which do not fit into either histological type and present a mixed pattern (intestinal and diffuse). Other histological classifications are used such as the Ming classification, based on the growth pattern (125), the Japan Research Society for Gastric Cancer (JRSGC) classification which individuates five common types (126) similarly to that of the World Health Organization (127). Our study in histopathology and molecular pathology of gastric carcinomas was based on Lauren classification. To note that the intestinal-type of gastric cancer is frequently accompanied by liver metastasis, while diffuse-type gastric cancer is characterized by peritoneal dissemination. Moreover, the intestinal adenocarcinomas have a better prognosis than the diffuse variant.

2.4. Overview of the molecular mechanisms of gastric malignancy

Many studies have attempted to "decode" the mechanism of gastric carcinogenesis. Correa in 1975 described a model in which the basic, sequential steps of stomach carcinogenesis are the following: atrophy (loss of glands), intestinal metaplasia, dysplasia and carcinoma (128). As in a "cascade", each step can be divided in smaller sub-steps in terms of extension of the mucosal surface involved, as well as in phenotypic and genotypic characteristics. *Helicobacter pylori* may also act as a promoter in the progression from normal to neoplastic epithelium, possibly by inducing a hyperproliferative state in the inflamed gastric mucosa. This model was supported by a recent study in which a cohort of 4.655 healthy subjects were monitored for 7.7 years by measuring blood pepsinogen levels (markers of atrophy) and anti-*H. pylori* antibodies (129).

The metaplasia/dysplasia/carcinoma sequence fits better the intestinal-type gastric cancer. Tahara proposed a model in which intestinal and diffuse gastric carcinomas can arise either *de novo* (61) or from precursor lesions, such as dysplasia or adenoma

(figure 7), which are actually considered as similar lesions by the Vienna classification (130). At the molecular level, the intestinal-type of gastric cancer seems to have different genetic origins and biological behavior with respect to the diffuse one. In fact, the intestinal-type develops by a cumulative series of genetic alterations similar to those in colorectal cancer, which are not found in the diffuse-type (61). Mixed gastric carcinomas composed of intestinal and diffuse components exhibit some but not all of the molecular events for each of the two types of stomach cancer (60, 61). It has been suggested that human telomerase reverse trascriptase (hTERT)-positive epithelial cells in normal gastric mucosa, intestinal metaplasia, and gastric adenoma may be viewed as epithelial "stem cells". Hyperplasia of hTERT-positive epithelial cells in intestinal metaplasia caused by *H. pylori* (Hp) may induce "chronic mitogenesis" which can facilitate especially the progression of the intestinal-type gastric cancer (61).

Other scientists have recently shown that although acute injury, acute inflammation, or transient parietal cell loss within the stomach do not lead to bone marrow-derived stem cell (BMDC) recruitment, chronic infection of C57BL/6 mice with *H. pylori*, a known carcinogen, induced repopulation of the stomach with BMDCs. Their findings suggested that stomach cancer may originate from bone marrow-derived stem cells (BMDCs) rather than from stomach stem cells and thus have broad implications for the multistep model of gastric cancer progression (131).

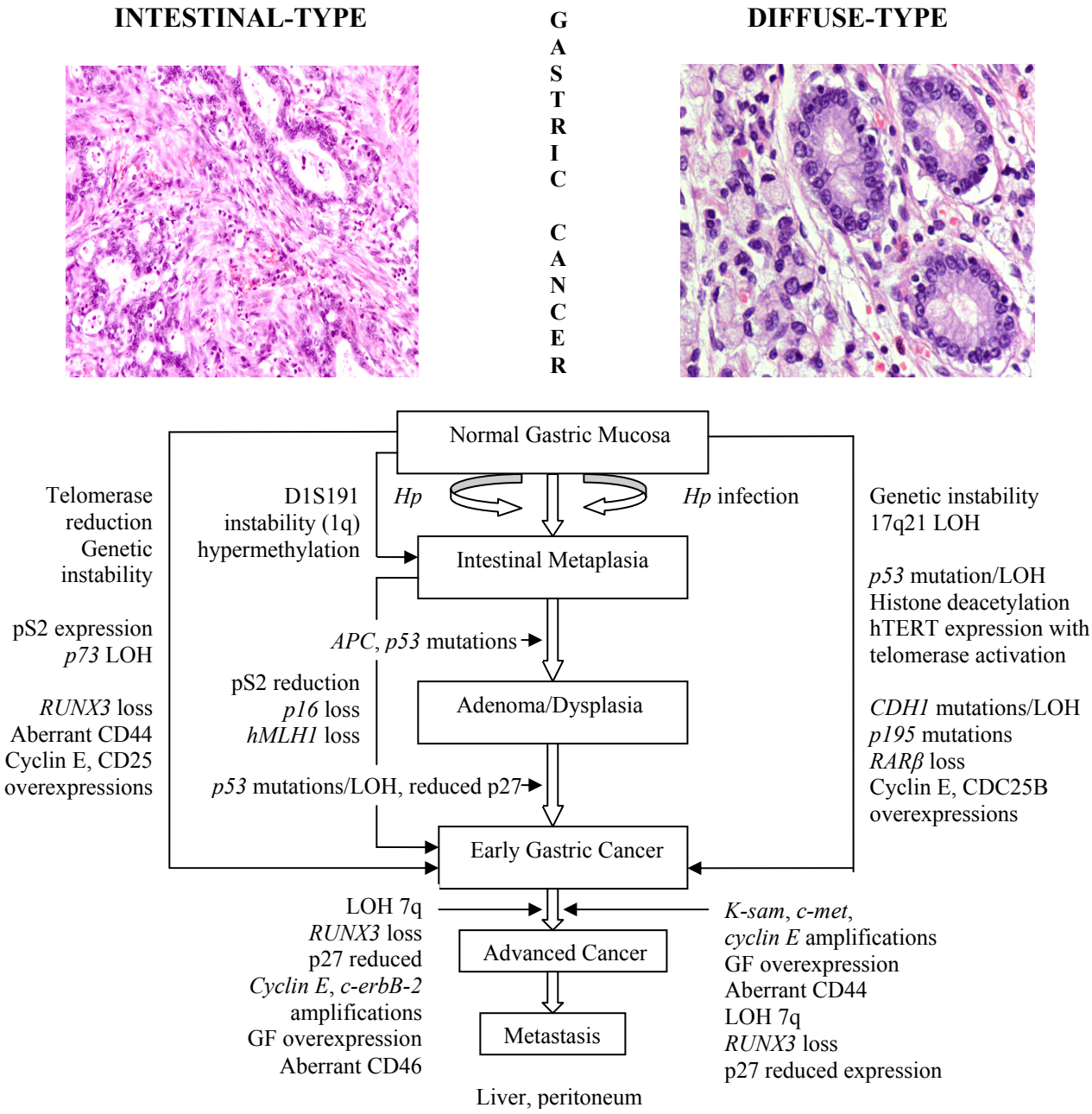

Figure 7. "Precancerous cascade" in the malignant progression of intestinal- and diffuse-types in gastric adenocarcinomas associated with genetic and epigenetic alterations, according to Corea *et al.*, 1975 [128], Yasui *et al.*, 2000 [60], Tahara, 2004 [61]. The images of intestinal- and diffuse-type gastric cancers were kindly provided by Dr. Carla Vindigni, Department of Human Pathology and Oncology, University of Siena, Siena, Italy.

3. Molecular characterization of gastric adenocarinoma

3. Molecular characterization of gastric adenocarcinoma

3.1. Expression patterns of cell-cycle-regulated proteins in gastric cancer and their prognostic significance

Cell kinetic data may be important indicators of clinical behavior in many types of cancer. Despite gastric cancer being common, its prognosis has not been improved significantly in recent years. Recently, greater insight has been gained into the biological properties of tumor cells, how they become malignant and what mechanisms they may use to invade and metastasize. This involves tumor suppressor genes, oncogenes, cell-adhesion molecules, cell-cycle regulators, growth factors and cytokines.

Our laboratory is primarily interested in understanding how Retinoblastoma-like 2 gene, *Rb2/p130* (OMIM *180203) (gene map locus 16q12.2) interacts in various types of cancer. Rb family proteins (pRb/p105, pRb2/p130 and p107) play a key role in cell cycle control and are involved in transcription repression and tumor suppression. pRb, pRb2/p130 and p107 interact with different E2F family factors and can inhibit E2F responsive promoters, interfering with progression of cell cycle, gene transcription, initiation of apoptotic process and cell differentiation (figure 8) (132, 133).

We have also investigated the Polycomb group Enhancer of Zeste 2, *EZH2* (OMIM *601573) (locus 7q35), a transcriptional repressor involved in controlling cellular memory and already linked to tumorigenesis in other organs, the "vascular endothelial growth factor", *VEGF* (OMIM *192240) (6p12), important during the angiogenic process, and of course the well known tumor suppressor *p53* (OMIM +191170) (locus 17p13.1) with a special attention to their relationships with other cell cycle regulators, and how all these molecules promote neoplastic conversion. The resulting paper (134) came quite contemporaneously with that one of Merola and Mattioli *et al.*, 2006 (135) investigating the

molecular events occurring in Barrett's metaplasia (BM). Among the most interesting results obtained, the high correlations found between expression of EZH2, the intestinal-type carcinoma and the risk of distant metastasis, point to a possible prognostic value of EZH2 in this type of cancer. The strong inverse correlation between $p27^{KIP1}$ expression and the risk of advanced disease, and positive correlations between $p21^{WAF1}$ or nuclear pRb2/p130 expression levels and low-grade (G1) gastric tumors, confirmed the traditionally accepted role for these tumor-suppressor genes in gastric cancer.

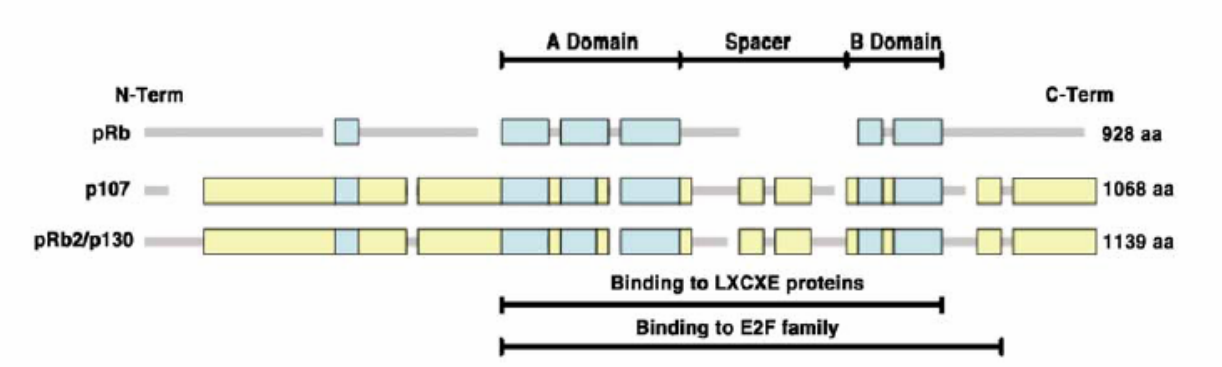

Figure 8. Amino-acid sequence homologies among the Rb family members. The highly homologous regions correspond essentially to the A and B domains, which represent the "pocket" (blue). p107 and pRb2/p130 share a higher sequence homology with respect to pRb/p105 (yellow). Non homologues sequences are represented in grey. *Source*: Caracciolo *et al.*, 2006 (review) (ref. 132).

3.1.1. Immunohistochemical analysis of pRb2/p130, VEGF, EZH2, p53, $p16^{INK4A}$, $p27^{KIP1}$, $p21^{WAF1}$, Ki-67 expression patterns in gastric cancer

Mattioli E*, **Vogiatzi P***, Sun A, Abbadessa G, Angeloni G, D'Ugo D, Trani D, Gaughan JP, Vecchio FM, Cevenini G, Persiani R, Giordano A, Claudio PP.

* Eliseo Mattioli and Paraskevi Vogiatzi contributed equally to this manuscript

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Immunohistochemical Analysis of pRb2/p130, VEGF, EZH2, p53, $p16^{INK4A}$, $p27^{KIP1}$, $p21^{WAF1}$, Ki-67 Expression Patterns in Gastric Cancer

ELISEO MATTIOLI,[1,2] PARASKEVI VOGIATZI,[1,3] ANG SUN,[1] GIOVANNI ABBADESSA,[1,4] GIULIA ANGELONI,[5] DOMENICO D'UGO,[6] DANIELA TRANI,[1,7] JOHN P. GAUGHAN,[8] FABIO MARIA VECCHIO,[9] GABRIELE CEVENINI,[10] ROBERTO PERSIANI,[6] ANTONIO GIORDANO,[1,11] AND PIER PAOLO CLAUDIO[1]*

[1]*Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania*
[2]*Department of Pathology, University of Bari, Ospedale Policlinico Consorziale, Italy*
[3]*Department of Molecular Biology, Medical Genetics Unit, University of Siena, Siena, Italy*
[4]*Department of Oncology and Hematology, Istituto Clinico Humanitas, Rozzano (MI), Italy*
[5]*Department of Cardiovascular Medicine, Catholic University Medical School, Campobasso, Italy*
[6]*Department of Surgery, Catholic University Medical School, Campobasso, Italy*
[7]*Department of Scienze Cardio-Toraciche e Respiratorie, Osp. A. Monaldi, Seconda Universita' degli Studi di Napoli, Italy*
[8]*Biostatistics Consulting Center, Temple University, School of Medicine, Philadelphia, Pennsylvania*
[9]*Divisione di Anatomia Patologica, Università Cattolica del Sacro Cuore, Roma, Italy*
[10]*Department of Chirurgia e Bioingegneria, University of Siena, Siena, Italy*
[11]*Department of Human Pathology and Oncology, University of Siena, Siena, Italy*

Although the considerable progress against gastric cancer, it remains a complex lethal disease defined by peculiar histological and molecular features. The purpose of the present study was to investigate pRb2/p130, VEGF, EZH2, p53, $p16^{INK4A}$, $p27^{KIP1}$, $p21^{WAF1}$, Ki-67 expressions, and analyze their possible correlations with clinicopathological factors. The expression patterns were examined by immunohistochemistry in 47 patients, 27 evaluated of intestinal-type, and 20 of diffuse-type, with a mean follow up of 56 months and by Western blot in AGS, N87, KATO-III, and YCC-2, -3, -16 gastric cell lines. Overall, stomach cancer showed EZH2 correlated with high levels of p53, Ki-67, and cytoplasmic pRb2/p130 ($P < 0.05$, and $P < 0.01$, respectively). Increased expression of EZH2 was found in the intestinal-type and correlated with the risk of distant metastasis ($P < 0.05$ and $P < 0.01$, respectively), demonstrating that this protein may have a prognostic value in this type of cancer. Interestingly, a strong inverse correlation was observed between $p27^{KIP1}$ expression levels and the risk of advanced disease and metastasis ($P < 0.05$), and a positive correlation between the expression levels of $p21^{WAF1}$ and low-grade (G1) gastric tumors ($P < 0.05$), confirming the traditionally accepted role for these tumor-suppressor genes in gastric cancer. Finally, a direct correlation was found between the expression levels of nuclear pRb2/p130 and low-grade (G1) gastric tumors that was statistically significant ($P < 0.05$). Altogether, these data may help shed some additional light on the pathogenetic mechanisms related to the two main gastric cancer histotypes and their invasive potentials.
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Gastric cancer is a common malignancy and still remains a major public health issue. The highest incidence rates are reported in Korea, Japan, and Eastern Asia. A high incidence is also observed in Eastern Europe and parts of Latin America, while in Western Europe and USA the disease is in constant decline. Despite the advance in therapeutic options, less than 20% of patients survive 5 years after diagnosis (Smith et al., 2006). Gastric cancer is usually sporadic, but familial aggregation of the disease may be seen in approximately 10% of the cases (Oliveira et al., 2006). Over 95% of gastric malignancies are adenocarcinomas. According to the widely used Lauren's 1965 classification, there are two types of gastric cancer: the

Eliseo Mattioli and Paraskevi Vogiatzi contributed equally to this manuscript.

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*Correspondence to: Pier Paolo Claudio, College of Science and Technology, Center for Biotechnology, Bio Life Sciences Building, Suite 333, 1900 North 12th Street, Philadelphia, PA 19122-6099. E-mail: claudio@temple.edu

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intestinal-type of adenocarcinoma, which follows the pathologic sequential steps of atrophic gastritis, intestinal metaplasia, dysplasia, carcinoma; and the less common diffuse-type, with worse prognosis and correlated with chronic gastritis (Tahara, 2004; Correa and Schneider, 2005; Smith et al., 2006). Distal gastric cancer (non-cardial) is often of the intestinal-type and predominates in developing countries, among blacks, and in lower socio-economic groups, whereas proximal tumors (many of which show diffuse-type histology) are more common in developed countries, among whites, and in higher socio-economic classes. While the incidence of the former is declining, that of the latter is not; in particular, the signet-ring subtype has been increasing. The main risk factors for distal gastric cancer include *Helicobacter pylori* infection and dietary factors, whereas gastroesophageal reflux disease and obesity play important roles in the development of proximal stomach cancer (Crew and Neugut, 2006).

At the molecular level gastric tumors arise from multiple genetic and epigenetic alterations that involve oncogenes, tumor-suppressor genes, cell-cycle regulators, cell adhesion molecules, DNA repair genes and from genetic instability, and its pathogenesis is still unknown (Tahara, 2004).

In this study we evaluated immunohistochemically the expression of various proteins, crucial to cell-cycle control (p53, $p16^{INK4A}$, $p27^{KIP1}$, $p21^{WAF1}$, and pRb2/p130), tissue development and differentiation (EZH2), and angiogenesis (VEGF), and Ki-67 in gastric cancer patients, in order to further elucidate the molecular mechanisms involved in gastric transformation and its malignant progression.

The *p53*, $p16^{INK4A}$, $p27^{KIP1}$, $p21^{WAF1}$, *RB2/p130* tumor-suppressor genes act by modulating cell proliferation via control of G_1 arrest checkpoint of cell-cycle (Ford et al., 2004).

