Genetic factors related to the histological and macroscopic lesions of the stomach

PhD thesis

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ABBREVIATIONS

A	Adenine
APC	Adenomatous Polyposis of the Colon
ARF	Alternative Reading Frame (p14)
ARMS	Amplification Refractory Mutation System
bp	Base Pair
BSA	Bovin Serum Albumin
C	Cytosine
C	Cysteine
cagA	Cytotoxic Associated Gene A
CI	Confidence Interval
CoCl2	Cobaltous Chloride
CS	Capillary DNA Sequencing
CYP2E	Cytochrome P450, Subfamily IIE
D	Aspartic acid
dATP	Deoxy Adenin TriPhosphate
dCTP	Deoxy Citosoine TriPhosphate
ddATP	DiDeoxy Adenin TriPhosphate
ddCTP	DiDeoxy Citosoine TriPhosphate
ddGTP	DiDeoxy Guanin TriPhosphate
ddNTPs	DiDeoxyNucleotides TriPhosphates
ddTTP	DiDeoxy Timine TriPhosphate
dGTP	Deoxy Guanin TriPhosphate
DNA	Deoxyribo Nucleic Acid
dTTP	Deoxy Timine TriPhosphate
E	Glutamic acid
EDTA	EthyleneDiamineTetraacetic Acid
G	Guanine
H	Histidine
H. pylori	Helicobacter pylori
HA	Heteroduplex Analysis
HAUSP/USP7	Ubiquitin-Specific Protease 7
HLA-DQ	Major Histocompatibility Complex, Class II, DQ

HLA-DR	Major Histocompatibility Complex, Class II, DR
I	Isoleucine
IHC	Immunohistochemistry
IL-1	Interleukin-1 gene
IL-10	Interleukin-10 gene
IL-1RN	Interleukin 1 Receptor Antagonist
IL-1β	Interleukin-1β gene
IL-8	Interleukin-8 gene
IM	Intestinal Metaplasia
IRF1	Interferon Regulatory Factor 1
K	Lysine
KLF6	Kruppel-Like Factor 6 gene
L	Leucine
LI	Labeling Index
LPS	Lipopolysaccharides
MDM2	Mouse Double Minute 2 Homolog
MgCl2	Magnesium Chloride
MHC	Major Histocompatibility Complex
NAT1	N-acetyltransferase-1 gene
NcoI	NcoI Restriction Endonuclease
NSAIDs	Non Steroid Anti Inflammatory Drugs
-OH	Hydroxyl group
OR	Odds Ratio
P	Proline
p53	Tumor Protein p53 gene
PBS	Protein Binding Site
PBS	Phosphate-Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PIK3CA	Phosphatidylinositol 3-Kinase, Catalytic, Alpha gene
PMN	Polymorphonuclear leucocytes
Q	Glutamine
R	Arginin
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid

RNase	Ribonuclease enzyme
S	Serine
SARS	Severe Acute Respiratory Syndrome
SH3	Src homology-3
SNP	Single Nucleotide Polymorphism
SSCP	Single-strand conformation polymorphism
SSPE	Sodium chloride/Sodium Phosphate/EDTA buffer
Streptavidin-HRP	Streptavidin-biotinylated horseradish peroxidas
T	Thymine
T	Threonine
TNF-α.	Tumor Necrosis Factor-α gene
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet radiation

INTRODUCTION

I. GASTRITIS AND PREMALIGNANT CONDITIONS OF GASTRIC CANCER

Macroscopic and microscopically detectable alterations of the stomach can play an important role in the development of gastric cancer. Gastritis, intestinal metaplasia (IM) and atrophy are very common abnormalities, which may cause serious complaints and furthermore, can transform into a malignancy.

The classification of the various forms of gastritis is imperfect in general and misleading occasionally. The Sydney classification of gastritis classify according to the localisation (i.e. pangastritis, gastritis of the body or the antrum) and according to etiology. Due to this classification the criteria of the gastritis can be endoscopic or histomorphologic (acute, chronic and chronically-active) and the grade is normal-, low-, middle- or high-grade [1].

Macroscopic alterations

Erosive Gastritis

Erosive gastritis is gastric mucosal erosion caused by damage to mucosal defenses. It is typically acute, presents with bleeding, but may be subacute or chronic with few only or no symptoms. Diagnosis is established by endoscopy. The causes of erosive gastritis include NSAIDs, alcohol, stress, and less commonly radiation, viral infection, vascular injury, and direct trauma. Superficial erosions and punctate mucosal lesions occur. Deep erosion, ulceration, and sometimes perforation may ensue in severe or in untreated cases. Lesions typically develop in the corpus, but the antrum may also be involved (Figure 1). The acute and chronic forms of erosive gastritis are diagnosed endoscopically.

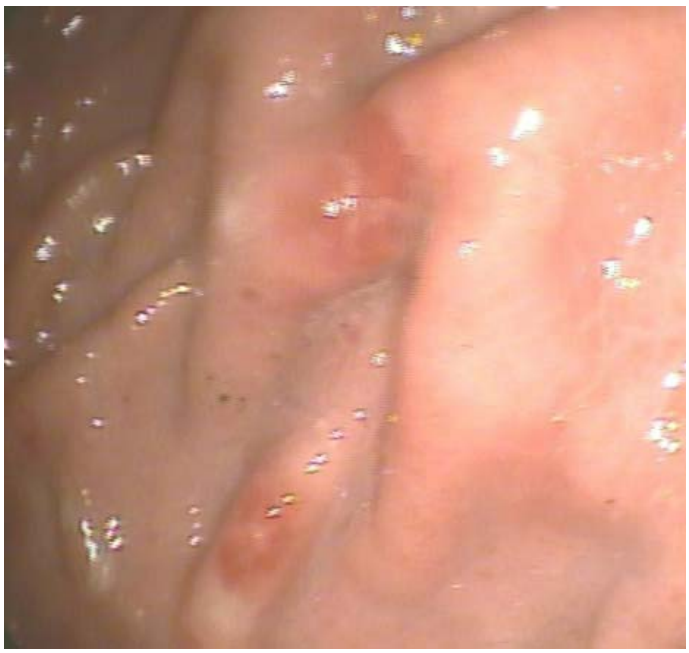


Figure 1 – Endoscopic appearance of erosive gastritis.
The hallmarks of erosive gastritis are mucosal lesions, which do not penetrate the muscular layer of the mucosa.

Non-erosive gastritis

The term "non-erosive gastritis" refers to a variety of histological abnormalities that are mainly the results of *Helicobacter pylori* (*H. pylori*) infection. Most patients are asymptomatic. Diagnosis is established by endoscopy and histology [2].