Abnormalities of the *p53* gene have been identified in many malignancies, including gastric carcinomas (Martin et al., 1992).

The production of p53 is increased in response to cellular insults or DNA damage, and p53 then induces cell-cycle arrest at the G_1/S-junction (Fenoglio-Preiser et al., 2003). The $p21^{WAF1}$ and $p27^{KIP1}$ genes produce proteins that are activated by p53 and induce cell-cycle arrest by inhibition of kinase activity of cyclin/cyclin-dependent kinase complexes regulating cell-cycle progression (Michieli et al., 1994; Wiksten et al., 2002). Several authors have reported that overexpression of $p21^{WAF1}$ and $p27^{KIP1}$ in gastric cancer results in improved outcome, although a few studies reported opposite results (Feakins et al., 2000; Kaye et al., 2000; Migaldi et al., 2001; Wiksten et al., 2002). More recently, Liu et al. (2001) suggested that combined examination of $p21^{WAF1}$, $p27^{KIP1}$, and p53 expression allows precise estimation of prognosis in patients with gastric cancer.

The $p16^{INK4A}$ was originally identified as a tumor-suppressor gene because frequently mutated in melanomas, has been shown to be involved in a broad range of tumors. Alterations of $p16^{INK4A}$ are reported in various human malignancies, and are exceeded in frequency only by the *p53* tumor-suppressor gene. In particular, $p16^{INK4A}$ inactivation has been identified as a possible event in malignant transformation of gastric mucosa (Myung et al., 2000). Recently, hypermethylation of the $p16^{INK4A}$ promoter was found more frequently in microsatellite instable gastric carcinomas (Kim et al., 2003).

VEGF is a homodimeric glycoprotein that functions as a mitogenic factor for endothelial cells. Secreted by many different cell types, its expression is upregulated by hypoxia. Many recent experimental data suggest a major involvement of this protein in tumor angiogenesis, a complex process of primary importance for neoplastic progression and metastatic spread. VEGF expression has been found recently highly expressed in intestinal-type gastric cancer with respect to that of diffuse-type gastric cancer ($P = 0.017$) (Chen et al., 2004). Recently, VEGF expression has been shown to be downregulated, at the transcriptional and translational levels, by Rb2/p130 and p53 expression, both in vitro and in vivo (Riccioni et al., 1998; Claudio et al., 2001).

pRb2/p130 is a member of the Retinoblastoma family of proteins which also includes pRB/p105 and p107. These nuclear proteins, also known as "pocket proteins" for their unique structure, negatively regulate the G_1-S cell-cycle transition by interacting with Cyclin–CDK complexes, modulating the activity of several transcription factors such as the E2Fs. Genetic and functional inactivation of pRb2/p130 allows cells to bypass the G_0/G_1 checkpoint, enabling them to undergo mitosis (Claudio et al., 2002). pRb2/p130 acts by repressing transcription via binding to E2F4 and E2F5 members of E2F transcription factors (Gaubatz et al., 2000), which have binding sites for promoters of genes important for progression of cells from G_1 to S phase. Recent investigations have suggested the importance of chromatin remodeling for the suppression of cellular proliferation mediated by the pocket proteins (Ferreira et al., 1998; Kuo and Allis, 1998; Stiegler et al., 1998; Ito and Adcock, 2002). In a recent study, we have also demonstrated that EZH2 interacts with pRb2/p130 both in vitro and in vivo and that the functional role of this protein–protein interaction is to interfere with the repressive activity of pRb2/p130 on cell-cycle-promoting genes, such as *Cyclin A*, underlying a new mechanism of inactivation of pRb2/p130 function depending on EZH2 expression (Tonini et al., 2004).

EZH2 is a newly identified nuclear protein that shows sequence homology to the "Enhancer of Zeste" protein of *Drosophila*, and is therefore considered a member of the Polycomb group of proteins (Varambally et al., 2002). Also EZH2 is thought to be involved in gene expression control by taking part in chromatin remodeling. EZH2 has been found widely expressed in developing embryos, and its expression decays upon tissue maturation and differentiation. In human pathology, EZH2 has been shown overexpressed in the most aggressive forms of prostate cancer, exhibiting correlation with poor clinical outcome, and thus acting as a novel marker of aggressiveness and unfavorable prognosis in this type of cancer (Varambally et al., 2002).

The Ki-67 is a commercially available monoclonal antibody that reacts with a nuclear antigen expressed in proliferating but not in quiescent cells. Expression of this antigen occurs preferentially during late G_1, S, G_2, and M phases of the cell cycle, while in cells in G_0 phase the antigen cannot be detected. Consequently, the antibody is used in tumor pathology to detect proliferating cells in neoplastic diseases. Ki-67 labeling index is calculated immunohistochemically evaluating the cell growth-related antigen Ki-67, using the monoclonal antibody MIB-1. Its positivity has been evaluated also in gastric cancer (Schipper et al., 1998; Igarashi et al., 1999).

In this study, we are describing for the first time the correlation between the expression levels of p53, $p21^{WAF1}$, $p27^{KIP1}$, $p16^{INK4A}$, pRb2/p130, VEGF, EZH2,

and Ki-67 in primary gastric cancers and their relative expression in gastric cancer cell lines.

MATERIALS AND METHODS

Patients and samples

Forty-seven patients (aged 48–86 years) underwent surgery at the Department of Surgery, Catholic University Medical School, Campobasso, Italy for malignant gastric tumors (34 males and 13 females) were enrolled in this study. Twenty-seven samples were intestinal-type and 20 were diffuse-type. Twenty-four patients were staged as I–II and 23 as III–IV according to the AJCC classification. Overall mean age of patients was 63 years (range 48–86). Mean age in the intestinal-type group was 68 years (range 48–83). Mean age in the diffuse-type group was 63 years (range 49–78).

Forty-one patients were evaluated with a mean follow-up of 56 months and a median of 37 months (range 8–146 months) because 6 patients were either lost at the follow-up or were deceased for causes other than gastric cancer. Follow-up was updated to June 2006. Patients signed an informed consent for the study that was reviewed by the Institutional Review Board.

Sample processing and histological diagnosis

All bioptic samples were formalin-fixed (for at least 24 h) and paraffin-embedded. Several 4-μm thick sections were cut from each specimen, mounted on glass, and dried at 37°C. Two sections of each sample were stained with Hematoxylin and Eosin and evaluated by a pathologist to confirm the diagnosis.

Immunohistochemistry

Several sections of each sample, cut from the same blocks, were used to perform immuhistochemical reactions, according to the following protocol. All sections were dewaxed in xylene and rehydrated through a sequence of decreasing concentration of alcoholic solutions; endogenous peroxydase activity was quenched by 0.5% hydrogen peroxide incubation for 30 min at room temperature. Sections were microwave-pretreated in 10 mM citrate buffer (pH 6.0) for antigen retrieval (three cycles of 5 min each at 650 W). After blocking with PBS-diluted normal serum, sections were incubated with primary antibodies according to the following conditions:

- anti p53 (Dako, Hamburg, Germany, clone DO7, mouse monoclonal antibody): incubated overnight at 4°C + 2 h at room temperature; dilution 1:50;
- anti $p21^{WAF1}$ (BD Biosciences Pharmingen, Franklin Lake, NJ, clone AB11, mouse monoclonal antibody): incubated overnight at 4°C + 2 h at room temperature; dilution 1:100;
- anti $p27^{KIP1}$ (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, mouse polyclonal antibody): incubated overnight at 4°C + 2 h at room temperature; dilution 1:10;
- anti $p16^{INK4A}$ (Santa Cruz Biotechnologies, Inc., rabbit polyclonal antibody): incubated 2 h at room temperature; dilution 1:200;
- anti EZH2 (Upstate, Lake Placid, NY, rabbit polyclonal antibody) incubated overnight at 4°C + 2 h at room temperature; dilution 1:50;
- anti VEGF (Upstate, clone AB-3, mouse monoclonal antibody): incubated overnight at 4°C + 2 h at room temperature; dilution 1:50;
- anti Rb2/p130 (Neomarkers, Inc., Union City, CA, mouse monoclonal antibody): incubated overnight at 4°C + 2 h at room temperature; prediluted (ready to use);
- anti Ki-67 (Dako, clone MIB-1, mouse monoclonal antibody): incubated 2 h at room temperature; dilution 1:100.

After two washes in PBS, sections were incubated with goat anti-mouse or anti-rabbit biotinylated secondary antibody for 30 min at room temperature. After two washes in PBS, sections were incubated with avidin–biotin–peroxydase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) and then washed two more times in PBS. The immunoreactivity was revealed using diaminobenzidine (DAB) as the final chromogen. Finally, sections were counterstained with Meyer's Hematoxylin, dehydrated through a sequence of increasing concentration alcoholic solutions, cleared in xylene, and mounted with epoxydic medium.

During each immunohistochemical assay proof slides were coupled with negative control slides on which the primary antibody was omitted.

Slides evaluation

Slides were evaluated by two different pathologists (EM and FMV), who assessed both percentage of positive neoplastic cells and staining intensity (rated in a three-step scale, low-, medium-, and high-intensity). Discrepancies in the evaluation were resolved by conjoined reobservation of the cases through a multi-headed microscope.

Each case was scored according to the formula:

$$\text{IS (index of staining)} = (i + 1) \times P_i$$

Where i = staining intensity (ranging 1–3) and P_i = percentage of positive cells.

Statistical analysis

Correlation analysis (Pearson and Spearman correlation coefficients) was used to evaluate statistical relationships among the candidate proteins and with clinicopathological parameters. Univariate and multivariate Cox proportional hazards analysis was used to relate protein levels to survival. Variables found to be significant by univariate analysis were entered in a multivariate model to evaluate their independent association with survival.

Cell culture

AGS, NCI-N87, KATO-III, and NL-20 cells were obtained by the American Type Culture Collection. The AGS (ATCC CRL-1739) cell line is from fragments of a primary gastric tumor, moderately differentiated, resected from a Caucasian, 54 years old female patient who had received no prior therapy and it is tumorigenic in athymic BALB/c mice. The NCI-N87 [N87] (ATCC CRL-5822) line is derived from a liver metastasis of a well-differentiated carcinoma (intestinal type) of a Japanese male prior to cytotoxic therapy, which is tumorigenic in athymic nude mice. The non-tumorigenic KATO-III (ATCC HTB-103) cell line is derived from pleural effusion, lymph nodes, and Douglas cul-de-sac from a signet ring carcinoma, poorly differentiated or diffuse type in a Japanese male of 55 years. YCC-2, YCC-3, YCC-16 were a kind gift of Dr. Sun Young Rha, Yonsei Cancer Metastasis Research Center (CMRC, Seoul, Korea). YCC-2 and -3 were derived from the ascite fluid, while the YCC-16 cells were from peripheral blood, of three different gastric cancer patients. NL-20 cells (normal lung epithelium) (CRL-2503) were utilized as normal control in Western blot analysis because they express wild-type p53. AGS cells were grown in Ham's F12 medium, the NCI-N87 and KATO-III cells were grown in RPMI 1640, the YCC lines in DMEM at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. All these mediums were supplemented with 2 mM L-glutamine and 10% fetal bovine serum. The NL-20 cells were grown in Ham's F12 medium with 1.5 g/L sodium bicarbonate, 2.7 g/L glucose, 2.0 mM L-glutamine, 0.1 mM non-essential amino acids, 0.005 mg/ml insulin, 10 ng/ml epidermal growth factor, 0.001 mg/ml transferrin, 500 ng/ml hydrocortisone, and 4% fetal bovine serum at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Western Blot analysis

Western blot analysis was performed on 50 μg of total protein lysate extracted from six gastric cell lines (AGS, NCI-N87, KATO-III, YCC-2, YCC-3, YCC-16) as described previously (Kim et al., 2005) using antibodies against Rb2/p130 diluted 1:500 (BD Biosciences Pharmingen), VEGF diluted 1:200 (Santa Cruz, CA), EZH2 diluted 1:250 (Abcam, Inc., Cambridge, MA), $p16^{INK4A}$ diluted 1: 200 (Santa Cruz), $p21^{WAF1}$

diluted 1: 500 (Santa Cruz), $p27^{KIP1}$ diluted 1: 200 (Santa Cruz), p53 clone D-11 diluted 1: 200 (Santa Cruz), and HSP-72/74 diluted 1:5,000 (Calbiochem, San Diego, CA).

RESULTS

Immunohistochemical analysis

The expression levels of $p16^{INK4A}$, $p21^{WAF1}$, $p27^{KIP1}$, pRb2/p130, VEGF, p53, EZH2, and Ki-67 were determined by immunohistochemistry in 27 cases of intestinal-type and in 20 cases of diffuse type gastric cancer (Fig. 1). The immunohistochemical assay revealed several significant differences in the expression of the investigated markers between our patients and are summarized in Table 1.

The immunostaining for each protein was also determined as positive or negative by a cutoff value determined as follows: p53, $p16^{INK4A}$, $p27^{KIP1}$, and $p21^{WAF1}$ and EZH2 staining was interpreted as positive when >10% of the tumor cells showed distinct nuclear staining and Ki67 when >25% showed distinct nuclear staining as previously reported (Al-Moundhri et al., 2005). In general,

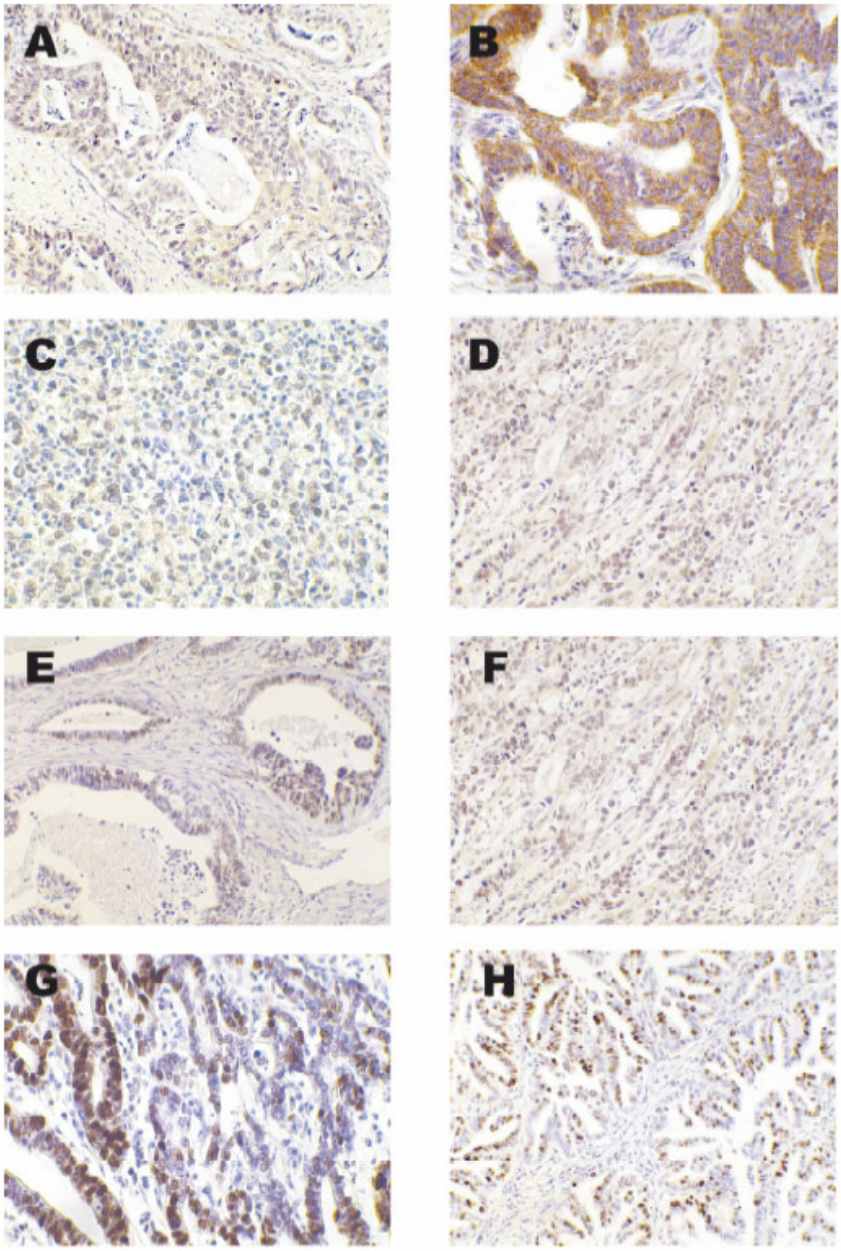

Fig. 1. Representative panel of immunohistochemical analysis of EZH2, VEGF, pRb2/p130, $p27^{KIP1}$, $p16^{INK4A}$, $p21^{WAF1}$, p53, and Ki-67 in gastric cancer. A: High expression levels of EZH2 in a gastric carcinoma, (B) Case showing extremely high expression of VEGF in the cytoplasm, (C) Low expression levels of pRb2/p130 in the nuclei of a primary gastric cancer, (D) High expression levels of $p27^{kip1}$, (E) High expression levels of $p16^{INK4A}$, (F) Representative case expressing high levels of $p21^{WAF1}$, (G) Case showing extremely high expression of p53, and (H) High levels of Ki-67 labeling index. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1. Correlation between overall series of protein expressions in gastric cancer

		Correlations with Pearson r			
		Low	Moderate	High	*P*-value
pRb2/p130 n	VEGF	0.296			<0.05
	EZH2	0.295			<0.05
	p21	0.380			<0.01
pRb2/p130 c	VEGF		0.520		<0.01
	EZH2		0.515		<0.01
EZH2	pRb2/p130 n	0.295			<0.05
	pRb2/p130 c		0.515		<0.01
	p53	0.361			<0.05
	Ki-67	0.357			<0.05
VEGF	pRb2/p130 n	0.296			<0.05
	pRb2/p130 c		0.520		<0.01
p27	p21	0.330			<0.05
p16	Ki-67	0.435			<0.01
	p53	0.464			<0.01
p53	EZH2	0.361			<0.05
	p16	0.464			<0.01
	Ki-67			0.990	<0.01
Ki-67	EZH2	0.357			<0.05
	p16	0.435			<0.01
	p53			0.990	<0.01

pRb2/p130 n: nuclear pRb2/p130.
pRb2/p130 c: cytoplasmic pRb2/p130.

the expression of the studied genes in our cases was as follows: $p16^{INK4A}$ (46.8%), $p21^{WAF1}$ (53.2%), $p27^{KIP1}$ (72.3%), p53 (55%), EZH2 (78.8%), and Ki-67 (38.3%).

Consistently with the results of a very recent publication (Matsukawa et al., 2006), the non-cancerous gastric mucosa showed faint or no EZH2 immunoreactivity restricted to the nuclei of glandular epithelial cells (data not shown). In the majority of the gastric cancers examined, high EZH2-specific nuclear immunostaining was found (78.8%). Positive statistically significant correlations were found between the expression levels of EZH2 and Ki-67, p53 and nuclear Rb2/p130 expression ($P < 0.05$). Stronger positive correlation ($P < 0.01$) was found between the expression levels of EZH2 and cytoplasmic pRb2/p130, even though a variable pRb2/p130 nuclear staining was also noted in all the samples analyzed.

Positive correlations were found between the expression levels of nuclear pRb2/p130 and $p21^{WAF1}$ or VEGF ($P < 0.01$ and $P < 0.05$, respectively). Stronger positive correlations were found between the expression levels of VEGF and cytoplasmic pRb2/p130 that were statistically significant ($P < 0.01$).

It has been reported by Al-Moundhri et al. and others that the growth inhibitory activity of $p21^{WAF1}$ and $p27^{KIP1}$, may be modulated in more advanced colorectal and gastric tumors through inactivation (Cheng et al., 1999; Al-Moundhri et al., 2005). Consistently with these previously published data we found a positive correlation between $p21^{WAF1}$ and $p27^{KIP1}$ in our gastric tumor samples ($P < 0.05$).

Additionally, we found positive significant correlations between the expression levels of Ki-67 and EZH2 or $p16^{INK4A}$ ($P < 0.05$ and $P < 0.01$, respectively). Interestingly, we found also a very strong positive correlation ($P < 0.01$) between Ki-67 and p53 expression levels.

Correlation of the biological parameters according to histological gastric tumor types

The specimens we examined were from patients affected by either diffuse (42.55%) or intestinal gastric cancers (57.45%) according to the Lauren's criteria.

TABLE 2. Correlations of protein expressions according to the intestinal-type of gastric cancer

		Correlations with Pearson r			
		Low	Moderate	High	*P*-value
pRb2/p130 c	VEGF	0.499			<0.01
	EZH2	0.412			<0.05
VEGF	pRb2/p130 c	0.499			<0.01
EZH2	pRb2/p130 c	0.412			<0.05
p53	Ki-67			0.986	<0.01
Ki-67	p53			0.986	<0.01

pRb2/p130 n: nuclear pRb2/p130.
pRb2/p130 c: cytoplasmic pRb2/p130.

Analyzing the data according to tumor type (intestinal vs. diffuse), we found some interesting and statistically significant correlations that were not present when considering the global patients' population and that are summarized in Tables 2 and 3. Considering the intestinal-type, we found the following correlations (Table 2): EZH2 was still correlated with the cytoplasmic expression of pRb2/p130 ($P < 0.05$). Moreover, some other correlations that were found at the global analysis were also confirmed. Specifically, cytoplasmic pRb2/p130 expression was correlated with VEGF expression ($P < 0.01$) whereas the expression levels of p53 were highly correlated with that of Ki-67 ($P < 0.01$).

Considering instead the diffuse-type, we found the following correlations (Table 3): cytoplasmic pRb2/p130 expression was still correlated with that of VEGF ($P < 0.05$) and EZH2 ($P < 0.01$). Interestingly in the diffuse-type, generally manifesting a more aggressive biological behavior, the correlation between nuclear expression levels of pRb2/p130 and EZH2 was lost. We also found that the EZH2 expression correlated with those of p53 and Ki-67 ($P < 0.01$). Finally, the correlations between $p21^{WAF1}$ and $p27^{KIP1}$ expressions levels and between p53 and $p16^{INK4A}$ or Ki-67 were still maintained ($P < 0.01$).

Interestingly, in the analysis of diffuse-type gastric cancer specimens, nuclear expression of pRb2/p130 and $p27^{KIP1}$ revealed a novel statistically significant relationship ($P < 0.05$), that was not found analyzing either the total patient population or just the intestinal histological type.

TABLE 3. Correlations of protein expressions according to the diffuse-type of gastric cancer

		Correlations with Pearson r			
		Low	Moderate	High	*P*-value
pRb2/p130 n	p27		0.504		<0.05
pRb2/p130 c	VEGF	0.466			<0.05
	EZH2		0.644		<0.01
VEGF	pRb2/p130 c	0.466			<0.05
EZH2	pRb2/p130 c		0.644		<0.01
	p53		0.583		<0.01
	Ki-67		0.584		<0.01
p27	pRb2/p130 n		0.504		<0.05
	p21		0.583		<0.01
p16	p53			0.749	<0.01
	Ki-67			0.749	<0.01
p53	EZH2		0.583		<0.01
	p16			0.749	<0.01
	Ki-67			1.000	<0.01
Ki-67	EZH2		0.584		<0.01
	p16			0.749	<0.01
	p53			1.000	<0.01

pRb2/p130 n: nuclear pRb2/p130.
pRb2/p130 c: cytoplasmic pRb2/p130.

Comparing the data of the two main histological groups, we found some protein expression hallmarks that could underline the fact that the diffuse more than the intestinal type of gastric cancer has been linked to genetic alterations. Intriguingly, we found that in patients with diffuse-type gastric cancer $p16^{INK4A}$ was expressed in 45% and p53 in 40% of the cases, and that the two protein expressions were also highly correlated ($P < 0.01$). Additionally, the percentage of diffuse-type tumor samples expressing high levels of Ki-67 was low (30%), but also in this case there was a highly statistical correlation with $p16^{INK4A}$ ($P < 0.01$).

Correlation of EZH2, $p16^{INK4A}$, $p21^{WAF1}$, $p27^{KIP1}$, pRb2/p130, VEGF, p53, and Ki-67 expression levels with clinicopathological parameters

Immunohistochemical expression levels and their associations with clinicopathological features in the 47 gastric cancers tissues samples were analyzed and are summarized in Table 4. Thirty-seven cases (78.8%) belonged to the high EZH2 expression group. Conversely, none of the corresponding normal mucosa expressed EZH2. Intestinal-type gastric cancers showed higher levels of expression of EZH2, VEGF, p53 ($P < 0.05$), and cytoplasmic pRb2/p130 or Ki-67 ($P < 0.01$). We also analyzed the clinicopathological features in relation to the proteins' expression using an ANOVA test considering the various tumor locations. We found that the expression of $p27^{KIP1}$ ($P < 0.001$), p53 ($P = 0.002$), and Ki-67 ($P = 0.009$) correlated with intestinal-type tumors independently of the various gastric tumor locations: antrum, cardias medium, medium antrum, cardias medium antrum, and medium. No associations were found between the diffuse-type and any of the considered biological parameters considering the various tumor locations using the ANOVA test.

Importantly, while we were submitting our manuscript we found that in accordance with a very recent publication (Matsukawa et al., 2006), tumors expressing high levels of EZH2 correlated with more aggressive biological behavior. In fact, high EZH2 expressing tumors were those presenting with distant metastasis including those along the hepatic ilum ($P < 0.01$).

Notably, high $p27^{KIP1}$ expression levels were correlated with lower risk of distant metastasis confirming a protective role for $p27^{KIP1}$ in gastric carcinomas ($P < 0.05$). Additionally, high $p21^{WAF1}$ and nuclear and cytoplasmic pRb2/p130 expressing tumors were classified as low-grade tumors (G1) ($P < 0.05$), confirming a protective role for these tumor-suppressor proteins in gastric tumors. Surprisingly, invasive tumors (T2-T4) showed higher expression levels of nuclear Rb2/p130 ($P < 0.05$).

TABLE 4. Correlations between biological and clinicopathological parameters in gastric cancer

		Correlations with Spearman r			
		Low	Moderate	High	*P*-value
pRb2/p130 n	G1	0.291			<0.05
	T > 1	0.323			<0.05
pRb2/p130 c	G1	0.340			<0.05
	Intestinal type	0.399			<0.01
VEGF	Intestinal type	0.334			<0.05
EZH2	Intestinal type	0.362			<0.05
	Metastasis	0.422			<0.01
p53	Intestinal type	0.370			<0.05
p27	Metastasis	−0.393			<0.05[a]
p21	G1	0.322			<0.05
Ki-67	Intestinal type	0.377			<0.01

pRb2/p130 n: nuclear pRb2/p130.
pRb2/p130 c: cytoplasmic pRb2/p130.
G1: G1 grade differentiated disease.
T > 1: T2, T3, T4.
[a]Inverse correlation.

Expression of $p16^{INK4A}$, $p21^{WAF1}$, $p27^{KIP1}$, pRb2/p130, VEGF, p53, and EZH2 in gastric cancer cell lines

In order to verify the immunohistochemical results, we decided to study the expression pattern of the same proteins considered in the immunohistochemical experiments in a series of Western blot analysis (Fig. 2). We studied the expression levels of $p16^{INK4A}$, $p21^{WAF1}$, $p27^{KIP1}$, pRb2/p130, VEGF, p53, and EZH2 in various gastric cell lines of Caucasian (AGS) or Japanese (N87, KATO-III), and Korean origin (YCC-2, YCC-3, and YCC-16). We found that $p16^{INK4A}$ was not expressed in AGS, N87, and KATO-III cells (Fig. 2A), while it was expressed abundantly in the cell lines of Korean origin (YCC-2, YCC-3, and YCC-16) (Fig. 2B). The NL-20 cells (human normal bronchial epithelium) were used as a control and showed high levels of $p16^{INK4A}$. On the other hand, we found that $p21^{WAF1}$ was not expressed in the cell lines of Korean origin (YCC-2, YCC-3, and YCC-16) even though these cells expressed p53 (Fig. 2B). The $p21^{WAF1}$ protein was instead expressed abundantly in the AGS cells, which express low levels of p53. The $p21^{WAF1}$ gene product was expressed less abundantly in N87 cells (Fig. 2A), which instead expressed a faster migrating form of p53 (*p53) when reacted with an antibody against p53 (clone D-11, which was raised using the entire p53 molecule as an antigen). The KATO-III cellular lysate did not reveal a band when reacted with an antibody against p53 because of a genomic deletion (Yokozaki, 2000).

All the different cell lines expressed $p27^{KIP1}$ at an abundant level. VEGF expression was also abundant in most of the cell lines. However, the N87 cells that showed high expression levels of hypophosphorylated form (active) of pRb2/p130 showed lower levels of VEGF when compared to the AGS and KATO-III cells, that expressed less abundant hypophosphorylated pRb2/p130 (Fig. 2A). Regarding the cell lines of Korean origin, it needs to be pointed out that the YCC-2 and -3 cells were derived from the ascite fluid, and YCC-16 cells derived from peripheral blood (Kim et al., 2005). Moreover, only the YCC-3 and -16 have been reported to grow in nude mice. In this respect, the YCC-2, -3, and -16 cells represent a progression model of gastric tumor aggressiveness. Analyzing the expression levels of VEGF and EZH2 in these cells, we found that there was an increasing expression level of these markers from the YCC-2 to the YCC-16 cells (Fig. 2B). The YCC-3 and -16 cells which are able to grow in nude mice, showed higher expression levels of VEGF and EZH2 when compared to the YCC-2 cells that were previously shown to bear lower biological aggressiveness. Additionally, the more biologically aggressive YCC-16 cells showed lower levels of hypo-phosphorylated pRb2/p130 and higher levels of VEGF, confirming previous data obtained in vitro (Claudio et al., 2001). HSP72/73 was used as a loading control.

DISCUSSION

Gastric carcinoma is a major cause of morbidity and mortality worldwide. The most reliable prognostic factors are tumor stage and completeness of excision. Tumor grade and histological type may be also useful

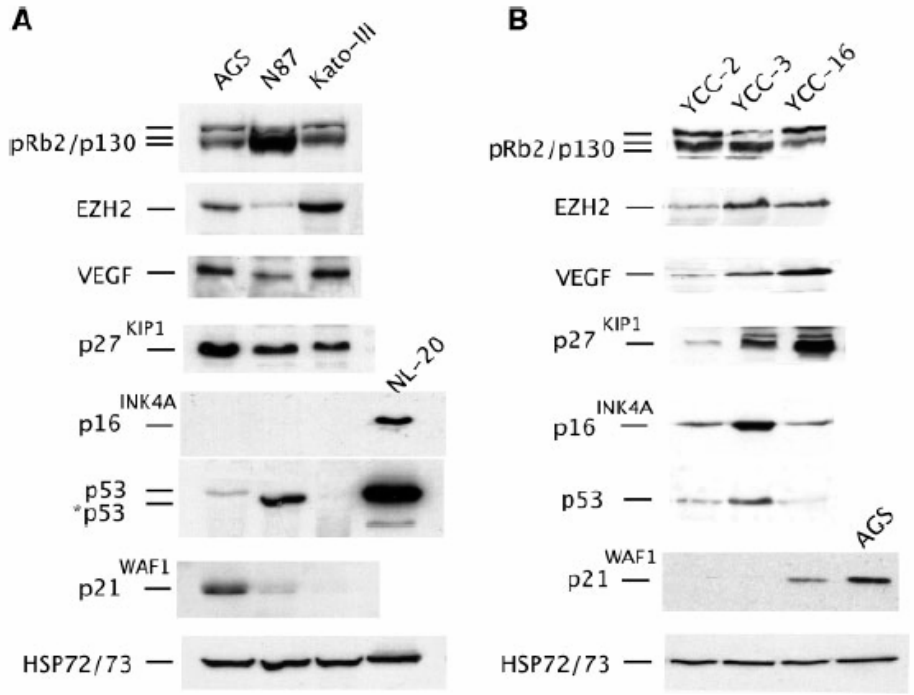

Fig. 2. Western blot analysis of various gastric cells lines. A: Western blot analysis of total lysates extracted from AGS, N-87, and KATO-III cells. On the left are indicated the antibodies used. *p53 indicates a faster migrating form of p53. Total lysates extracts from NL-20 cells were used as a control for the p16^{INK4A} antibody reaction. HSP72/73 was used as a loading control. B: Western blot analysis of total lysates extracted from YCC-2, YCC-3, and YCC-16 cells. On the left are indicated the antibodies used. Total lysates extracts from AGS cells were used as a control for the p21^{WAF1} antibody reaction. HSP72/73 was used as a loading control.

factors. Although previous reports are conflicting, immunohistochemical studies are important in helping finding potential novel prognostic factors, since they may help predict not only baseline life expectancy, but also tumor response to specific anticancer drugs. This will be especially true with further uncovering of the contribution to disease progression by old and new proteins and their interacting pathways. In an era where many effective but expensive tests are being developed and used by the research community, immunohistochemistry still remains a widely used and affordable technique in clinical settings. The development of human cancers including gastric cancer is a multistep process and phenotypic changes during cancer progression reflect the sequential accumulation of genetic alterations in cells. We have performed a series of protein expression analysis by immunohistochemistry or Western blot of various proteins involved in the cell cycle and in angiogenesis in primary gastric tumor samples and in established gastric cancer cell lines. Cellular proliferation follows an organized and timely regulated progression through the cell cycle, which is controlled by protein complexes composed of cyclins and cyclin-dependent kinases (CDKs) (Tonini et al., 2002). The cell-cycle progression is determined by checkpoints between early and late G_1 phases, and between the S- and G_2/M-phases. A major contribution for cell-cycle regulation is due to the cdk inhibitors (CKIs) such as p16^{INK4A}, p21^{WAF1}, and p27^{KIP1}. The p21^{WAF1} protein is transactivated and mainly controlled by p53 and its activation leads to G_1-phase arrest of the cell cycle by inhibiting the kinase activity of cyclin-dependent kinase complexes regulating cell-cycle progression. The p16^{INK4A} protein is overexpressed in cells defective in pRb function, and it may participate in a feedback loop wherein repression of p16^{INK4A} expression by pRb may allow CDK4 to phosphorylate and inhibit pRb. The p16^{INK4A} protein inhibits specifically CDK4 and CDK6, and by inhibiting their activity it inactivates pRb. The p27^{KIP1} protein is a negative regulator implicated in G_1 phase arrest by inhibiting cyclin E–CDK2, cyclin A–CDK2, and cyclin D–CDK4 complexes, and abrogating their activity. Therefore, deregulation of any of these molecules may result into an uncontrolled proliferation.

The immunohistochemical assay revealed several significant differences in the expression of the investigated markers among our patients some of which were confirmed at the Western blot analysis. Abnormalities in p16^{INK4A} and pRb are not infrequent in gastric cancer. Lack of p16^{INK4A} expression is often due to hypermethylation of the *p16^{INK4A}* promoter region. Previous studies have also shown that deletion of the *p16^{INK4A}* gene is associated with the degree of differentiation and metastasis of gastric cancer. In our cases instead p16^{INK4A} expression was not correlated to any of the available clinicopathological parameters. Additionally, 46.8% of our cases expressed high levels of p16^{INK4A}. Serrano et al. (1993) have proposed that physiological inactivation of pRb during the G_1-phase leads to increased p16^{INK4A} expression in order to limit CDK4 activity. Inactivation of pRb would also stimulate cell to increase p16^{INK4A} expression in an attempt to inhibit CDK4. This would establish a negative feedback in which pRb negative tumors would have high levels of p16^{INK4A}, while pRb positive tumors might require decreased amounts of functional p16^{INK4A} in order to achieve a level of CDK4 activity sufficient for pRb inactivation. This could be the scenario of the pRb status, at least half of our patient population.