Microscopic alterations of the stomach

Chronic gastritis, atrophic gastritis (atrophy) and IM are considered precancerous lesions, because of their frequent association with adenoma and well-differentiated adenocarcinoma [3]. This type of gastric cancer, classified as intestinal, generally results from the multistep progression of chronic gastritis, atrophic gastritis, and IM or dysplasia into cancer [4] (Figure 2). This series of changes in gastric carcinogenesis is often initiated by *H. pylori* infection.

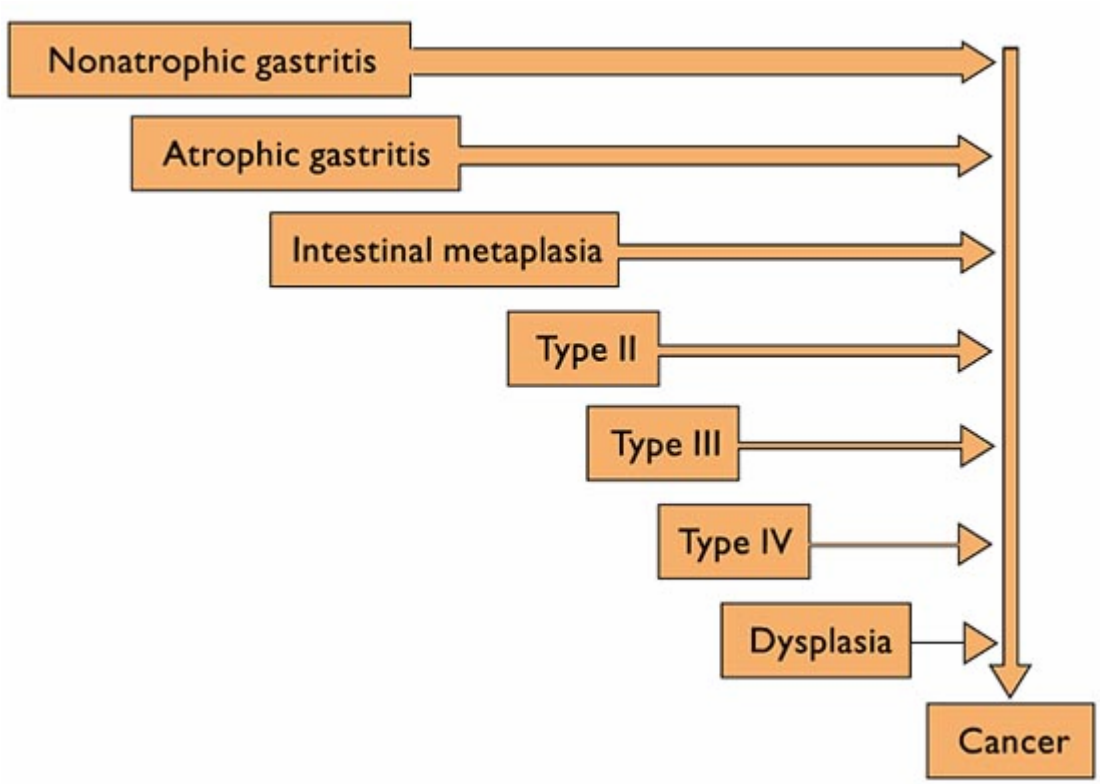


Figure 2 – Stages of cancer development from premalignant lesions.

Source: Graham D, Genta R. Atlas of Infectious Diseases: Intra-Abdominal Infections, Hepatitis, and Gastroenteritis. Edited by Gerald Mandell (series editor), Bennett Lorber. 1997 Current Medicine, Inc.

http://www.images.md/users/image_show.asp

Gastritis

Gastritis is inflammation of the gastric mucosa caused by any of several conditions, including infection, drugs, stress, and autoimmune phenomena. Many cases are asymptomatic, but dyspepsia and gastrointestinal bleeding sometimes occur. Gastritis can be further classified histologically as acute or chronic, based on inflammatory cell type. No classification scheme matches perfectly with the pathophysiology; a large degree of overlap exists.

Chronic gastritis implies some degree of atrophy or metaplasia. It predominantly involves the antrum or the corpus [2] (Figure 3).

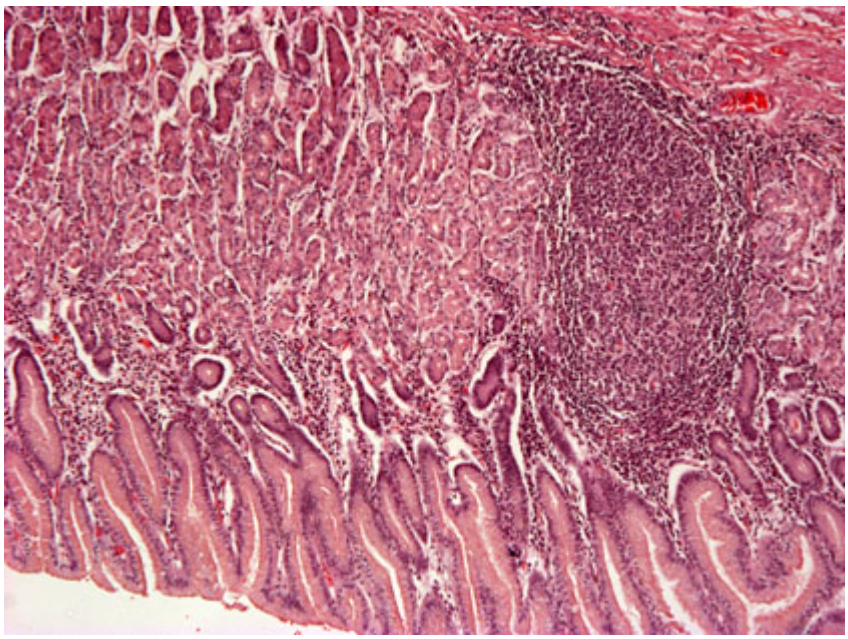


Figure 3 - Moderate chronic inflammation of the gastric mucosa.
40x magnification, HE-staining.

Atrophy

Atrophic gastritis is characterized by loss of specialized glandular tissue, for example, the disappearance of oxyntic glands from the gastric corpus [5]. The development of gastric atrophy was recognized as a critical step in the Correa pathway to intestinal-type gastric cancer, and accumulating evidence indicates that gastric atrophy is much more consistently associated with gastric cancer, than IM. Therefore, gastric atrophy appears to be a better indicator of gastric cancer risk, than IM [6]. Atrophy is generally present as either a multifocal or a diffuse pattern in gastric tissue (Figure 4).