In accordance with Al-Moundhri et al. (2005) we have also found that most of the tumor samples examined expressed high levels of $p21^{WAF1}$ and $p27^{KIP1}$. In our study we have also found that high levels of $p21^{WAF1}$ significantly correlated with those of $p27^{KIP1}$, and that high-levels of $p27^{KIP1}$ inversely correlated with the presence of metastasis confirming a role for $p27^{KIP1}$ as a tumor-suppressor gene in this type of cancer. Moreover, we also found that high levels of $p21^{WAF1}$ expression directly correlated with differentiation-grade 1 (G1) gastric cancers. The fact that we found high-levels of $p21^{WAF1}$ and $p27^{KIP1}$ expression may be explained as follows. It has been proposed that the growth-inhibitory activity of the $p27^{KIP1}$ and $p21^{WAF1}$ may be modulated in advanced tumors through their inactivation (Polyak et al., 1994; Cheng et al., 1999). Western blot analysis of a panel of gastric carcinoma cells lines demonstrated that the biologically more aggressive cell lines AGS and YCC-16 contained more $p27^{KIP1}$. To note that the AGS and YCC-16 cell lines are able to grow if injected subcutaneously in nude mice. Additionally, the AGS cell line of Caucasian origin showed a linear increase in $p21^{WAF1}$ and $p27^{KIP1}$, that is, in agreement with our immunohistochemical results. Surprisingly, we observed that while all the cell lines tested showed low levels of p53 expression, the N87 cells expressed abundantly a faster migrating form of p53 (labeled *p53 on Fig. 2A) when reacted with the antibody against p53 (clone D-11, which was raised using the entire p53 molecule as an antigen). We are currently investigating the significance of the apparent increased stability of p53 in this cell line and its genetic structure and therefore we can only speculate that the faster migrating form of p53 could be the result of a possible genetic alteration not infrequently found in the p53 family of proteins in many types of cancer (Murray-Zmijewski et al., 2006).

Another important step in gastric carcinogenesis is the upregulation of the angiogenic factor VEGF, which usually is associated to more aggressive tumors (Fondevila et al., 2004). We found higher VEGF expression levels in the intestinal-type of gastric carcinomas in accordance with previously published data (Chen et al., 2004). High levels of VEGF expression were also found in the cell lines tested. In particular, the Korean cell lines (YCC-2, -3, and -16) demonstrated a progressive increase of VEGF expression passing from the less to the more aggressive biological phenotype.

Importantly, tumors that expressed high levels of VEGF also expressed higher levels of cytoplasmic pRb2/p130 ($P < 0.01$) providing a link between the two proteins also in gastric carcinomas. The role of pRb2/p130 in the cytoplasm, if any, is still unknown and the significance of the potential link of the direct correlation between the expression of pRb2/p130 in the cytoplasmic compartment and that of VEGF in this type of cancer, remains to be elucidated.

Cytoplasmic, but also nuclear pRb2/p130 expressions were also found significantly correlated to differentiation-grade 1 (G1) tumors indicating the lack of a role for this protein in less differentiated tumors. Additionally, high cytoplasmic pRb2/p130 expression was found significantly correlated to the intestinal type, which develops following the pathologic sequential steps of atrophic gastritis, intestinal metaplasia, dysplasia, and carcinoma. We did not find cytoplasmic pRb2/p130 correlated to the less common diffuse-type, which presents with worse prognosis, develops after chronic gastritis and for which there are evidence of genetic predisposition (Caldas et al., 1999; Tahara, 2004; Correa and Schneider, 2005; Smith et al., 2006). Distal gastric cancer (non-cardial) is often of the intestinal-type and predominates in developing countries, among blacks, and in lower socio-economic groups, whereas proximal tumors (many of which show diffuse-type histology) are more common in developed countries, among whites, and in higher socio-economic classes. The main risk factors for distal gastric cancer include *Helicobacter pylori* infection and dietary factors, whereas gastro-esophageal reflux disease and obesity play important roles in the development of proximal stomach cancer (Crew and Neugut, 2006). More studies are needed to assess the role of these proteins in the pathogenesis of gastric cancer especially with respect to the presence or not of *Helicobacter pylori* infection and dietary factors in the patients screened. Unfortunately, we did not have available these clinicopathological data at the time of our study and therefore we can not discuss the possible implications and modulations of molecular events due to the presence of *Helicobacter pylori*.

Additionally, cytoplasmic, but also nuclear pRb2/p130 expressions were also found significantly correlated to that of the newly identified human nuclear protein (EZH2) that shows sequence homology to the "Enhancer of Zeste" protein of *Drosophila*, and is therefore considered a member of the Polycomb group, which maintains homeotic gene repression and is thought to control gene expression by regulating chromatin (Varambally et al., 2002). A very recent publication reported that EZH2 expression is restricted to gastric cancer cells and that non-cancerous gastric mucosa showed faint or no EZH2 immunoreactivity restricted to the nuclei of glandular epithelial cells (Matsukawa et al., 2006). In the present study, we found that 78.8% of the cases expressed high levels of EZH2 and that the expression levels of EZH2 protein determined by Western blot analyses were in good agreement with those of the immunohistochemical analysis. Additionally, we found that EZH2 correlated with higher levels of p53 and Ki-67. Interestingly, these three proteins as well as cytoplasmic pRb2/p130 and VEGF were significantly associated with intestinal-type gastric cancer. In particular, high levels of EZH2 expression in gastric cancer tissues were significantly associated with the presence of metastasis indicating a possible role of this protein in gastric tumor spread. These results are in agreement with other reports indicating the re-expression of this protein in different human cancers (Varambally et al., 2002; Kleer et al., 2003; Dukers et al., 2004; Arisan et al., 2005; Gil et al., 2005; Weikert et al., 2005; Matsukawa et al., 2006), but pointing out that in the specific case of intestinal-type of gastric malignancy its re-expression occurs in tight correlation with the presence of metastasis, a factor that influence its prognosis. This study represents another evidence that the immunohistochemical investigation of various genes' expression could be of aid in understanding and predicting the aggressiveness of gastric malignancies. The fact that EZH2 could be a marker of aggressive subgroups in several cancers as well as in gastric neoplasias may be of significant practical interest, since the polycomb proteins have been proposed recently as candidates for targeted therapy in breast cancers (Takeshita et al., 2005). Additionally, the fact that EZH2 and Ki-67, pRb2/p130, and p53 showed tight correlations in their expressions, suggests that their association should be further studied as possible predictive factors in gastric malignancies.

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3.2. Tumor suppressor genes in gastric cancer: old molecules, new understanding

The observation that mutations in tumor suppressor genes can have haploinsufficient as well as gain of function and dominant negative phenotypes has caused a reevaluation of the "two-hit" model of tumor suppressor inactivation. The two-hit model was derived from mathematical modeling of cancer incidence. Subsequent interpretations implied that tumor suppressors were recessive, requiring mutations in both alleles. This model has provided a useful conceptual framework for three decades of research on the genetics and biology of tumor suppressor genes. Indeed, p53 is an example of "old" molecule which follows the "two-hit" dogma. Recently it has become clear that mutations in tumor suppressor genes are not always completely recessive. Haploinsufficiency occurs when one allele is insufficient to confer the full functionality produced from two wild-type alleles. It can be partial or complete and can vary depending on tissue type, other epistatic interactions, and environmental factors. In addition to simple quantitative differences (one allele versus two alleles), gene mutations can have qualitative differences, creating gain of function or dominant negative effects that can be difficult to distinguish from dosage-dependence. Like mutations in many other genes, tumor suppressor gene mutations can be haploinsufficient, dominant negative or gain of function in addition to recessive. Thus, under certain circumstances, one hit may be sufficient for inactivation. In addition, the phenotypic penetrance of these mutations can vary depending on the nature of the mutation itself, the genetic background, the tissue type, environmental factors and other variables (136). This received complexity pushed our curiosity to better understanding the role of genes still to be fully explored , such us *RUNX3*, in gastric cancer. The challenge for the future will be to incorporate the old knowledge with the new findings, which may ultimately provide both a better understanding of disease development, as well as a foundation for novel strategies for gastric cancer diagnosis and therapy.

3.2.1. The Limitless Role of p53 in Cell Cycle Machinery: Good News or Bad News?

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Journal Club

The Limitless Role of p53 in Cell Cycle Machinery

Good News or Bad News?

Paraskevi Vogiatzi[1,2]
Marco Cassone[1]
Giovanni Abbadessa[1,3]
Pier Paolo Claudio[1,*]

[1]Sbarro Institute for Cancer Research and Molecular Medicine; College of Science and Technology; Temple University; Philadelphia, Pennsylvania, USA

[2]Department of Molecular Biology; Medical Genetics Unit; University of Siena; Siena, Italy

[3]Department of Oncology and Hematology; Istituto Clinico Humanitas; Rozzano, Italy

*Correspondence to: Pier Paolo Claudio; College of Science and Technology; Center for Biotechnology; Bio Life Sciences Building Suite 333; 1900 North 12th Street; Philadelphia, Pennsylvania; 19122-6099; Tel.: 215.204.9523; Fax: 215.204.9522
Email: claudio@temple.edu

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NOTE

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ABSTRACT

The p53 tumor suppressor gene acts as a great protagonist in deciding how cells undergo either cell cycle arrest or apoptosis after experiencing various stress signals, including DNA damage, hypoxia, oncogene activation, and hyperproliferation. Research on p53 is in steady expansion, as evidenced by the continual flood of papers claiming novel mutations, gain or loss of p53 functions, and gene interactions. The latest study carried out by Spurgers of Texas University and his Colleagues (J Biol Chem 2006; 281:25134-42) emphasizes the strong impact of p53 in the complicated machinery that regulates cell cycle progression. In this paper, microarray data and well-evaluated statistical procedures on PC3 and LNCaP prostate carcinoma cells, open new perspectives in p53 mechanisms and bring the simultaneous identification of novel p53-repressed cell cycle genes, hopefully providing significant improvements in the study of DNA damage response, multistep carcinogenesis, and treatment rationales and outcomes.

INTRODUCTION

p53, or TP53, also known as "cellular gatekeeper" or "guardian of the genome" because of its role in fitting cellular response to DNA-damaging agents, conserving stability by preventing genome mutation, is definitely the most popular molecule in the cell cycle scenario.[1,2] In fact, in 1993, the Editor of the Science journal selected p53 as the "Molecule of the Year".[3] The p53 protein was independently discovered in the late 1970's by Levine[4] of Princeton University, Lane[5] of the University of Dundee, Scotland, and Old[6] of the Memorial Sloan-Kettering Cancer Center in New York City, as a cellular ~53 kDa nuclear phosphoprotein bound to the hexameric DNA helicase, large T antigen of Simian Virus SV-40. It was initially described as an oncogene with weak oncogenic properties, but in the late 1980's was identified as a tumor suppressor gene in colon cancer.[7] The protein is called "p53" because of its molecular mass and its gene locus[8-10] is 17p13.1 (OMIM 191170). The open reading frame of p53 is 393 amino acids long,[10] with the central region (from about 100 to 300) containing the DNA-binding domain. It was recently demonstrated on the mouse p53 core domain that p53 dimer-dimer contacts are less frequently mutated in human cancer than intra-dimer contacts.[11] The p53 protein is present in all normal cells but at low concentrations, mainly because of its relatively short half life (~20 minutes).[1] After 28 years of intensive study, *p53* is considered the most commonly mutated gene in human cancer, contributing to Li-Fraumeni Sydrome (LFS1) (OMIM #151623), and has also been associated with several nonneoplastic diseases among which atherosclerosis and neurological degenerative disorders such as Alzheimer's disease, Parkinson's disease, ataxia-telangectasia, Huntigton's disease and prion-borne diseases (reviewed by Royds and Iacopetta Ref. 12). More than 21,000 mutations in the *p53* gene are reported in IARC (International Agency for Research on Cancer) TP53 database (www-p53.iarc.fr), and in UMD (Universal Mutation Database), thus claiming a possible role in about 50% of human cancers and about 30% of all human diseases associated with mutations.[13]

ESSENTIAL ROLE OF P53 IN ALL CELL-CYCLE TRANSITIONS

p53 is a potent transcription factor and a pleiotropic regulator of diverse biological effects. It modulates several cellular functions such as cell cycle, apoptosis, DNA repair, differentiation, and angiogenesis (reviewed by Laptenko and Prives Ref. 14). Remarkable advances and a few surprises can be found regarding p53 role in senescence,[15] the cytoskeleton and cell motion (reviewed in ref. 16), the mitochondrial respiration,[17] and the size and life span of mammals.[18]

Spurgers and coworkers' investigation was carried out under the core theme of identifying novel p53-responsive genes.[19] Adenoviral p53 gene transfer was performed in the p53-null PC3 prostate carcinoma cells. A total of 111 genes among 14,500 genes targeted by the Affymetrix U133A microarray were significantly repressed by p53 expression, and this result has been confirmed by reverse transcription-polymerase chain reaction (RT-PCR) of 20 randomly selected genes. 41% of the repressed genes are cell cycle components. In LNCaP prostate carcinoma cells, which show wild-type p53, using p53-specific siRNA, the authors increased the transcription of the same genes. These experiments demonstrated also that these genes were regulated in a p53 dependent manner, which did not involve p21.

This study adds value to previous efforts which used DNA microarrays[20] in demonstrating that p53 is one of the most powerful molecules of the cell cycle machinery (reviewed in ref. 21). In this report, among the 111 downregulated genes studied, 45 genes are cell-cycle elements. According to Spurgers et al,[19] the majority of them were involved in the M-phase during the spindle formation (18 genes described), or in the DNA-synthesis phase (16 genes found), which is known to be the most vulnerable period of the cell cycle. The study design is impeccable regarding techniques and statistical data analysis, though semi-quantitative RT-PCR was performed in 20 randomly chosen genes and not in all 45 genes. Clearly, these in vitro findings may not accurately represent the molecular events taking place in the actual tissue from which cells were derived, and correlations with other cancers are not possible at the present moment. The wide range of biological effects due to p53 can in part be explained by its transcriptional activation of the expression of a number of target genes including *p21^{WAF1}, GADD45, 14-3-3 sigma, bax, Fas/APO1, KILLER/DR5, PIG3, Tsp1, IGF-BP3* and others (reviewed in ref. 22). Some p53-repressed genes require the transactivation of *p21^{WAF1}*, a cyclin-dependent kinase inhibitor, which can facilitate the formation of RB/E2F complexes that act to repress E2F target genes.[22] Interestingly, in the Spurgers and colleagues' study[19] a large number of p53-repressed, cell-cycle regulatory genes are E2F transcription factor targets; however after DNA damage if the p21 protein activity is prevented using siRNA, these cell cycle genes are still repressed. This finding, in accordance to other precedent reports,[23,24] is pertinent to a p53 regulatory mechanism which is p21-independent.

The study by Spurgers et al.[19] not only provides the demonstration of novel p53 repressed genes, but also points on the links to the NF-Y transcription factor. Among the studied genes, *cyclinB2, CDC2*, and *CDC25C* repression was NF-Y dependent, confirming previous data on G_2 phase cell cycle arrest.[25] In addition, the authors provide the first evidence that CDC25A is a target of p53-dependent transcriptional repression. Specifically, they hypothesized that CDC25A may be the target of two arms of the DNA damage response, the rapid degradation via the proteasome, and p53-dependent repression at the transcriptional level (Fig. 1).

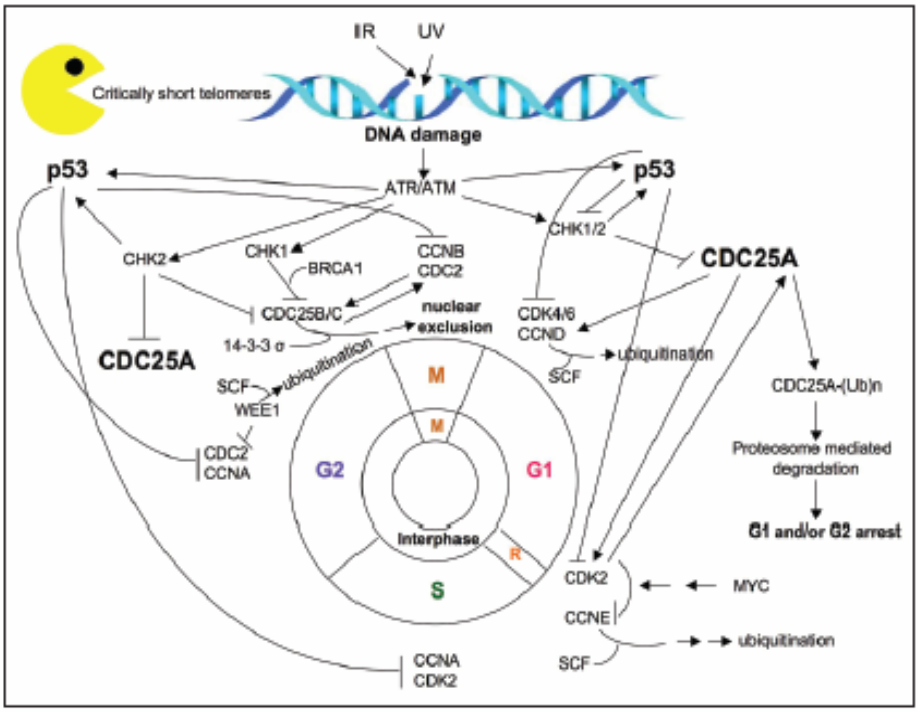

Figure 1. Ataxia-Telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) are members of the phosphatidyl inositol 3-kinase-like family of serine/threonine protein kinases (PIKKs), and play important roles in the cellular response to DNA damage. Activation of ATM by ionizing radiation results in the activation of signal transduction pathways that induce cell cycle arrest at G_1/S, S and G_2/M. In this figure emphasis is given to CDC25 phosphatases, which are involved in a complex web of cellular interactions wherein they function to regulate cell-cycle progression under normal cell growth and in response to DNA damage.

In Spurgers and collaborators' work,[19] p53 is found at the promoters of *cyclinB2, CDC2*, and *CDC25C* before and after DNA damage, at regions that harbor CCAAT sequence elements but no consensus p53 DNA binding sites (mapped by in ref. 26). This could be explained by the fact that even in the absence of consensus DNA binding sites, p53 may directly or indirectly repress the promoter of these regulated genes.