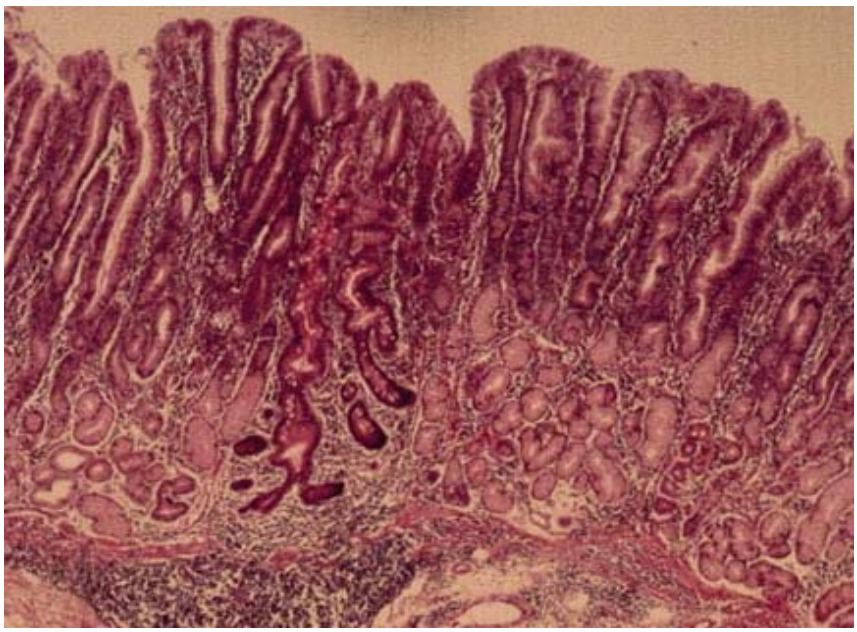


Figure 4 – Multifocal atrophic gastritis with intestinal metaplasia.
40x magnification, HE-staining.

Intestinal metaplasia

Metaplasia is a potentially reversible change from a fully differentiated cell type to another, indicating adaptation to environmental stimuli. In the stomach, intestinal type metaplasia is the most common form. IM is considered a precursor of gastric cancer, especially of its intestinal type; however, the progression of IM to gastric cancer is not obligatory. It is defined as replacement of the gastric mucosa with intestinal-type epithelium. IM is initiated by gastric stem cells; this process is usually triggered by persistent irritation of the gastric mucosa [7]. This series of changes in gastric carcinogenesis are often triggered by *H. pylori* infection. Gastric IM is considered a precancerous lesion because of its frequent association with adenoma and well-differentiated adenocarcinoma [8].

The classification of IM is time-consuming and most laboratories do not perform this routinely, although it would be important in establishing the diagnosis. Determining the type of IM does not probably add further prognostic value to the biopsy; however, the extent and location of IM may identify patients with a high cancer risk [9]. Types III and IV of IM have been shown as more specific markers of premalignancy, with relevance especially to the early and intestinal types of cancer [10].

Additionally, a high PCNA-labeling index in the gastric mucosa, along with a histological diagnosis of incomplete IM, could constitute a reliable prognostic marker of the severity of the lesion [11] (Figure 5).

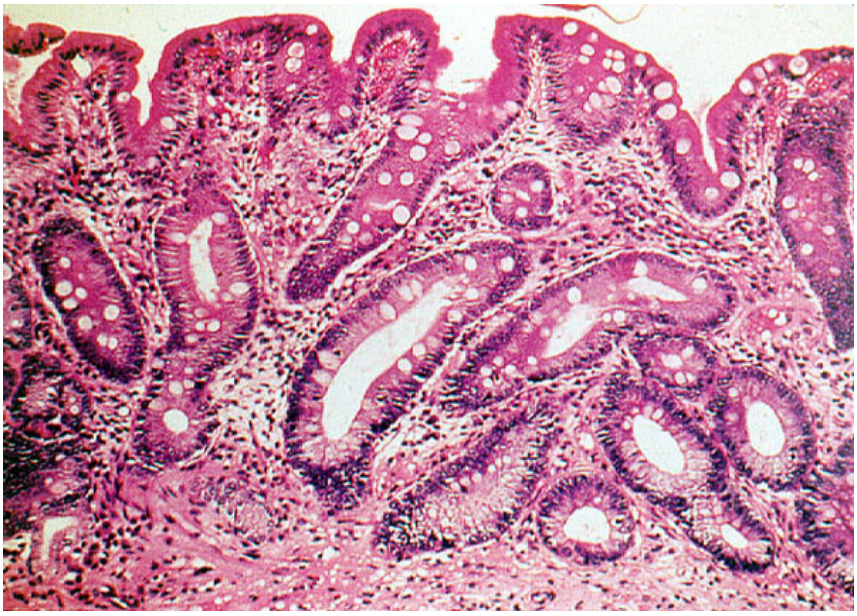


Figure 5 – Gastric intestinal metaplasia.
40x magnification, HE-staining.

Dysplasia

Dysplasia can be defined as abnormalities of cytology and/or histological architecture that are considered neoplastic, but do not meet the criteria of unequivocal carcinoma [12]. Epidemiological studies have shown that IM and dysplasia in the stomach carry a high risk of progression to cancer [13]. Early diagnosis of gastric cancer through the timely recognition of premalignant lesions such as IM and dysplasia is very important. Follow up of low-grade dysplasia and surgical treatment of high-grade dysplasia are both mandatory.

II. THE ROLE OF *H. PYLORI* INFECTION IN GASTRIC DISEASE

H. pylori is a microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa in approximately 50% of the world's population; it is a primary pathogen of benign and malignant gastroduodenal diseases [14] [15]. *H. pylori* resides in the mucous layer covering the gastric epithelium and its presence is almost invariably associated with inflammatory infiltration of the lamina propria. As the epithelial layer is intact,

secondary mediators recruit inflammatory cells into the mucosa. *H. pylori* secretes factors, peptides and lipopolysaccharides (LPS), which are chemotactic for neutrophils and monocytes.

In vivo, H. pylori infection of the gastric mucosa induces the production of various cytokines, including interleukin-1β, -6, -8 and TNF-α. IL-1 or TNF-α alone and γ-interferon in synergism with TNF-α can induce IL-8 production in gastric cell lines. Enhanced IL-8 production may also ensue as a result of *H. pylori* infection and secondary to the accompanying up-regulation of IL-1 or TNF-α. Prolonged production of IL-8 by gastric epithelial cells could result in the recruitment of neutrophils and lymphocytes into infected tissues and therefore, might play a role in the immunopathogenesis of *H. pylori* infection [16] (Figure 5).

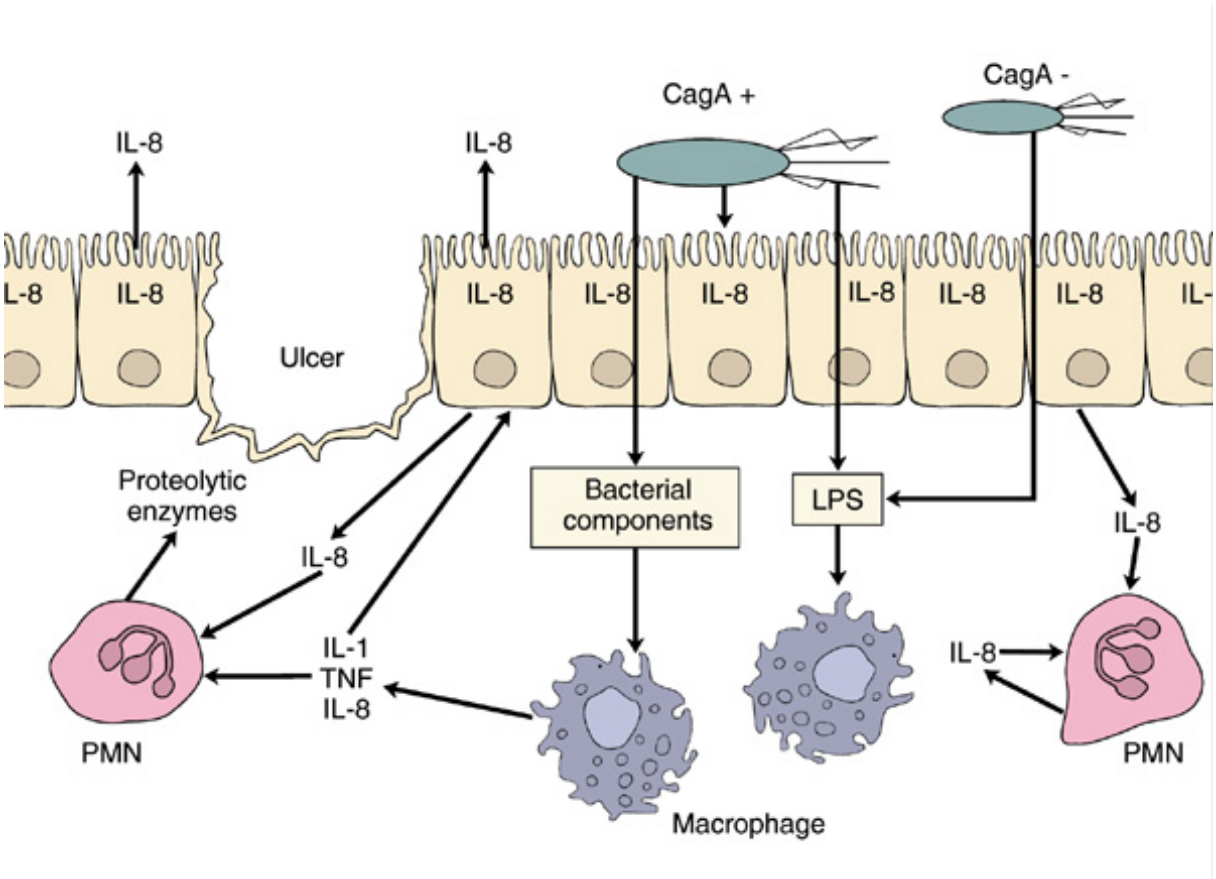


Figure 5 –The mechanism of *H. pylori* induced production of the cytokine IL-8 by gastric epithelial cells.

Source: Walker W. Gastroenterology and Hepatology: Stomach and Duodenum. Edited by Mark Feldman (series editor), Mark Feldman. ©1996 Current Medicine Group LLC.

H. pylori infection is an established risk factor of gastric cancer, but gastric cancer occurs in only a very small proportion of individuals infected with this microorganism [17]. Some evidence for a genetic susceptibility to *H. pylori* infection has been presented. Infection by *H. pylori* may cause gastric cancer by inducing hyperproliferation of gastric cells, interfering with antioxidant functions, and increasing the production of reactive oxygen species and nitric oxide, which may be responsible for oxidative DNA damage. It is suggested that *H. pylori* strain and host genotypes probably influence the risk of carcinogenesis by differentially affecting host inflammatory responses and epithelial cell physiology [18].

H. pylori infection is a risk factor for gastric cancer also because of the atrophy, IM and dysplasia that develop in chronic infection. Factors influencing the risk of these conditions in *H. pylori* infection include the patient's age at the onset of the infection and the presence of cagA – a marker of more pathogenic *H. pylori* strains [19]. Infection by cagA-positive *H. pylori* strains is associated with an increased prevalence and greater intensity of antral atrophy and IM, in addition to more severe gastritis [20].

III. KNOWN OR ASSUMED RELATIONSHIP BETWEEN GENETIC ALTERATIONS OF THE HOST AND *H. PYLORI* INFECTION AND GASTRIC DISEASE, AS WELL AS THEIR ROLE IN INFLAMMATORY AND MALIGNANT PROCESSES

Gastric cancer and premalignant conditions are very frequent causes of morbidity. They are in the focus of various studies, and many mutations are known as genetic factors influencing the development of the cancer of the stomach.

Although bacterial factors have an important role in the pathogenesis of the disease, most evidence suggests that host factors are paramount determinants of progression to gastric cancer. Although most patients infected with H. pylori will newer develop gastric cancer [21] the risk of developing this malignancy is increased up to 3-fold in individuals with a first-degree relative with gastric cancer, and familial clustering is evident in 10% of cases [22]. Infection with *H. pylori* is likely to pervade the whole family; however, even if *H. pylori* infection is controlled, a family history of gastric cancer remains a risk factor for developing the disease, suggesting that hereditary susceptibility indeed exists [6].

Polymorphisms of genes encoding proteins involved in the immune mechanisms, which determine the intensity of the inflammatory response to *H. pylori* infection, may contribute to inter-individual variation in the severity of the infection and progression of gastric lesions [23].