The *CDC25* gene was first identified as a regulator of the cell cycle in yeast and Drosophila. Its product is a tyrosine phosphatase that dephosphorylates specific tyrosine/threonine residues on cyclin-dependent kinases (CDKs). In most eukaryotic organisms at least three splice variants have been identified: CDC25A, CDC25B, CDC25C, which are essential regulators of G_1-S, G_2-M cell cycle transitions, and mitosis, where all three isoforms show specificity towards CDK1-CCNB (reviewed in ref. 27). Regarding NF-Y or nuclear transcription factor Y, it recognizes a CCAAT motif upstream of higher eukaryotic gene promoters. Elcon et al.[28] demonstrated that NF-Y-binding sites cooccur significantly in promoters of cell-cycle-regulated genes in all the stages of the cell cycle, but have preference for G_2 and G_2/M phases.

Spurgers et al.[19] have considerably improved our understanding of the function of p53 in cell cycle checkpoint circuits. Their work adds further complexity to our knowledge of the possible cross talks among p53, p21 and NF-Y. In recent publications some controversial data emerged. On one side, some authors underlined the relationship between p53 and NF-Y: Ceribelli et al[29] showed a large overlap between p53 and NF-Y targets; Testoni and Mantovani[30] demonstrated in non-neoplastic diseases, such as AEC (Ankyloblepharon-Ectodermal dysplasia-Clefting) and EEC (Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate) syndromes, that NF-Y may be a molecular target of p63, a member of p53 family. Yun et al.[31] still recognized NF-Y involvement in transcriptional repression but in a p53-p21 signaling pathway. On the other side, p53-mediated repression of cyclin B1 (CCNB1) was found independent of p21 and NF-Y.[32] It was also reported that p73α and p73β isoforms can decrease the levels of CCB1 depending on the presence of Sp1-binding sites and independently of the NF-Y binding sites.[33]

Even if our information regarding p53 regulatory mechanisms is sometimes contradictory, elegant reports on gene therapy and pharmacological products inhibiting p53 demonstrate a marked progress in its application to clinical settings. Efforts have been directed at restoring wild-type p53 by retrovirus or adenovirus (Ad5CMV-p53), and destructing wild-type-p53-deficient cells by modified adenovirus (ONYX-015). Small-molecule modulators of p53 inhibit p53-MDM2-binding (Gankyrin,[34] RITA,[35] and Nutlins[36]); others act by direct activation (polyamines) or reactivate wild-type p53 in mutant cells, as for PRIMA-1,[37] CP31398, Ellepticine and Pifithrine. Regarding CDC25 phosphatases and p53, there are several CDC25 inhibitors. Among them, NSC663284, NSC95397, and BN82685 are quinone-derived compounds that are probably the most potent CDC25 inhibitors to date.[27] They are equally active in all CDC25 phosphatases in vitro and may cause cell cycle arrest, depending on the cell type examined.[27] Like for PRIMA-1 molecule, the above CDC25 inhibitors are toxic at high doses. The aforementioned compounds are among the first of a series of cell-based drug strategies and pharmacological refinements which may improve preclinical testing and efficacy of therapeutic protocols in cancer patients.

CONCLUDING REMARKS

DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage. Originally, a checkpoint was defined as a specific time in the cell cycle, in which the integrity of DNA was "checked" before allowing progression through the cell cycle. Today, the term "checkpoint" has a broader significance and is applied to entire ensemble of cellular responses to DNA damage, and p53 justifies this last meaning because it is involved in the arrest of cell cycle progression, induction of DNA repair, and apoptosis. In the Spurgers et al.'s study,[19] it becomes even clearer that p53 monitors the integrity of the genome assuring a constant surveillance, operating during the entire cell cycle. Many questions are raised from this study. The elementary goal is, since p53 is such a proficient molecule in several distinct points of the cell cycle, to continue to search which other p53-dependent genes are involved and their possible mechanisms of action in diverse cancer cell lines. DNA microarrays study global transcription profiles, and can also be developed with the additional aim to identify common p53 mutations with good specificity. Needless to say, this technique cannot characterize some pivotal events of the regulatory networks that underlie the transcriptional modulation, such as the actual protein turnover, alternative splicing, conformational changes, post-transcriptional modification and subcellular localization. However, DNA microarrays are at present the only means of screening all the human genes from a single template, allowing us to take a panoramic, although short-timed snapshot of our whole genome at work. Accompanied with other techniques, DNA microarrays undoubtedly contribute to cancer research and will enhance the characterization of more genes and their functions, and foster the drug discovery process.

So, good news is tempered by bad ones: good news is the continuing work leading to new findings on the mechanisms of action of p53, and the improved reliability of microarray approaches; bad news is the complexity of the p53-mediated network (reviewed in ref. 38), the cytotoxicity that CDC25's inhibitors show at high doses. Examples of "obstacles" in this field are the multiple p53 family isoforms (reviewed in ref. 39 and 40), the CDC25 isoforms and probably even the differences between cell types, not to mention, of course, that not all cancer events are explained as p53-dependent processes.[33,41,42] Results from recent analyses of genome-wide data expand our understanding of the role of p53 in shaping the human genome and offer insights into potential therapeutic strategies. Since the cell cycle is undoubtedly a p53 family affair, we must not only develop drugs, but also improve our understanding of how they work and how they can be improved. Based on a deeper knowledge of p53 biology, for instance, it is expected that a pan-CDC25 inhibitor would block cell cycle progression. This paper will renew the challenge to interpret new information about p53, working towards a more coherent picture of this protein and further development in cancer therapy.

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3.2.2. How Does the Human RUNX3 Gene Induce Apoptosis in Gastric Cancer? Latest Data, Reflections and Reactions.

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Journal Club

How Does the Human RUNX3 Gene Induce Apoptosis in Gastric Cancer?

Latest Data, Reflections and Reactions

Paraskevi Vogiatzi[1,2,3]
Giulia De Falco[1,3]
Pier Paolo Claudio[3]
Antonio Giordano[1,3,*]

[1]Department of Human Pathology and Oncology; [2]Department of Molecular Biology; Medical Genetic Unit; University of Siena; Siena, Italy

[3]Sbarro Institute for Cancer Research and Molecular Medicine; College of Science and Technology; Temple University; Philadelphia, Pennsylvania USA

*Correspondence to: Antonio Giordano; Sbarro Institute for Cancer Research and Molecular Medicine; College of Science and Technology; Temple University; Bio Life Science Bldg. Suite 333; 1900 N. 12th Street; Philadelphia, Pennsylvania 19122 USA; Tel.: 215.204.9520; Fax: 215.208.9522; Email: giordano@temple.edu

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KEY WORDS

Runt-related (RUNX), RUNX3, FoxO3a/FKHRL1, tumor suppressor, gastric cancer

ABSTRACT

RUNX3 is the oldest known gene in the RUNX family. Data have demonstrated its function to be thoroughly involved the neurogenesis of the dorsal root ganglia, T-cell differentiation and tumorigenesis of gastric epithelium. As a TGF-β target, RUNX3 protein is believed to be involved in TGF-β-mediated tumor suppressor pathway; however, little is known about its role in apoptosis. According to recent data reported by Yamamura et al., (*J Biol Chem* 2006; 281:5267-76), RUNX3 interacts with FoxO3a/FKHRL1 expressed in gastric cancer cells to activate *Bim* and induce apoptosis. The cooperation between RUNX3 and the PI3K/Akt signaling pathway component FoxO3a/FKHRL1 suggests the putative role of *RUNX3* in the homoeostasis of gastric cells and in stomach cancer control. Here we discuss recent breakthroughs in our understanding of the mechanisms of *RUNX3* in gastric malignancy and comment on possible future trends and perspectives.

INTRODUCTION

Gastric cancer is a leading cause of death from cancer worldwide.[1] Although many scientific advances have been made in this research arena, major questions are still unresolved.

It is a particularly exciting time for research on the *RUNX3* gene, with as many as 153 papers published to date, of which 42 focus on its relationship with gastric tumor. The novel finding of cytoplasmic retention of RUNX3 needs to be emphasized. While previous reports focused on RUNX3 genetic and epigenetic alterations, Ito et al.[2] posed further interesting questions about "how" and "where" altered *RUNX3* gene or protein levels may contribute to gastric tumorigenesis. Also, Chi et al.[3] suggested that at least part of the tumor suppressor activity of *RUNX3* is due to its ability to induce *CDKN1A* (*p21*$^{WAF1/Cip1}$) expression. Finally, Yamamura et al.[4] showed that RUNX3 works together with FoxO3a/FKHRL1 in the induction of apoptosis by activating *Bim* and may play an important role in tumor suppression of gastric cancer (Fig. 1).

RUNX3 encodes the DNA binding subunit of the heterodimeric transcription factor PEBP2/CBF, and is regulated by the TGF-β/Smad pathway. RUNX3 has frequently been reported to be epigenetically silenced in many types of cancers,[5-16] including stomach cancer. Ito et al.[2] discovered that RUNX3 is inactivated in more than 80% of gastric cancers not only by gene silencing but also by protein mislocalization.

Notably, in 1994, Levanon et al.[17] shed light on the human runt-related transcription factor 3 (RUNX3) gene, and one year later Bae et al.[18] better defined its chromosomal locus at 1p36.13-p36.11, a region that has been suggested to be a tumor suppressor locus in various cancers. The same year, Avraham et al.[19] mapped the homologous gene to mouse chromosome 4. In 2002, it was reported that *RUNX3* may harbor dominant-oncogene properties in T-cell lymphoma,[20] while it seems to exert tumor suppressor properties in gastric cancer.[5,21,22]

RUNX3 belongs to a small family of transcription factors (RUNX), whose name originates from the "runt domain" (RD), found in the *runt* gene of *Drosophila*.[23] The RUNX mammalian family includes three genes, *RUNX1*, *RUNX2* and *RUNX3*; by sequence analysis *RUNX3* was demonstrated to be the oldest of the three genes, and is found in Cnidarians, the most primitive animals, where it regulates the growth of their primitive gastrointestinal system. *RUNX3* is the smallest in size and simplest gene in the RUNX family, and maintains extensive structural similarities with the other family members. All RUNX genes are regulated at the transcriptional level by two promoters, P1 at 3', and P2 at 5', are TATA box-less, harbor multiple TSSs and share the runt domain. The P2 promoter of *RUNX3* is almost ubiquitously expressed, including in stomach cancer cells, whereas P1 is only known to be expressed in a few tissues, such as the thymus and ovary. This may generate confusion with the widely used Rel homology domain and, in fact, it

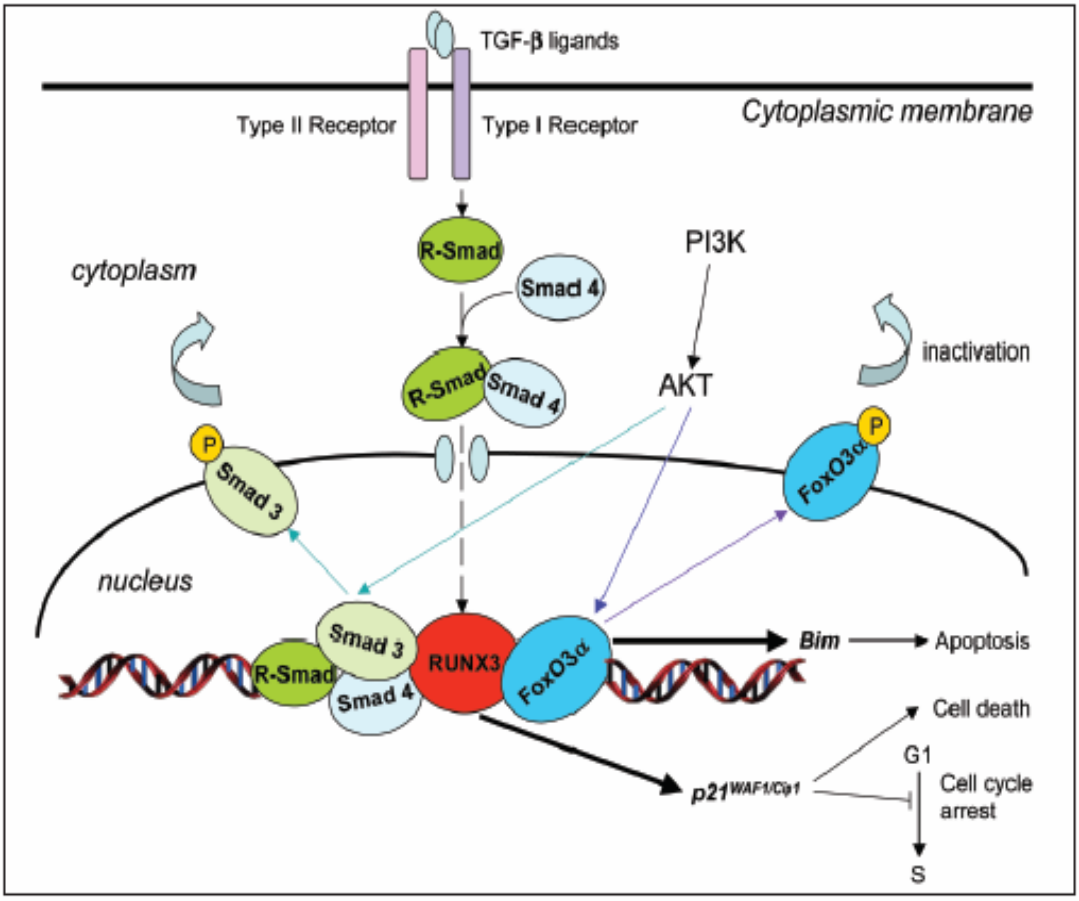

Figure 1. Epithelial growth suppression induced by TGF-β signaling pathway in gastric cancer. The receptor complex consists of two types of transmembrane serine/threonine kinases (type I and type II). R-Smad complexes (receptor-regulators Smad 2/3 with common-partner Smad or Smad 4) are released by the receptors of type I, and translocate into the nucleus, where they cooperate with sequence-specific transcription factors to regulate gene expression. Here emphasis is placed on RUNX3, which cooperates with the transcriptional factor FoxO3α to induce apoptosis in gastric cancer cells. Akt phosphorylates FoxO3α and exports it from the nucleus to the cytoplasm. On the other hand, Akt phosphorylates also Smad3, sequestering it in the cytoplasm. It is also indicated here that RUNX3 overexpression may stimulate TGF-β-dependent endogenous p21 induction, inhibiting cell growth and causing arrest of the cell cycle in early G1 phase.

must be noted that some researchers use the term RHD erroneously in reference to the Runt domain (RD). The RUNX proteins, with their runt domain, bind to the RUNX DNA-motif of the promoter of target genes and allow protein-protein interactions with core-binding factor-β (CBF-β). In this way, RUNX proteins either activate or repress transcription through interactions with other transcription factors and co-activators or co-repressors.

RUNX1 is involved in the process of hematopoiesis and individuals who inherit heterozygous mutations in this gene develop acute myeloid leukemia. *RUNX2* is required for osteogenesis. *RUNX3* instead appears to be expressed in many cell types including mesenchymal cells, blood cells, and dorsal root ganglion neurons; it seems to be especially prominent in epithelial cells of the adult gastrointestinal tract and in hematopoietic cells.

Intestinalization of gastric epithelium is closely associated with gastric carcinogenesis. In this respect, it is worth noting that *RUNX3* is downregulated in intestinal metaplasia.[5] A critical question is whether *RUNX3* inactivation induces intestinal metaplasia.

The *RUNX3* gene is located on 1p36, where loss of heterozygosity (LOH) is often reported in many types of cancers including stomach, colon and pancreatic cancer. Therefore, a major tumor suppressor has been predicted to reside on 1p36. On the other hand, the TGF-β-mediated signal transduction pathway is regarded as a tumor suppressor pathway, since receptors and Smad proteins are often altered in many different types of cancers (reviewed by Akhurst, Derynck).[24] Therefore, *RUNX3* may be the long sought-after tumor suppressor on 1p36 and the RUNX3 protein may be a target of the TGF-β-mediated tumor suppressor pathway (reviewed by Ito and Miyazono).[25] If this model is correct, *RUNX3* could be involved in many cancers in addition to gastric cancer. Studies to further examine this possibility are under way in various laboratories.

Many findings support the role of *RUNX3* as a tumor suppressor gene in gastric tumors even though it does not correspond to the traditional view of tumor suppressor genes such as Rb1 and p53, which follow the "two hit hypothesis" of Knudson.[26] Knudson's model supposes that a gene's mutation of both alleles is required to cause a tumor. Recent reports describe haplo-insufficient genes requiring inactivation of only one allele, and genes inactivated not by mutation but rather epigenetic hypermethylation. Also, cytosolic accumulation can be an alternative way of inactivation, and this may

be the case of *RUNX3*.[2] Better understanding nuclear-cytoplasmic shuttling could offer useful molecular markers and potential therapeutic drugs, and may open new horizons in cancer therapy.

These findings are extremely intensive; nevertheless, results from another group exclude *RUNX3* as a tumor suppressor gene in early-onset gastric carcinomas, which may display molecular characteristics distinct from gastric carcinomas occurring at a later age.[27]

RUNX3 is expressed by normal gastric epithelial cells, but expression is undetectable in all gastric cancer cell lines tested, and it is reduced in primary gastric cancer samples. *RUNX3* expression is reduced in 40% of early-stage carcinomas, and in nearly 90% of advanced cases by hemizygous deletion and hypermethylation of its promoter.[5]

IS *RUNX3* A TUMOR SUPPRESSOR?

When subcutaneously injected into nude mice, *RUNX3*$^{-/-}$ p53$^{-/-}$ gastric epithelial cells developed tumors, while *RUNX3*$^{+/+}$ p53$^{-/-}$ cells did not. This is in agreement with the hypothesis that *RUNX3* is a tumor suppressor gene whose loss of function may cause tumorigenesis (reviewed by Fukamachi).[28] However, one of the pitfalls of this experiment is the lack of mice injected with *RUNX3*$^{-/-}$ p53$^{+/+}$ cells since the authors reported that p53$^{+/+}$ cells exhibited senescence in early passages. More robust data could have been achieved by inclusion in the experiment of a *RUNX3*$^{-/-}$ cell line with an inducible p53.