Mutations of APC and other genes

Inactivation of the APC gene is seen in about 20% of early sporadic gastric cancers [24]. The APC gene appears to play a key role in the initiation of a subset of sporadic gastric cancer as well as of colon carcinoma. Loss of heterozygosity at chromosomes 1p, 5q, 7q, 11p, 13q, 17p, and 18p (possible sites of tumor suppressor genes) has been observed in a high proportion of gastric cancer cases [25] [26].

In a human gastric cancer cell line, Nozawa et al. [27] found a point mutation in the IRF1 gene. In a set of 80 sporadic gastric cancers, Cho et al. [28] identified 4 missense mutations in the KLF6 gene – these mutations were absent from corresponding normal tissue, and this suggests somatic mutation. In addition, 16 of 37 (43.2%) informative cases exhibited allelic loss at the KLF6 locus. Mutations were detected also in the PIK3CA gene in 12 of 185 (6.5%) gastric carcinomas [29].

Polymorphisms of the IL-1β gene

The search for genetic factors predisposing to gastric cancer has also capitalized on the observation that patients who progress to gastric atrophy and subsequently cancer, secrete lower levels of gastric acid, compared to patients with duodenal ulcer. Thus, initial genetic appraisal of families with an increased incidence of precancerous changes focused on IL-1β, a well-known proinflammatory cytokine, which is also a powerful inhibitor of acid secretion in the stomach. The IL-1β gene cluster includes both IL-1β and IL-1RN, the gene that encodes the naturally occurring IL-1 receptor antagonist. An increased risk for gastric cancer associated with proinflammatory IL-1β polymorphisms has now been confirmed in many populations throughout the world [30] [31] [32]. More recent studies also detected genetic polymorphisms of TNF-α and IL-10, which – when combined with proinflammatory IL-1β gene cluster polymorphisms – result in a high-risk genotype with a 27-fold or greater risk of developing gastric cancer [33]. Studies have also identified the presence of the –251T allele, located in the IL-8 promoter, as a significant risk factor of gastric cancer [34] [35]. These observations

provide strong evidence for the role of host genetics in progression to gastric cancer and corroborate the relationship between the inflammatory response and the development of gastric atrophy [6].

An increased gastric cancer risk is associated not only with IL-1β but also with NAT1 variants, which may account for up to 48% of attributable risk of gastric cancer. Polymorphisms at the HLA-DQ, TNF-α and CYP2E genes may confer some protection against gastric cancer [36].

Polymorphisms of the IL-8 gene

IL-8, also known as neutrophil-activating peptide-1, is secreted by several types of cells in response to inflammatory stimuli [37]. It has many different functions, including G-protein coupled receptor protein signaling, angiogenesis, calcium-mediated signaling, cell cycle arrest, cell motility, cell-cell signaling, chemotaxis, neutrophil activation and chemotaxis or regulation of cell adhesion [38].

The IL-8 T-251A is a functional polymorphism apparently correlated with the pathogenesis of *H. pylori* related diseases. Compared to the T allele, the A allele is associated with an elevated level of IL-8 in the gastric mucosa, and the presence of this allele is associated with a higher inflammatory score [39]. Ohyauchi et al. investigated the effect of the T-251A of IL-8 on the risk of *H. pylori* related gastroduodenal disease, and concluded that presence of the A allele may be associated with progression to *H. pylori* positive gastric atrophy, and may increase the risk of gastric cancer and ulcer in Japanese people [40].

Polymorphisms of the TNF-α gene

TNF-α is a multifunctional, pro-inflammatory cytokine, which provides a rapid form of host defense against infection, but may be fatal in excess. Various interferons have been shown to exert anti-tumor effects synergistically with TNF-α *in vitro* [41]. Appraising the importance of genetic variation in the TNF-α gene to disease susceptibility or severity is complicated by its location within the MHC, a highly polymorphic region that encodes numerous genes involved in immunologic responses [42].

Five polymorphisms were identified in the TNF-α gene. Polymorphisms in the promoter region, at positions -238 and -308 have been reported to be relevant to

differences in transcriptional activity [43]. G-308A polymorphism causes elevated expression of the TNF-α protein. An association has been found between these variations in the promoter region and susceptibility to infectious diseases [44].

Genetic alterations of the p53 gene

The p53 gene is located on the short arm of chromosome 17. The gene is 20303 base pairs long, contains 10 coding exons (Figure 6), and encodes a 393 amino acid large protein, which is characteristically active in tetramer form. This protein binds to p53 binding sites (PBS) and activates the downstream genes, which arrest growth and/or invasion.

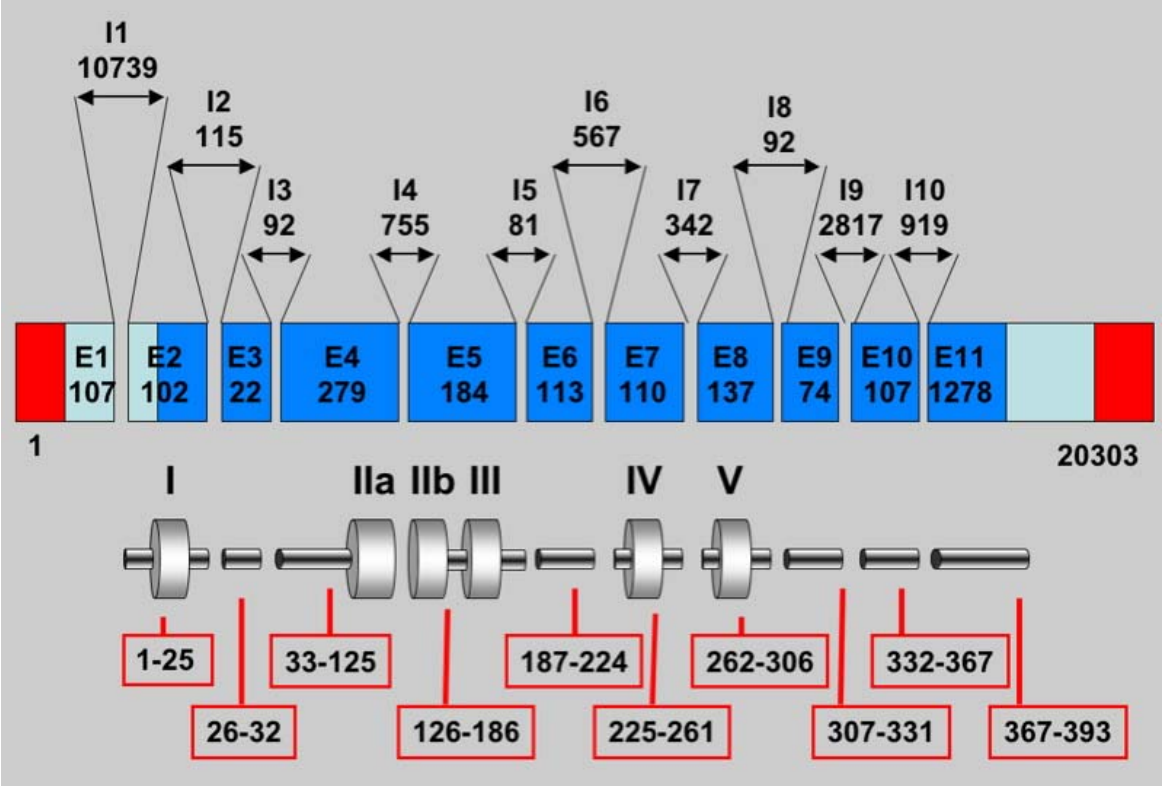


Figure 6 – Organization of the human p53 gene.
http://p53.free.fr/p53_info/p53_gene.html

The p53 tumor suppressor gene plays an important role in the cell cycle. The p53 protein arrests the cell cycle or induces apoptosis in response to DNA damage, thereby allowing the injured cells to be destroyed or repaired before DNA synthesis is re-

initiated [45]. The p53 tumor suppressor protein is of critical importance in the control of cell growth and in the maintenance of genomic stability. It is present at low concentrations in normal cells. The low concentration is maintained by MDM2-mediated ubiquitination and resulting proteolysis. The HAUSP/USP7 ubiquitin hydrolase is thought to regulate MDM2 and to help stabilizing p53. When cells are subjected to stress such as hypoxia or UV radiation, the p53 gene is activated, the levels of its protein product increase and its ubiquitin-dependent degradation is blocked. The resulting elevation of p53 protein level either leads to the arrest of the cell cycle or induces apoptosis. Arrest of the cell cycle allows DNA repair to proceed before mitosis. If the damage is severe, p53 activation results in apoptosis. Hence, functional p53 confers protection against tumor growth [46] (Figure 7).

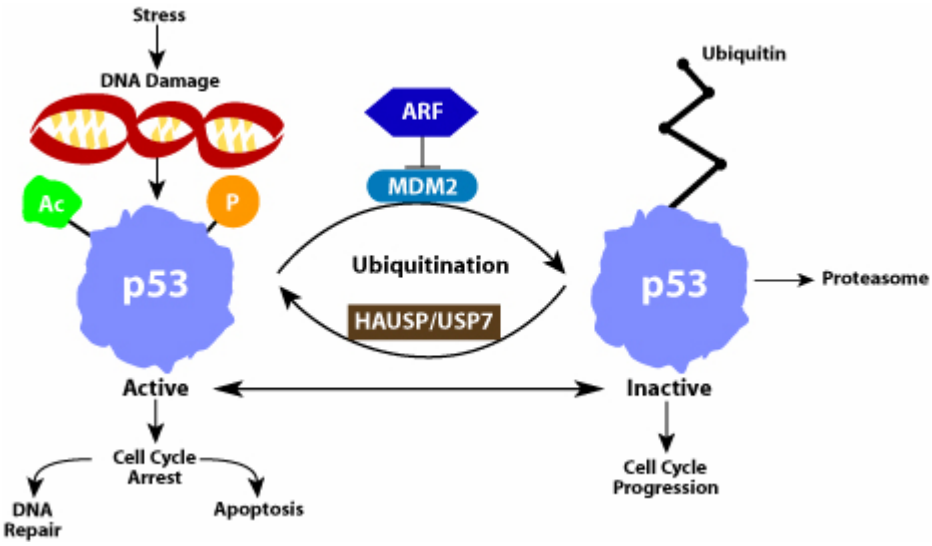


Figure 7 – The p53 gene a "gatekeeper" of life and death.
http://www.emdbiosciences.com/html/cbc/p53_apoptosis_cancer_spotlight.htm

Mutations of the p53 gene are found in more than 50% of all human cancers, belonging to more than 50 different cell and tissue types. This suggests that there is a powerful selection for loss of p53 activity during tumor development. Although p53 mutations do occur in gastric cancer, the role of p53 as a predisposition gene appears to be confined to the Li-Fraumeni syndrome only.

Mutations of the p53 gene in gastric cancer

The development and progression of gastric cancers are generally driven by an accumulation of genetic alterations, which have been detected also in precancerous lesions such as IM adjacent to gastric carcinoma. Among genetic alterations, mutations of the p53 gene seem to be the key factors in the development of gastric cancer.

The most thoroughly examined regions of the p53 gene are exons 5 through 8. In intestinal type gastric cancer, the mutation hotspot is codon 175 (exon 5), which is one of the most heavily affected codon in all types of cancers, along with codon 245, 248, 273 and 282 (exon 7 and 8) mutations. Additionally, codons 173 and 213 may also be affected in gastric cancer. The most common mutations in this type of gastric cancer are C:G→A:T transitions at CpG islands and A:T→T:A transversions [47].

In precancerous IM, codons 248 through 250 (exon 7) of the p53 gene may also be affected [48].

Polymorphisms of the p53 gene

Only two polymorphisms, proline (CCG) to serine (TCG) at codon 47 (exon 4) [49] and arginine (CGC) to proline (CCC) at codon 72 (exon 4) [50], alter the amino acid sequence of p53 protein. Codon 34 (CCC-CCA, proline) [51] and 36 (CCG-CCA, proline) polymorphisms [52], located also in exon 4, do not change the amino acid sequence. The P47S variant is a rare polymorphism affecting a codon conserved during evolution.

Codon 72 is located in the proline-rich region, which is necessary for the protein to induce apoptosis. This polymorphism may affect the structure of the putative SH3-binding domain.

These two polymorphic variants of p53 appear functionally distinct. The difference that the R72 variant induces apoptosis markedly better than the P72 variant may also influence cancer risk [53]. Many studies have investigated the association between p53 polymorphisms and the increased risk for different cancers. Most of the studies reported so far have failed to reveal any effect of the R72P polymorphism on cancer risk. The R72P codon variation may influence progression during gastric carcinogenesis [54]. The R/R genotype may be associated with the development of distal gastric cancer [55].