This "new generation" of tumor suppressor genes, when characterized could be possible drug targets of small molecules or demethylating agents to guide protein reexpression.[21] A variety of detection methods of DNA methylation (e.g., original MSP, quantitative MSP, COBRA, bisulfite sequencing, and so on) may allow investigation of a larger number of CpG islands. But, to prevent confusion, a certain degree of uniformity is necessary for the evaluation of the experimental results, as is the use of gene-specific, not global demethylating agents with gene-specific control of DNA methylation.

Additionally, new question marks in this field of research originate from the observation that a retrovirus activates RUNX3 in T-cell lymphomas in mice.[29]

There are many uncertainties when inferring gene function through the interpretation of knockout phenotypes. For example, the association of *RUNX3* deficiency with defects in cytotoxic T-cell development[30] may also cause secondary phenotypes that are not linked to RUNX3 activity (reviewed by Levanon et al.).[31]

However, a widespread involvement of *RUNX3* in different cancers is certain. Therapeutic approaches focused on the cross-talk between RUNX3 and PI3K/Akt and between RUNX3 and TGF-β might contribute to improve gastric cancer control. Future studies aimed to focus more closely on RUNX3 in the TGF-β pathway, and to investigate the gene in "Smad-dependent" and "Smad-independent" signaling pathways should be encouraged.

Future perspectives and challenges will be provided by the investigation of the interaction between *RUNX3*, the *Drosophila caudal*-related homeobox transcription factor, *Cdx2* and Wnt signaling.[28]

Another recent study in Drosophila shows the interaction between the GATA factor (Serpent) and RUNX factor (Lozenge).[32] This finding defines a new perspective for research using human homologues.

Other unresolved question is how RUNX3 regulates gastric epithelial differentiation through other genes such as *hedgehog*, *neurogenin 3* and *intestinal trefoil factor* which, when deleted, result in intestinal metaplasia of gastric epithelial cells in mice (reviewed by Yuasa).[33]

From the first gastric biopsy by Billroth to the recent molecular findings on gastric malignancy, a great deal has been learned; however, the mechanisms characterizing the relationship between gene expression and gastric cancer still need to be deciphered. In the plethora of interacting signaling factors in stomach cancer, recent technical advances such as microarray technology, proteomics, and gene therapy could offer real hope to patients and families for a personalized diagnosis and therapy.

The mechanisms by which *RUNX3* exhibits its functional properties are slowly on their way to being clarified. Gene therapy is a new therapeutic tool but for the moment with relatively poor therapeutic outcome, and it is not restricted to a specific tumor. Anti-sense RNA for *RUNX3* may be of potential clinical value in the gene therapy of gastric cancer.

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4. Prevention of gastric cancer

4. Prevention of gastric cancer

Primary prevention of gastric cancer includes avoiding exposure to known cancer-causing agents, enhancement of host defense mechanisms, modifying lifestyle and chemoprevention. A better diet, containing fruits, fresh vegetables and green tea which are rich in antioxidants, combined with regular physical activity, moderate alcohol consumption and abstinence from smoking, remain the underpinnings of a healthy lifestyle. Many food pyramids have been published, based on the traditional Mediterranean diet (figure 9) which is associated with lower death rates and longer life expectancy (137). Green tea has been claimed to be a potentially effective protector from gastric cancer, thanks to it multiple protective properties: antibacterial, antiviral, antioxidative, antitumor, antiarteriosclerotic and antimutagenic. Anyway, its use is still much discussed. A recent study shows that the green tea extract exerts *in vitro* an effect on cytoskeletal actin remodeling and this provides further support for the use of green tea as a chemopreventive agent (138). The protective activity of green tea *in vivo* is not demonstrated (139), although a recent study conducted on 72.943 Japanese subjects showed a significant inverse association between its consumption and distal gastric cancer in women (140).

The trefoil (TFF) proteins, in particular TFF1, TFF2, TFF3, seem to reduce the risk of carcinogenesis associated with chronic persistent inflammation in the gastrointestinal tract since they act as mucosal protectors and repair lesions (141).

Resveratrol, a dietary phyto-chemical, exerts its antiproliferative action by interfering with the action of endogenously produced reactive oxygen. These data are supportive of the nitric oxide action against reactive oxygen; therefore, a resveratrol-rich diet may be chemopreventive against gastric cancer (142).

It is known that insufficient intake of vitamins A and C could increase the risk of gastric cancer. Fenretinide or N-(4-hydroxyphenyl) retinamide (4-HPR) is a vitamin A

analogue and one of the most promising retinoids for chemoprevention because it has proven to be less toxic than many other vitamin analogues (143). The analogous of vitamin A are currently being tested in a number of chemoprevention trials for breast, prostate, cervical, skin, ovarian, lung cancer and transitional cell carcinoma of the bladder (143). *In vitro* studies revealed that 4-HPR acts at least in part via the RARβ receptor in the BGC823 stomach adenocarcinoma cell line; therefore, 4-HPR could represent a useful tool as a chemopreventive drug for gastric carcinoma therapy (144).

Recently, it has been hypothesized that estrogen may prevent the development of gastric cancer since patients with prostate cancer exposed to estrogen therapy showed to be at a reduced risk of developing stomach tumors (145).

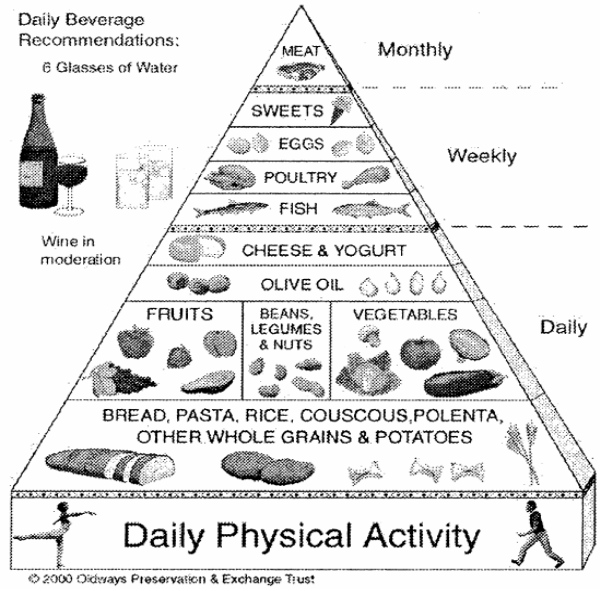

Figure 9. The traditional healthy Mediterranean diet pyramid. *Source*: Gifford, 2002 (ref. 137).

5. Gastric cancer: new therapeutic options

5. Gastric cancer: new therapeutic options

Although techniques for early diagnosis and treatment regimens of gastric cancer have been established, due to the complexity of its genesis and clinico-pathological variability, the efficacy and safety of treatment are still low, especially for advanced tumors. Over the last decade, new drugs and therapeutic approaches have been the object of intense investigations by pharmaceutical and biotechnology companies. Novel vaccines, chemotherapeutic drugs and gene therapy have been developed.

5.1. Traditional therapeutic approaches

5.1.1. Novel vaccines

Phage-display technology is now well established as an important experimental approach in designing new reagents for diagnosis of diseases and development of novel vaccines (146). Recent experiments suggest that a vaccinal approach using a nano-delivery system carrying a tumoral epitope and CpG oligodeoxynucleotides (CpG ODN) as adjuvants may have important implications for gastric cancer therapy (147).

5.1.2. Novel adjuvants

Heat shock proteins are found to be overexpressed in human gastric cancer and seem to play an important role in the progression of this cancer; as such, they could be targets of novel therapeutic approaches. The heat shock protein of oncophage HSP96 can be used as an adjuvant to enhance the immunogenicity of cancer peptide antigens in gastric cancer (148). Recently, it has been shown that anti-sense HSP70 oligomer can abrogate heat shock protein HSP70 expression in human gastric cancer cells, induce apoptosis and inhibit

cell proliferation, thus suggesting that HSP70 is required for the proliferation and survival of human gastric cancer cells under normal conditions (149).

5.1.3. Surgical strategies

Patients with gastric carcinoma are surgically treated via gastrectomy, which could be total or partial. However, total gastrectomy is still associated with a risk of mortality and postoperative complications in 2-4% and 10-20% of patients, respectively (150). In order to define subgroups of patients at a very low or very high risk of tumor recurrence after radical surgery for gastric cancer, a scoring system obtained with a regression model on the basis of follow-up data has been suggested (151). It has been proposed that laparoscopic subtotal gastrectomy for distal gastric cancer is a safe oncological procedure with additional benefits such as reduced blood loss, shorter time to resumption of oral intake and earlier discharge from hospital (152). In recent years, prophylactic gastrectomy has been considered for early detection and curative resection of diffuse-type gastric cancer in patients carrying CDH1 germline mutations.

5.1.4. Chemotherapeutic treatments and their combinations

Single agents such as epirubicin, mitomycin, doxorubicin, cisplatin, etoposide (VP-16), fluorouracil, irinotecan (CPT-11), hydroxyurea, taxanes and the nitrosoureas have low response rates (15% to 30%), brief duration of response, few complete responses and little impact on survival.

Combinations of drugs are more widely used than single agents, largely because of higher response rates, more frequent complete responses and the theoretical potential of longer survival. Drug combination therapy has been shown to improve median survival by about six months in patients with metastatic disease. Chemotherapeutic drug combinations mostly used in clinical practices can be: leucovorin, etoposide and fluorouracil (ELF) or methotrexate, fluorouracil, leucovorin, doxorubicin (FAMTX) or hydoxyurea,

leucovorin, fluorouracil and cisplatin. The last treatment has been shown to have slightly better results since the response rate is 62% and the median survival time is 11 months (153). Other modalities of combined therapies such as radiotherapy and leucovorin/fluorouracil chemotherapy have been applied depending on patient response and tolerance. Over the last few years, the use of preoperative or neo-adjuvant chemotherapy has been found to convert unresectable tumors to resectable ones. Biological therapy with trastuzumab, a recombinant humanized anti-HER-2/neu, combined with chemotherapeutic agents (i.e., doxorubicin) can be applied in gastric cancer treatment successfully (154).

Other non-chemotherapeutic drugs have been recently used in clinical practice. Of these, non-steroidal anti-inflammatory drugs (NSAIDS) are used for prevention or regression of cancer since they may target the cycloxygenase-2 (COX-2) enzyme (155).

Other studies have reported that immunological therapy using a combination of antibodies against the receptors of vascular endothelial growth factor (VEGF) and epidermal growth factor (EFG) could represent a powerful tool for the therapy of gastric cancer since either anti-VEGF or anti-EFG or their combination could effectively inhibit tumor cell growth. These findings support the hypothesis that inhibiting multiple biological pathways that mediate tumor growth is an effective therapeutic strategy for the treatment of gastric cancer (156).

Conventional adjustments in the dose of chemotherapeutic treatment could be ineffective in preventing toxicity and response variability in gastric cancer patients. New strategies for individualizing treatment for cancer patients are becoming an emerging issue in clinical practice. Pharmacogenetics could be an important source of information in this respect, by clarifying the complex correlation existing between an individual genetic profile and the response to therapy in terms of toxicity and activity. It is currently speculated that some host gene polymorphisms involved in metabolism, cellular transport and interaction

with molecular targets of the drugs used in gastric cancer therapy might be prognostic factors in the clinical outcome of specific chemotherapeutic treatments.

Several polymorphisms (i.e., genetic mutations with a frequency >1%) in normal population have been described for genes encoding proteins involved in the metabolism of the drugs, employed in the treatment of gastric cancer. The efficacy of 5-fluorouracil (5FU) treatment is dependent on the mutational status of thymidilate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) genes. Some polymorphisms detected in these genes seem to be implicated in the development of toxicity to 5FU. The polymorphisms of X-ray cross-complementing group 1 (XRCC1), excision cross-complementing (ERCC1) and glutathione S-transferase (GSTP1) genes have been described to have a role in the development of pharmacoresistance to platinum derivatives. Other polymorphisms, such as those of the 5, 10 methylenetetrahydrofolate reductase (MTHFR) gene, seem to be involved in methotrexate (MTX) metabolism as well as the uridine diphoshate-glucuronosyltransferase 1 A1 (UGT1A1), which is involved in irinotecan metabolism. Other genes, such as multi-drug resistance gene (MDR1) and MDR-associated protein (MRP2), are involved in response to irinotecan as well as anthracyclines transport. Overall, these findings suggest that the clinical applications of pharmacogenetics could represent a powerful tool in determining the appropriate drug and dose to be used in each individual patient with gastric cancer (157).

5.2. Gene therapy: Is it promising?

Knowledge of molecular mechanisms governing malignant transformation brings new opportunities for therapeutic intervention against cancer using novel approaches. One of them is gene therapy. This new discipline is based on the transfer of genetic material to an organism with the aim of correcting a disease. The genes can be delivered directly into the subject, using a variety of vehicles named vectors (*in vivo* gene therapy), or delivered into cells isolated *in vitro* that are subsequently introduced into the organism (*ex vivo* gene therapy). Today, cancer treatment is the most frequent application of experimental gene therapy approaches. The known genetic alterations that give rise or contribute to the malignant transformation of cells in gastric cancer are increasing in number and this provides multiple candidate targets for gene therapy intervention. Of course, as we will explain later, special approaches be required to target selected genes, and these approaches often differ from gene therapy applied in monogenic diseases. Specifically, the lack of safe and efficient therapeutic options against stomach cancer is fostering the development of new gene therapy applications for this disease.

5.2.1. On the road to personalizing gene therapy in gastric cancer: proposals for transforming dreams into reality

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Review

On the road to personalizing gene therapy in gastric cancer: proposals for transforming dreams into reality

Paraskevi Vogiatzi[1,2], Marco Cassone[1], and Pier Paolo Claudio[1,#]

[1]Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, PA, USA

[2]Department of Molecular Biology, Medical Genetics Unit, University of Siena, Siena, Italy

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 # Correspondence to:
Pier Paolo Claudio, M.D, Ph.D.
College of Science and Technology,
Center for Biotechnology,
Bio Life Sciences Building, Suite 333,
1900 North 12th Street,
Philadelphia, PA 19122-6099.
Phone: 215 204 9523
Fax: 215 204 9522
E-mail: claudio@temple.edu

Abstract

Gene therapy was proposed many decades ago as a more straightforward and definitive way of curing human diseases, but only recently technical advancements and improved knowledge have allowed its active development as a broad and promising research field. After the first successes in the cure of genetic and infectious diseases, it has been actively investigated as a means to decrease the burden and suffering generated by cancer. The field of gastric cancer is witnessing an impressive flourishing of studies testing the possibilities and actual efficacy of the many different strategies employed in gene therapy, and overall results seem to be two-sided: while original ideas and innovative protocols are providing extremely interesting contributions with great potential, more advanced-phase studies concluded so far have fallen short of expectations regarding efficacy, although invariably demonstrating little or no toxicity. An overview of the major efforts in this field is provided here, and a critical discussion is presented on the single strategies undertaken and on the overall balance between potentiality and pitfalls.

Introduction

Despite a gradual decline in incidence in many Western countries, gastric cancer remains a common lethal malignancy (1). More than 50% of gastric cancer patients are in metastatic stage at the time of diagnosis and, despite therapeutic advances in the past 15 years, overall prognosis remains very poor. Conventional treatment approaches such as chemotherapy, radiotherapy, hormone therapy, and reductive surgery have been used to combat the disease, but often with poor results. In response to a truly urgent need, the researchers are trying to provide more effective therapies to significantly improve survival time. Gene therapy refers to a large spectrum of treatment options for many disorders not limited to cancer. As expected, paradigms for gene therapy are mainly derived from experience with treatment of inherited genetic disorders, and for diseases such as cystic fibrosis (CF), Duchenne's muscular dystrophy, hemophilia, beta-Thalassemias, and Severe Combined Immune Deficiency (SCID) (2-6), realistic options for treatment have been established.

Gene therapy has been proposed as a “definitive solution” in cancer care due to its potential ability to terminate tumor cells by reverting their very first malignant features. This research field is witnessing a growing number of technical advancements and new applications, though results obtained so far are prominent in preclinical stages only, with little therapeutic benefits in clinical trials. Even when applied to the cure of a single disease such as gastric cancer, gene therapy encompasses a broad range of potential new therapeutics, and needs to be explained and discussed with a critical and prudent approach, presenting both its promises and pitfalls. Specifically, in this review article, we summarize developments in the overall picture of gene therapy that have paved the way to clinical trials in the gastric cancer field and some of the challenges and possible limitations in this cancer type. Using such knowledge we discuss novel engineering techniques as well as targeting strategies and possible combinatory regimens towards improvement of the therapeutic efficacy.

Gene therapy can be classified according to its different end results. In “gene replacement”, the malignancy harbouring a defective tumor suppressor gene is treated by the transfer of a normal or super-active version of the gene. “Gene silencing” encompasses a series of different strategies used to impair the expression of oncogenes. Among these, “antisense therapy” is obtained by introducing complementary oligonucleotides which bind to the mRNA or DNA and prevent translation or transcription, respectively. “Cytotoxic gene therapy” fosters the tumor cells to produce an enzyme capable of converting a non-toxic compound or "prodrug" into a physiologically active agent or cytotoxic agent. “Immunotherapy” improves the host's immune response to antigens beared by a particular tumor, while in “drug resistance reversal” a cascade of events leading to overexpression of resistance proteins, such as drug efflux pumps, is modulated and brought to normal levels.

Historical background

Ferrari and colleagues (7) used a retroviral vector to generate peripheral blood lymphocytes capable of transducing adenosine deaminase (ADA), an important enzyme in the purine catabolic pathway. Those lymphocytes, obtained from patients affected by ADA-negative SCID, were injected into SCID mice restoring immune competence. The experiments demonstrated that gene transfer is necessary and sufficient for development of specific immune functions *in vivo* and has therapeutic potential. Blaese et al (8) reported results of the first gene therapy clinical trial for ADA-deficient SCID, concluding that gene therapy was a safe and effective option for the treatment of some patients with this severe immunodeficiency disease. In fact, *ex vivo* retroviral-mediated transfer of the *ADA* gene was performed on the T cells of 2 children with mild SCID: a 4-year-old and a 9-year-old started gene therapy September 14, 1990 and January 1991, and received a total of 11 and 12 infusions, respectively. After 2 years of gene treatment both patients presented normalized blood T lymphocytes counts, and integrated vector and ADA gene expression in T cells persisted.