Shepherd et al. examined exons 5-9 by sequencing and codon 72 by restriction enzyme digestion in 217 gastric cancer patients [56]. Mutations were present in 3.2% of tumors and two polymorphic sites were found at codons 36 and 72. Codon 72 polymorphism was very common. Genotype frequencies were R/R (54%), R/P (33%), and P/P (14%). The genotype of the polymorphic site varied with race ($p = 0.001$): 64% of whites had the R/R genotype, compared with 24% of blacks. The difference in genotype by site, sex, or histological tumor type was not statistically significant ($p = 0.067$). By contrast, polymorphisms at codon 36 were found in 2 patients only. The prevalence of heterozygous P36P polymorphism located in the coding sequence is 4% [57].

Unfortunately, only limited literature is available on the prevalence of R72P polymorphism in gastric cancer preceding IM [55].

IV. OVERVIEW OF METHODS FOR THE DETERMINATION OF POLYMORPHISMS AND MUTATIONS

Heteroduplex Analysis (HA)

HA is a commonly used mutation-screening method, popular because of its simplicity. The technique is based on conformational differences in double-stranded DNA caused by the formation of heteroduplex molecules. These molecules have a mismatch in the double-strand, causing a distortion in its usual conformation. This altered conformation can be detected on polyacrylamide gels, as its migration is slower, than that of the corresponding homoduplex molecules. Heteroduplexes are generated during PCR of a heterozygous individual or by adding mutant and wild-type DNA in the same PCR reaction or by denaturing and renaturing a mixture of mutant and wild-type DNA. Both mutant and wild-type samples are run on the same gel to analyze differences in mobility. Heteroduplex molecules with as little as a single mismatch can show a different mobility in a gel, compared to homoduplex molecules [58].

Denaturing gradient gel electrophoresis

Denaturing gradient gels are used to detect non-RFLP polymorphisms. The small (200-700 bp) genomic restriction fragments are run on a low to high denaturant

gradient acrylamide gel. Initially, the fragments move according to molecular weight, but as they progress into higher denaturing conditions, each (depending on its sequence composition) reaches a point where the DNA begins to melt. Partial melting severely retards the progress of the molecule in the gel, and a mobility shift is observed. It is the mobility shift, which can vary for slightly different sequences (depending on the sequence, as little as a single bp change can cause a mobility shift). Alleles are detected by differences in their mobility [59].

Single-strand conformation polymorphism (SSCP)

SSCP is the electrophoretic separation of single-stranded nucleic acids, based on subtle differences in sequence (often just a single bp), which result in diverse secondary structure and a measurable dissimilarity in mobility through a gel. The mobility of double-stranded DNA during gel electrophoresis is dependent on strand size and length, but it is relatively independent of the particular nucleotide sequence. The mobility of single strands, however, is noticeably affected by very small changes in sequence, possibly one changed nucleotide out of several hundred. Small changes are noticeable because of the relatively unstable nature of single-stranded DNA. In the absence of a complementary strand, the single strand may experience intrastrand base pairing, resulting in loops and folds that give the single strand a unique three-dimensional structure, regardless of its length. A single nucleotide change can dramatically affect the strand's mobility through a gel by altering intrastrand base pairing and the resulting three-dimensional conformation. SSCP analysis offers an inexpensive, convenient, and sensitive method for determining genetic variation [60].

Similar to RFLPs, SSCPs can detect allelic variants of inherited, genetic traits that can be used as genetic markers. Unlike RFLP analysis, however, SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments [61]. As a mutation scanning technique, though, SSCP is more often used to analyze the polymorphisms at single loci [62].

Real-time PCR methods

Real-time PCR is able to detect sequence-specific PCR products as they accumulate in “real-time” during the PCR amplification process. As the PCR product of interest is produced, real-time PCR can detect their accumulation and quantify the

number of substrates present in the initial PCR mixture before amplification began. In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of emitted fluorescence at each cycle, it is possible to monitor the PCR reaction during the exponential phase. Plotting the log of the starting amount of template versus the corresponding increase in the fluorescence of the reporter dye during real-time PCR reveals a linear relationship [63].

There are a few different variations of the procedure, which allow specific detection using target-specific hybridization probes. A mutation or polymorphism present in the DNA prevents hybridization of specific target probes and accordingly, the expected signal is missing. It is also possible to design specific probes for mutations, and in this case, the signal confirms the presence of the suspected genetic alteration.

The advantages of using real-time instead of traditional PCR are numerous. First, there is no need for post-PCR processing such as RFLP, SSCP, or electrophoresis to determine the genotype. The increase in the fluorescent signal is proportional to the number of amplicons generated, and this allows a kind of quantification, which is much more stringent, than that seen during traditional PCR of the original sample [64].

RFLP

Restriction fragment length polymorphism (RFLP) is a laboratory technique for comparing DNA molecules based on their differing nucleotide sequences. The technique is used in genetic fingerprinting, paternity testing, and for the determination of known mutations or polymorphisms. First, purified DNA may be amplified by PCR. Then, the DNA is cut into restriction fragments using suitable endonucleases, which only cut the DNA molecule at specific sequences (restriction sites) that they recognize. These sequences are specific to each enzyme, and can be four, six, eight, ten or twelve bp long. The more base pairs the restriction site contains, the more specific it is – and the higher will be the probability that the enzyme recognizes it as a site to be cut. The restriction fragments are then separated according to length by agarose gel electrophoresis. The distance between the restriction sites varies between individuals, due to polymorphisms, insertions, deletions or transversions. This results in variable-

length fragments, as well as the position of certain amplicons differs between individuals (thus polymorphism) [65].

Capillary sequencing

The key principle of the capillary DNA sequencing (CS) method (or dye-terminator sequencing) is the use of labeled dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. This chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, standard deoxynucleotides (dATP, dGTP, dCTP and dTTP), and fluorescently labeled modified nucleotides (ddATP, ddGTP, ddCTP, or ddTTP) that terminate DNA strand elongation. The dideoxynucleotides are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand therefore terminates its extension, resulting in various DNA fragments of varying length. The dideoxynucleotides are added in a concentration lower than that of the standard deoxynucleotides, in order to allow strand elongation sufficient for sequence analysis. Each of the four dideoxynucleotide chain terminators is labeled with different fluorescent dyes fluorescing at different wavelengths. Automated DNA sequencers carry out DNA size separation by capillary electrophoresis, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms (Figure 8).