Only 2 DNA-based pharmaceuticals have received approval from regulatory agencies: an antisense oligonucleotide formulation, called Vitravene (USA, 1998) and an adenoviral gene therapy product, called Gendicine (China, 2004). Fomivirsen sodium, or Vitravene produced by Isis Pharmaceuticals, an inhibitor of immediate early region 2 (IE2) of human cytomegalovirus (CMV), is active against both sensitive and ganciclovir or foscarnet-resistant mutants of CMV (9), and is used for the local treatment of CMV retinitis in AIDS patients. Gendicine, manufactured by SiBiono Genetech, contains an adenovirus expressing p53, known to trigger apoptosis (10). The approval was based on a large, randomized clinical trial in patients with late-stage head and neck squamous-cell carcinoma (HSNCC) and showed a three-fold increase in complete responses when an Ad-p53 injection was combined with chemo- and radio-therapy.

To date, there have been more than 31,000 publications in the PubMed database regarding the field of gene therapy. Overall, 65% of clinical trials were performed in the US and only 29% in Europe, of which 12% took place in UK, and 6.5% in Germany. Gene therapy has been applied more frequently in cancer diseases (67%), and less frequently in monogenic diseases (8.7%), vascular diseases (8.7%), and infectious diseases (6.6%). The main viruses used as potential vectors for transducing genes into cancer cells are adenoviruses and retroviruses (25% and 24% of clinical trials, respectively). The most widely studied non-viral vectors are liposomes (8.3% of clinical trials). Another approach is the use of direct injection of plasmid DNA (17% of clinical trials), but this technique can only transfect cells immediately adjacent to the injection site, so only a small number of cells can be treated. Latest data released by the *Gene Medicine Journal* Website in 2006 (http://www.wiley.co.uk/genetherapy) show that only 1% of all clinical trials are in phase II/III, and 2.1% are in phase III.

Gene therapy applications in stomach cancer

Gene replacement

Many approaches other than surgery, radiations, and conventional chemotherapy have been suggested as potential candidates for the future treatment of gastric cancer. Among gene therapies, introducing tumor suppressors that may be inactivated in tumors would be a straightforward option. TP53 or p53 protein is a notable key molecular node, which involves several pathways including cell cycle, apoptosis, DNA repair, differentiation, angiogenesis, the cytoskeleton and cell motion, the mitochondrial respiration and cellular senescence (11, 12). The *p53* gene is critical for the suppression of tumorigenesis and this property has proven useful in gene therapy applications. Introduction of the *p53* tumor suppressor gene via a recombinant adenovirus inhibits the growth of gastric cancer cells *in vitro* and *in vivo* (13, 14). Very recently, Takimoto and colleagues (15), investigated the combination effect of adenoviral vector carrying wild type p53 (Ad-p53) with histone deacetylase inhibitors (HDACI) and sodium butyrate, on xenografted human gastric cancer cells (KATO-III) and hepatocellular carcinoma cells (HuH7) in nude mice. They confirmed an increased expression of Coxsackie adenovirus receptors with an associated increment of transgene (X-gal) expression with sodium butyrate treatment in KATO-III cells, and highlighted the role of sodium butyrate as a powerful enhancer of p53 gene therapy for cancer.

$p16^{INK4A}$ is a known cell cycle regulator and a tumor suppressor gene, frequently mutated in various human malignancies, including gastric cancer, with a frequency exceeded only by the *p53* gene. Jeong and collaborators in 2003 (16) demonstrated that the replacement of exogenous wild-type $p16^{INK4A}$ delays gastric cell proliferation and promotes chemosensitivity.

The tumor suppressor gene *PTEN* is mutated in a variety of human cancers. The growth regulatory functions of PTEN are primarily mediated via its lipid phosphatase activity, which specifically reduces the cellular levels of phosphatidylinositol 3,4,5-trisphosphate. Genetic approaches have revealed a surprising diversity of global and cell type-specific PTEN-regulated functions that appear to be primarily controlled by modulation of a single phosphoinositide (17). *PTEN* is described as

"mutated in multiple advanced cancers 1" in OMIM database, and Hang et al., 2005 (18) demonstrated for the first time its regulatory role in human gastric growth. They showed that adenovirus-mediated transfer of PTEN inhibited cell growth and induced apoptosis in gastric cell lines and in human gastric tumor xenografts.

The proapoptotic BCL2-associated X protein (BAX) induces cell death by acting on mitochondria. It has been shown that adenovirus mediated transfer of pro-apoptotic *Bax* gene was successful *in vitro* and *in vivo*, and may prove effective in gene therapy of stomach cancers (19).

The caspases are key effector components of apoptosis. A cascade of protease reactions is believed to be responsible for the apoptotic changes observed in mammalian cells undergoing programmed cell death. This cascade involves members of the aspartate-specific cysteine proteases of the ICE/CED3 family, also known as the caspase family. The adenovirus mediated gene transfer of *caspase-8* in gastric cancer cell lines induces apoptosis in detached carcinoma cells and shows potential activity against dissemination of gastric and possibly other carcinoma cells (20). Fu et al., in 2003 (21) showed that the recombinant expression of *caspase-3*, which works in a common pathway, can induce apoptosis on the SGC7901 gastric cell line.

Recently it has been shown the introduction of the tumor suppressor gene Fhit, which is frequently inactivated in gastric carcinomas, decreases the sensitivity to carcinogens and induces apoptosis in stomach tumors *in vivo* (22), confirming that the increasing number of genes and mechanisms targeted by gene replacement approach is undoubtedly good news in this young research field.

Gene silencing approaches

Double-stranded RNA (dsRNA)-depending posttranscriptional double-stranded RNA, better known as RNA interference (RNAi), is a very promising gene-silencing technique suggested by a physiological regulatory phenomenon first recognized in *Caenorhabditis elegans* (23). RNAi technique has been used to knock out genes in many embryo and animal cell lines such as Hela, HEK293, and P19 (24, 25). In RNAi, dsRNA degrades homologous mRNA and hence blocks the

expression of the corresponding gene. Although the mechanism of RNAi has not been fully elucidated, RNAi shows great value in functional genomics studies and gene therapy as a simple and effective gene knock-out tool.

The tumor suppressor gene *E-cadherin* or *CDH1* is a specific calcium ion-dependent cell adhesion molecule. Germline CDH1 mutations are observed in siblings with an inherited susceptibility to diffuse gastric cancer (OMIM 192090). Zheng et al., 2005 (26) suppressed CDH1 expression and tumor invasion in MKN45 gastric cell line by RNAi technique, demonstrating the metastatic ability of *CDH1* and the potent role of this genetic approach.

A novel approach for ameliorating chemotherapy of gastric carcinoma through the X-linked inhibitor of apoptosis (XIAP) was recently evaluated *in vitro*. The down-regulation of XIAP via antisense RNA led to apoptosis of gastric cancer lines, correlating with cellular p53 status and activation of caspase-3 (27).

The RNAi technology, and small RNAs (guide RNAs or siRNAs) design is a hot spot for research, but variability in laboratory design schemes is a major drawback that needs to be addressed. Fire et al., in 1998 (23) showed that the dsRNA targeting intron and promoter sequence had no interference effect in *C. elegans*. Elbashir et al., 2002 (28) indicated that 5' and 3' UTRs should be avoided in designing siRNA, taking into consideration the copious protein-binding regions. These protein and translation initiation complexes will affect the combination between siRNA endonuclease complex and mRNA, hence yielding no detectable interference. In plants, dsRNA targeted promoter sequence showed specific inhibition of gene expression and induction of methylation of sequence of interest (29). The siRNAs, which usually range from 21 to 25 nucleotides, depending on the species of origin, have the significant disadvantage that their effects are transient persisting approximately 1 week. The long (~500 nucleotides) dsRNAs could produce stable silencing in embryonic mammalian cells, but their utility is limited because of the restriction of cells that lack endogenous, non-specific responses to dsRNA. Paddison et al., in 2002 (30) proposed short hairpin RNAs (stRNAs) as valid experimental tools produced exogenously or *in vivo* from RNA polymerase III

promoters. The stRNAs permit the creation of continuous cell lines in which the target gene suppressed is stably maintained by RNAi and may be useful for the construction of transgenic animals. On this direction, Silva and colleagues, in 2005 (31), generated large-scale arrayed, sequence-verified libraries comprised of more than 140,000 second-generation short hairpin RNA expression plasmids, targeting the most known and predicted genes in human and mouse genomes available to the scientific community for investigation of individual gene functions and genomic approaches.

Other gene silencing approaches include the use of dominant negative mutant alleles, which bind a protein complex inhibiting protein function, the anti-sense oligonucleotides, and the ribozymes. Min et al., 2005 showed the blockade by adenovirus-mediated expression of a truncated dominant negative insulin-like growth factor (IGF) I receptor which sensitized the gastric cancer cells to chemotherapy and suppressed their peritoneal dissemination *in vivo* (32).

The idea of using an anti-sense oligonucleotide (ASO) arised three decades ago (33); however, it may still make "sense" in therapeutic strategies. Kim and colleagues in 2004, observed that administering bcl-2 anti-sense oligonucleotides induced a downregulation of the anti-apoptotic protein bcl-2 and thus increased significantly the sensitivity of stomach cancer to chemotherapeutics *in vivo* (34). Unfortunately, only a small number of anti-sense oligonucleotides are currently used in clinical trials, including gastric cancer, because of some toxicity observed in patients, such as impairment of complement and coagulation cascades, thrombocytopenia, hyperglycemia, and hypotension, attributable to the chemistry of the ASOs (35).

Bi et al. in 2001 demonstrated that reversion of the malignant gastric phenotype can be achieved through inhibition of the oncogene c-*erb*B2/neu-encoded protein p185 by the specific ribozyme RZerb2 very efficiently both *in vitro* and *in vivo* (36). The finding that ribozymes have RNA catalytic activity is undoubtedly another important step in gene therapy. In theory, ribozymes have a specific advantage over anti-sense oligonucleotides; since each ribozyme's enzymatic activity should result in cleavage of multiple copies of the mRNA target, while anti-sense oligonucleotides

might be expected to interact with only one molecule of mRNA target. However, attempts to introduce modifications that improve the stability of ribozymes also increased the affinity for substrate mRNA (i.e. hybridization strength), thus causing a strong reduction of the catalytic activity (37). This may explain the few reports of ribozymes use in clinical trials; however, alternative modifications or new RNA-based enzymes yet to be described may change the destiny of ribozymes in therapeutic applications.

Cytotoxic gene therapy

In cancer research, the transfection of genes capable of sensitizing malignant cells to a drug, or activating prodrugs into their effective form, is expected to greatly increase the efficacy and selectivity of therapy. The most frequently used suicide gene/prodrug system is the herpes simplex virus (HSV) thymidine kinase (HSV-tk)/ganciclovir (GCV) system that can convert the prodrug GCV (non-toxic) into phosphorylated GCV (active agent). The phosphorylated GCV inhibits the synthesis of DNA in the cells and enhances the destruction of cancer cells via apoptotic and non-apoptotic mechanisms. Indeed, Kwon et al. in 2003 showed that the *bfl*-1 antiapoptotic gene could be used in combination with HSV-tk/GCV to enhance host immune response against colon cancer (38). Chung-Faye et al. in 2001 proposed the recombinant expression of the bacterial enzyme nitroimidazole reductase gene with the prodrug CB1954 in a phase I study as a valid treatment of gastric cancer (39). Ueda et al. in 2001 showed that double transduction by the recombinant adeno vector-expressing carcinoembryonic antigen and cytosine deaminase renders gastric cancer more sensitive to 5-fluocytosine *in vitro* and *in vivo* than the single infection (40). Zhang et al. in 2006 demonstrated that the recombinant retroviral expression of both HSVtk and TNF-alpha genes enhanced the anti-tumor effect *in vivo*, though it didn't produce a significant difference in cell survival rate *in vitro* (41). Although a few reports have been published so far, this area of gene therapy is promising and it should be explored more since it can take advantage of knowledge and

expertise already developed with conventional drugs using the broad variety of physiological mechanisms that can be exploited to create new active therapeutic interactions.

Viral therapy

Adenoviral vectors (Ads) used in clinical protocols mainly target cancer diseases, gastric cancer included, because they have the ability to infect also post-mitotic tissues and are produced at titers high enough to transduce efficiently cells. New recombinant adenoviral vectors address the major disadvantages of those of first-generation also because of the improved safety and capacity to accommodate larger DNAs, and for the reduced inflammatory response (42). The oncolytic viral strategy is exploited using lytic viruses genetically engineered such as adenoviruses, which kill the host cells during their lytic replication cycle and are different from the "classic" gene therapy viruses, which work as gene delivery agents and do not replicate (43-44). Following a success in preclinical studies, they are now under evaluation in clinical trials.

ONYX-015 is a fusion of adenoviruses 2 and 5 and it is to date the most frequently used in clinical trials. Two phase I/II clinical trials showed the efficacy of ONYX-015 in metastatic gastrointestinal cancer (45, 46).

Heideman et al. in 2002 examined re-targeting an adenovector lacking native binding activity to gastric cancer-specific epithelial cell adhesion molecule (EpCAM), by coupling it to a bi-specific single-chain antibody directed at EpCAM; this indirect targeting strategy resulted in higher selectivity for primary cancer cells, with conserved activity (47).

Other experimental approaches of viral therapy targeting cell to cell interaction molecules in gastric cancer have been recently reported. The gene transfer of CD80, a ligand of CD28, into gastric cancer cells using an adenoviral vector *in vitro* and *in vivo* has shown potential efficacy *in vivo* (48).

Adenoviral-mediated gene transduction of NK4, an antagonist of hepatocyte growth factor (HGF), inhibits both peritoneal metastasis and intra-tumor vessels in gastric cancer *in vivo*, regardless of the

level of *cmet/* HGF receptor expression in the tumor cells, and especially in the early stages of peritoneal metastasis (49).

More recently, *in vitro* and *in vivo* use of cycloxygenase-2 tumor-specific promoter-driven conditionally replicating adenoviruses (COX-2 CRAds) with 5/3 chimeric fiber modification has been shown to be a potentially valuable tool for viral therapy of gastric cancer (50).

Immunotherapy

Immunomodulatory gene therapy consists in the induction of cellular immune responses to metastatic lesions. Immunotherapy is usually performed by injecting into the skin of the patient a suspension of irradiated tumor cells that have been transduced with a cytokine to stimulate a systemic immune response against tumor specific antigens. Unfortunately, the fact that only a few tumor-specific antigens act as recognition targets, the activity limited to low tumor burden, and the high financial and labor cost, are at present important obstacles to immunotherapeutic applications.

Tanaka et al. in 2002 evidenced that transfection of the adhesion molecule *ICAM-1* gene to cancer cells *in vitro* and *in vivo* can be effective against the peritoneal metastasis of gastric carcinoma (51). In a later report, Tanaka et al. in 2004, constructed an adenoviral vector, AdICAM-2, that encodes the full-length human ICAM-2 gene under the control of the cytomegalovirus promoter, and investigated its antitumor effects *in vitro* and *in vivo* (52). They concluded that their gene therapy approach might be advantageous for the cure of human scirrhous gastric carcinoma, which develops peritoneal dissemination with high frequency.

Shi et al. in 2005 constructed a nanovaccine coencapsulated with the gastric cancer specific antigen MG7 mimotope peptide and adjuvant CpG oligodeoxynucleotides (CpG ODN 1645) using new nanotechnology as nanoemulsion (53). They evaluated its immunocompetence *in vivo*, concluding that this vaccinal approach may have important implications for gastric cancer therapy. The same scientific group more recently suggested that vaccines based on MG7-Ag mimotope are antigenic and protective against gastric cancer, and that the heterologous primer-boost strategy (both the oral

DNA vaccine and the adenovirus vaccine) increases the efficacy of MG7-Ag mimitope DNA vaccines (54).

Drug resistance transfer

Multidrug resistance (MDR) is characterized by structural and functional resistance of the cells to unrelated drugs and is a serious impediment in chemotherapeutic management of cancer. In a recent study on gastric and pancreatic carcinoma cells, Zhou et al. (2006) demonstrated that adenovirus-mediated enhancement of the c-Jun NH2-terminal kinase (JNK) reduces the level of P-glycoprotein in a dose- and time-dependent manner (55). The MDR phenotype is often caused by drug efflux pumps in the plasmatic membrane of cancer cells. P-glycoprotein, encoded by *mdr1* gene, is the best-characterized drug efflux pump. The authors suggest that adenoviral JNK increases the activator P-glycoprotein binding activity in the MDR cells, and that the decrease of the P-glycoprotein expression level is associated with drug accumulation in the cells, enhancing the sensitivity of the MDR cells to chemotherapeutic agents.

Anti-angiogenesis gene therapy

Angiogenesis plays a pivotal role in facilitating growth and metastasis of solid tumors. The vascular endothelial growth factor (VEGF) is a specific and critical regulator of angiogenesis, seriously considered in angiogenesis-based treatments. Stoeltzing and colleagues in 2004 demonstrated that the direct suppression of Hif-1α decreased the VEGF expression inhibiting gastric tumor growth *in vivo* (56). Anti-angiogenic therapy can be based on transduction in the tissues surrounding the tumor to create an anti-angiogenic environment, rather than on transduction of target genes into cancer cells. Sako et al. in 2004, found that the soluble VEGF receptor sFlt-1 transduced by an adenovector in peritoneal mesothelial cells is able to inhibit the peritoneal dissemination of gastric cancer *in vivo* and to increase the survival of treated animals (57).

Clinical perspectives and future challenges of gene therapy

The first two SCID patients cured by gene therapy are now leading normal lives. Although gene therapy has great promise, many scientific obstacles remain before it becomes a practical form of therapy for most cancers. In the case of gastric cancer, advances in current treatment modalities have been recorded, but the overall clinical outcome so far remains dismal. Some researchers feel it is too early to witness an invasion of the cancer market with products based on gene therapy. The small number of marketed gene therapy products is not necessarily a negative sign, since this is due in part to a severe licensing system. Large-scale production and administration of these agents will require the development of new technology and additional training for physicians and paramedical staff. Coordinated, multidisciplinary efforts engaging clinicians in surgical, medical, and radiation oncology, basic scientists and medical statisticians is necessary. Clinicians will have the important task of enrolling patients in phase I and II trials to test the safety and potential anti-tumor efficacy of new agents or, more likely, of the combination of agents in the optimal temporal relationship to the core surgical operation. Bench research on antisense therapy, monoclonal antibodies, immunotherapy, and biological agents, such as the interferons, interleukins, and vaccines must go on in order to provide new potential agents to be tested.

Regarding the state of the art of antisense therapy as a genetic tool, synthetic siRNAs have been widely tested *in vitro,* but demonstrated a lot of limitations *in vivo*. They have low transduction efficiency, a short half-life, and preferentially target the liver after systemic application; for this reason they are of little use for systemic cancer gene therapy (37). These obstacles may however be overcome by the expression of siRNAs from targeted viral vectors (58).

The minor toxicities observed in clinical trials of anti-sense oligonucleotides have been readily reversed. This suggests that at least some gene therapy medications can be safely administered to cancer patients. New generations of modified anti-sense oligonucleotides could further reduce or eliminate the undesirable effects.

Future modifications to the basic immunotherapy may include combination with cytokines or other stimulatory molecules to increase tumor vaccine efficiency and induce antitumor activity both *in situ* and systemically.

Future work must be directed toward parallel developments in the sophistication of ribozymes structure, anti-sense oligonucleotides, and vector designs. At present, formivirsen is the first and only US Food and Drug Aministration (FDA)-approved ASO drug. Phase I trial in humans using AVI-4126, a third generation ASO, which targets *c-myc* mRNA, administered intravenously, demonstrated no toxicity and to be a promising new and safe therapeutic strategy for prostate cancer (59).

Some physicians and scientists have an a priori negative point of view regarding gene therapy, but we would like to suggest a more careful examination of all the advantages and disadvantages, considering the gene therapy recent history and the fact that the negative results of early clinical trials reflect in large part the still embryonic nature of the field. There is no shortage of ideas and applications for gene therapy, but there are important limitations which include low efficiency of gene transfer, poor specificity of response, lack of truly tumor-specific targets, and of course, our incomplete understanding of transcription control (60). The results of some early clinical trials should not be viewed negatively, but instead as reflecting a healthy scientific process and thus helping to define the hurdles that we must overcome in order to succeed with gene therapy**.** The latest progress in developing new systems of *in vivo* gene delivery is promising for the treatment of a number of malignancies, including gastric cancer. Immunotherapy (52) or the latest adenoviral vectors (47) may allow individualized gastric therapy based on the histology or the site of the gastric tumor.

Peer review is essential to evaluate the scientific and ethical basis of gene-therapy studies and clinical trials. It is equally important that scientists present and publish the results of such studies in a balanced way and temper our enthusiasm with practical reality. Despite some pitfalls, the promise

of gene therapy is intact and will likely become part of the overall multimodality approach to treating cancer. The low levels of toxicity observed to date in cancer gene therapy research indicate that combination with conventional treatments can be obtained without increasing treatment-related morbidities. This said, should we abandon the hope for a more effective therapy in cancer obtained using multimodal approaches?

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5.3. Diagnostic and therapeutic applications of epigenetics

5.3.1. Epigenetic therapy program in gastric carcinoma: where do we stand?

DNA methylating markers have been proposed for risk assessment, early detection, prognostic evaluation and as therapeutic targets. Demethylating agents, such as 5-aza-cytidine (azacitidine) or 5-aza-2′-deoxycytidine (decitabine) are DNA methyltransferase inhibitors and have been shown to increase expression of *de novo* methylation–silenced genes and to induce cell differentiation, apoptosis and growth suppression in a variety of cancers by global hypomethylation. They are active only in S-phase cells, where they serve as powerful mechanism–based inhibitors of DNA methylation. Moreover, the demethylating effect of 5-aza-2′-deoxycytidine seems to be universal, affecting all human cancer lines. On the other hand, demethylating drugs may have toxic effects on normal cells. Covalent attachment of the various DNA methyltransferases to DNA might be responsible for the citotoxicity of these agents, particularly at high doses (158).

DNA methyltransferase (DNMT) inhibitors reactivate gene expression *in vitro* in various gastrointestinal malignancies and it has been shown that histone deacetylase (HDAC) inhibitors reinforce this effect. In fact, many tumor suppressor genes are methylated in gastric cancer and their re-expression using the inhibitors of DNMT and HDAC could represent an innovative therapeutic approach in the treatment of this tumor. Recently growth inhibition effect of SK-7041 and SK-7068, HDAC inhibitors was documented, related with the induction of aberrant mitosis in human gastric cancer cells. Moreover, SK-7041 had a significant antitumor activity in human gastric xenograft model *in vivo* (159).

Trichostatin A (TSA), a deacetylating agent, can inhibit cell growth and induce apoptosis of gastric carcinoma cells through modulation of the expression of cell cycle regulators and apoptosis-regulating proteins. A recent study successfully used a combination of trichostatin A with demethylating agents in the treatment of gastric cancer, inducing specific apoptotic response in gastric tumor cells (119).

It has been proposed that the demethylation can be one of the cancer-preventive mechanisms in stomach cancer. However, despite these evidences pointing on possible clinical applications of chemicals with demethylating activities, their use in clinical practice needs careful evaluation due to difficulties in correct targeting. Inducing DNA hypomethylation may also have short-term anticancer effects, but there is also the risk to a late increase in the speed of tumor progression (160).

6. Future aims and perspectives

6. Future aims and perspectives

Data accumulated during the last years has considerably increased our knowledge on the mechanisms involved in gastric carcinogenesis. The acknowledged bacterial etiology of a portion of gastric cancer is offering opportunities to advance our understanding in the development of this disease. Several experiments demonstrated that chronic *H. pylori* infection models of Mongolian gerbils led to development of gastric carcinoma (161-163). Anyway, an important question to address for the scientific community is why Mongolian gerbil is the only species in which carcinogenesis has been experimentally induced by infection with *H. pylori*. Clarifying the etiology of gastric cancer could shed light into the pathogenesis of other cancers, especially those in which chronic active inflammation is suspected to play a role, such as carcinoma of the cervix, liver and large bowel, and perhaps even prostate cancer. Intervention to cure the infection at an earlier stage in high risk populations, before atrophy and metaplasia take place, may reduce cancer risk even more markedly than has been reported in trials conducted to date. It is also possible that supplying adequate antioxidants have a role in cancer prevention (164).

Our recent publication (134) was focused on studying for the first time a specific panel of cell-cycle regulators, taking into account the two different histomorphological entities of stomach cancer (124). A very important result is that EZH2 may have a prognostic value, especially for the intestinal-type of gastric cancer which is still the more common. Furthermore, we consider this as just the beginning of an accurate evaluation of the role of Rb2/p130 in this disease, with the aim to better clarify its mechanism in gastric malignancy.

Combining human polymorphisms of susceptibility and bacterial virulence assessment may further identify groups at the higher risk. Our group is highly involved in gastric cancer research in tight collaboration with the pathologists and specialist surgeons

in Siena (Italy) and Philadelphia (USA). In particular studies are ongoing on *CDH1* genetic and epigenetic alterations on gastric cell lines and patients' tumor samples.

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7. Acknowledgements

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9. Curriculum Vitae et Studiorum

9. *Curriculum Vitae et Studiorum*

PARASKEVI VOGIATZI, M.D.

Born in Psachna (Euboea) Greece on the 28th November of 1970. Greek citizen, resident in Rome, Italy. Married with Marco Cassone, Italian citizen, M.D, Ph.D.

Work Address:
Sbarro Institute for Cancer Research and Molecular Medicine
College of Science and Technology
Temple University
BioLife Science Bldg. Suite 333
1900 N 12th Street
Philadelphia PA 19122
U.S.A
vogiatzi@unisi.it
vogiatzi.paraskevi@temple.edu
Pvogiatzi@shro.org

EDUCATION

Secondary School Diploma in "Biology and Medicine", obtained at the "ΓΕΝΙΚΟ ΛΥΚΕΙΟ ΨΑΧΝΩΝ", in Psachna (Euboea) Greece.

Medical Degree in Medicine and Surgery at "La Sapienza" University, Rome Italy. Experimental Thesis on Progressive Ossific Fibrodysplasia (F.O.P) at the Cytogenetics Unit, Pediatric School Umberto I Hospital, La Sapienza University with Prof. Lida Bruni.

Qualification in Medicine by State Medical Examination (Esame di Stato), Siena, Italy.

Computer Science Operator diploma issued by the region of Tuscany.

LANGUAGES

Greek (mother tongue), Italian (excellent), English (very good).

EMPLOYMENT HISTORY

First employment at Performance, a private medical rehabilitation centre in Siena, working with Dr. Franco Merlo and Prof. Francesco Ferrari.

In 2001-2002 at the Department of Internal Medicine at Siena General Hospital "Le Scotte" in the group managed by Dr. Fulvio Bruni, Dr. Luca Puccetti and under the direction of Prof. Alberto Auteri.

In 2002-2003 I have worked for Prof. A. Renieri. I have spent several months working on the diagnosis of Autosomal Recessive Polycystic Kidney Disease (ARPKD), Retinoblastoma, COL4A3 (large screening), COL4A4, COL4A5, Alport Syndrome genes, Alzheimer Syndrome, Oculopharyngeal Muscular Dystrophy, and Genetic Counselling. My research in 2003-2004 was focused on Cytogenetics (particularly in Rett Syndrome and Mental Retardation) and of course Alport Syndrome.

Since 28th October 2004 I started to work on the molecular characterization of gastric adenocarcinoma. My tutor in the academic year 2004-2005 was Dr.Caterina Cinti, Ph.D., IFC-CNR, Siena, Italy.

My tutors for the academic year 2005-06 were Dr. Giulia De Falco, Ph.D., Department of Human Pathology and Oncology, and Dr. Pier Paolo Claudio, M.D., Ph.D., Associate Professor of Biotechnology, Temple University, Philadelphia, USA.

Principle techniques used in my research:

- Extraction of DNA (using Phenol, Kit of Qiagen, "Salting out" DNA extraction)
- PCR
- Electrophoresis in agarose 1,2%, 2%
- Enzymatic Resrictions
- Single Strand Conformational Polymorphism (SSCP) in Hoefer for different diseases
- Acrylamide Gel Electrophoresis and Silver Staining: Linkage Analysis of Autosomal Dominant Polycystic Kidney Disease
- SSCP in Gene-Phor (Pharmacia Biotech) for S.Alport (COL4A5 gene)
- Automatic Sequencing (Applied Biosystems, ABI PRISM 310)
- Denaturing High Performance Liquid Chromatography (DHPLC) (Transgenomic); last training course on the Transgenomic WAVE^{R} System and Navigator^{TM} Software: 07/14-15/2004.
- Cytogenetics
  Karyotype following standard protocol and detection of all chromosome abnormalities as reported by G-banded chromosome analysis.
  Last training course on the techniques of Molecular Cytogenetics as FISH (Fluorescent In Situ Hybridization) and CGH (Comparative Genomic Hybridization) in National Health Institute (Istituto Superiore di Sanità) in Rome on 13-17 September 2004. My tutors were: Dr. Domenica Taruscio, MD, Responsible for National Register for Rare Diseases, and Dr. Giovanna Floridia.
- Course of cDNA- microarray on 09/23-24/2004 by Genetix.
- Course for using Personal Molecular Imager^{R} FX on 10/11/2004 by BIO-RAD.
- Immunohistochemistry
- Cultures of tumoral cells
- Extraction of proteins
- Western Blot
- ChIP Assay (training course on 9/28-10/28/2005 at the Temple University, Sbarro Institute, Philadelphia, USA; my tutor was Dr. Marcella Macaluso).
- FACS Analysis
- MTT Cell Proliferation Assay
- MSP Analysis for the detection of the gene promoter hypermethylation
- Real Time PCR Course (Applied Biosystems, University of Pennsylvania, Philadelphia, PA, USA on 4/26/2006)
- Orientation in Animal Facility (rat and rodents), IACUC Training, Temple University, Philadelphia, PA, USA on 9/5-16/5/2006)

PUBLICATIONS

Research papers

1. Chiara Pescucci, Francesca Mari, Ilaria Longo, **Paraskevi Vogiatzi**, Rossella Caselli, Elisa Scala, Cataldo Abaterusso, Rosanna Gusmano, Marco Seri, Elena Bresin, and Alessandra Renieri. Autosomal-dominant Alport syndrome: Natural history of a disease due to COL4A3 or COL4A4 gene. *Kidney International* 2004;65:1598-603.

2. Eliseo Mattioli*, **Paraskevi Vogiatzi***, Ang Sun, Giovanni Abbadessa, Giulia Angeloni, Domenico D'Ugo, Daniela Trani, John P. Gaughan, Fabio Maria Vecchio, Gabriele Cevenini, Roberto Persiani, Antonio Giordano, and Pier Paolo Claudio. Immunohistochemical analysis of pRb2/p130, VEGF, EZH2, p53, $p16^{INK4A}$, $p27^{KIP1}$, $p21^{WAF1}$, Ki-67 expression patterns in gastric cancer. *J Cell Physiol* 2007;210:183-191.
*** The two Authors contributed equally to this manuscript**

Reviews

1. **Paraskevi Vogiatzi**, Marco Cassone, and Pier Paolo Claudio. On the road to personalizing gene therapy in gastric cancer: proposals for transforming dreams into reality. *Drug News Perspect* November 2006

2. Giovanni Abbadessa, **Paraskevi Vogiatzi**, Lorenza Rimassa, and Pier Paolo Claudio. Targets of antiangiogenic drugs currently used for colorectal cancer: what else can we hit to prolong responses? *Drug News Perspect* March 2007

3. **Paraskevi Vogiatzi**, Carla Vindigni, Franco Roviello, Alessandra Renieri, and Antonio Giordano. Deciphering the Underlying Genetic and Epigenetic Events Leading to Gastric Carcinogenesis. *J Cell Physiol* (accepted paper)

Journal Clubs

1. **Paraskevi Vogiatzi**, Giulia De Falco, Pier Paolo Claudio, Antonio Giordano. How Does the Human RUNX3 Gene Induce Apoptosis in Gastric Cancer? Latest Data, Reflections and Reactions. *Cancer Biol Ther* 2006;5:371-374.

2. **Paraskevi Vogiatzi**, Marco Cassone, Giovanni Abbadessa, Pier Paolo Claudio. The Limitless Role of p53 in Cell Cycle Machinery: Good News or Bad News? *Cancer Biol Ther* 2006;5:1090-1093.

Commentaries

1. Tanaka Y, Miyamoto S, Suzuki SO, Oki E, Yagi H, Sonoda K, Yamazaki A, Mizushima H, Maehara Y, Mekada E, Nakano H. Clinical Significance of Heparin-Binding Epidermal Growth Factor-Like Growth Factor and A Disintegrin and Metalloprotease 17 Expression in Human Ovarian Cancer. *Clin Cancer Res* 2005;11:4783-4792.

   Commentary by **Paraskevi Vogiatzi.** *The Women's Oncology Review* 2005;5:145-147. Publisher: Taylor & Francis Ltd.

2. Sauer MK, Andrulis IL. Identification and characterization of missense alterations in the BRCA1 associated RING domain (BARD1) gene in breast and ovarian cancer. *J Med Genet* 2005;42:633-638.

   Commentary by **Paraskevi Vogiatzi**. *The Women's Oncology Review* 2005;5:213-216.

3. Zorn KK, Bonome T, Gangi L, et al. Gene expression profiles of serous, endometrioid, and clear cell subtypes of ovarian and endometrial cancer. *Clin Cancer Res* 2005;11:6422-30.

   Commentary by **Paraskevi Vogiatzi** and Giulia De Falco. *The Women's Oncology Review* 2005;5:219-221.

4. Wang C, Fan S, Li Z, Fu M, Rao M, Ma Y, Lisanti MP, Albanese C, Katzenellenbogen BS, Kushner PJ, Weber B, Rosen EM, Pestell RG. Cyclin D1 Antagonizes BRCA1 Repression of Estrogen Receptor α Activity. *Cancer Res* 2005;65:6557-6567.

   Commentary by **Paraskevi Vogiatzi**. *The Women's Oncology Review*, March 2006.

5. Moscova M, Marsh DJ, Baxter RC. Protein chip discovery of secreted proteins regulated by the phosphatidylinositol 3-kinase pathway in ovarian cancer cell lines. *Cancer Res* 2006;66:1376-83.

   Commentary by **Paraskevi Vogiatzi** and Francesca Sanseverino. *The Women's Oncology Review*, March 2006.

Book Chapter (invited)

Paraskevi Vogiatzi, Maria Irene Scarano, and Pier Paolo Claudio. Epigenetic targets and drug development.

Book "Epigenome: from the control of cell growth to cancer", Wiley Publisher *in press*.

Oral Presentation

F. Mari, C. Pescucci, I. Longo, **P. Vogiatzi**, R. Caselli, E. Scala, A. Renieri. High phenotypic and incomplete penetration variability in dominant autosomic Alport's Syndrome. Sixth National Conference of the SIGU, Italian Society of Human Genetics, Verona, 24-27 September 2003.

Poster Presentations

1. C. Pescucci, F. Mari, I. Longo, **P. Vogiatzi**, R. Caselli, E. Scala, C. Abaterusso, R. Gusmano, M. Seri, N. Miglietti, E. Bresin, A. Renieri. Autosomal dominant Alport syndrome: natural history of a disease due to COL4A3 or COL4A4 gene. EUROPEAN HUMAN GENETICS CONFERENCE 2004 (ESHG) June 12-15, 2004, Munich, Germany.

2. F. Ariani, E. Scala, R. Caselli, C. Pescucci, I. Longo, F. Mari, I. Meloni, D. Giachino, M. Bruttini, **P. Vogiatzi**, G. Hayek, M. Zappella, A. Renieri. STK9 è mutato nella variante di sindrome di Rett con spasmi infantili. Seventh National Conference of the SIGU-Italian Society of Human Genetics, Pisa, 13-16 October 2004.