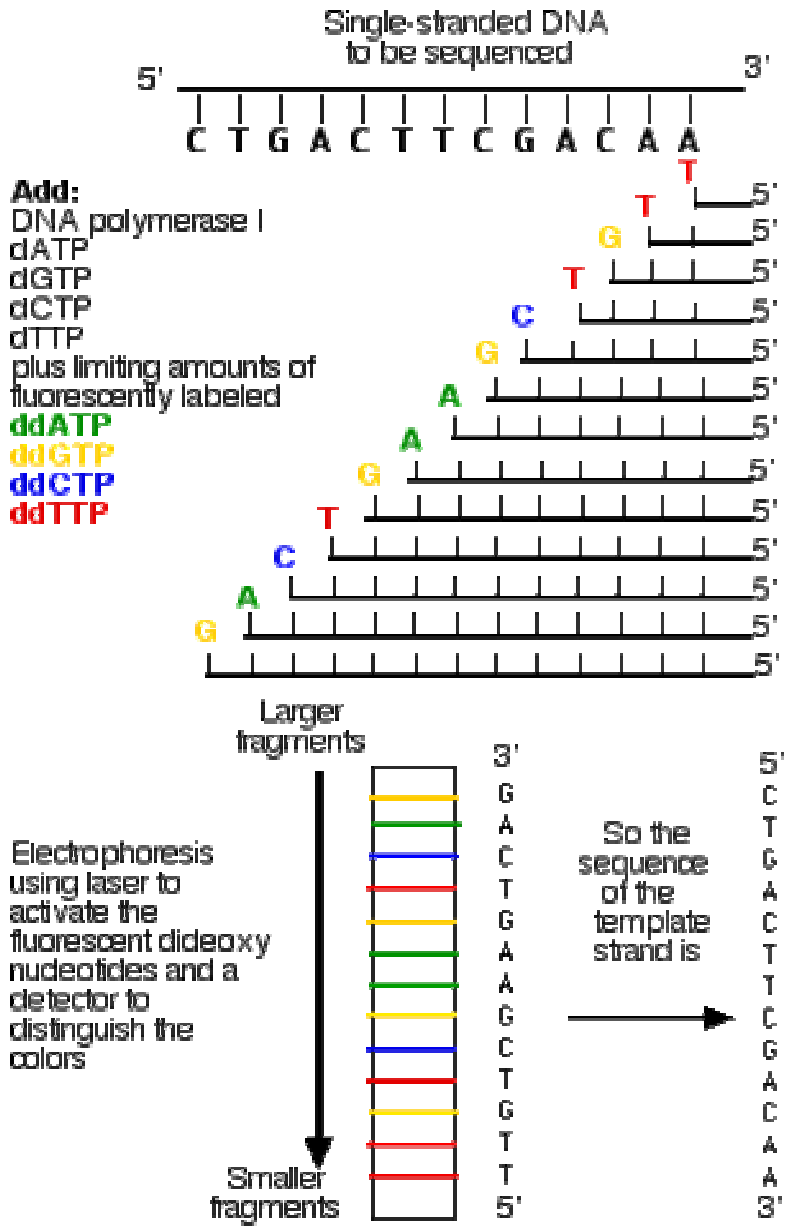


Figure 8 – Scheme of capillary electrophoresis.

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/D/DNAsequencing.html

Sequencing reactions by thermocycling, cleanup and re-suspension in a buffer solution before loading onto the sequencer are performed separately. This method is attractive because of its greater expediency and speed. Currently, it is the mainstay of automated sequencing with computer-controlled sequence analyzers. The dye-terminator sequencing method, along with automated, high-throughput DNA sequence analyzers, is used in the vast majority of sequencing projects, as it is both easier to perform and less expensive than most previous sequencing methods [[66]].

DNA-based microarrays

The most reliable method for mutation detection and polymorphism screening is the "gold-standard" – capillary sequencing (CS). The chip technology should become a novel alternative to CS, and could prove very useful for screening. The main advantage of chip technology, in comparison to traditional CS, is its rapidity and large capacity.

DNA microarray is a high-throughput technology, which consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides ("features"), each containing picomoles of a specific DNA sequence. This can be a short section of a gene or other DNA element that are used as probes to hybridize a DNA sample (known as the "target") under high-stringency conditions. Probe-target hybridization is usually measured and quantified by fluorescence-based detection of fluorophore-labeled targets, in order to determine the relative abundance of nucleic acid sequences in the target.

DNA microarrays are suitable even for scanning the entire sequence of a genome, to identify genetic variation at certain locations.

SNP microarrays

The SNP microarray is a specific type of DNA microarrays, designed to identify genetic variation in individuals and across populations [67]. Short oligonucleotide arrays can be used to detect SNPs responsible for the susceptibility to hereditary diseases. Generally known as genotyping applications, DNA microarrays are suitable in this fashion for forensic applications, genotyping, rapid screening for or measurement of genetic predisposition to disease, or for identifying DNA-based drug candidates. These SNP microarrays are being used also for the profiling of somatic mutations in cancer, that is, the loss of heterozygosity events, amplifications, and deletions of DNA regions. Amplifications and deletions can also be detected by comparative genomic hybridization in conjunction with microarrays, but may be of limited use in detecting novel copy number polymorphisms by probe coverage.

Resequencing microarrays

Resequencing arrays have been developed to sequence well-known portions of the genome of individuals. These arrays may be used to evaluate mutations or polymorphisms in various individuals and cancers [68].

Some of them offer the possibility to determine a custom-selected region of the genome of a desired species. The GeneChip CustomSeq Resequencing Array (Affymetrix, Santa Clara, CA, USA) allows genotyping 30.000 base pairs of any genes, selected by the user.

Most of them are designed by the array companies, and as such, are able to genotype only a well-determined part of the genome or the whole-genome of a species. Examples include the GeneChip Human Mitochondrial Resequencing Array 2.0 (Affymetrix, Santa Clara, CA, USA), which genotypes the whole DNA of human mitochondria, the GeneChip SARS Array (Affymetrix, Santa Clara, CA, USA) – a standard assay for complete sequence analysis of the corona virus Severe Acute Respiratory Syndrome (SARS), or the GeneChip p53 Probe Array (Affymetrix, Santa Clara, CA, USA), which genotypes the whole coding region of the human p53 gene.

The GeneChip p53 Probe Array is a resequencing tool, which performs sequence variation analysis (identifies nucleotide base changes such as point mutations and polymorphisms) on exons 2 through 11, and interrogates flanking intron sequences for spice junction analysis of the human p53 gene. The GeneChip probe arrays are manufactured using a technology that combines the photolithographic method with combinatorial chemistry. Over 50.000 different oligonucleotide probes can be synthesized in a 1.2 cm by 1.2 cm area. Each probe type is located in a specific area of the probe array, called a "probe cell". In an approx. 50 μm by 50 μm GeneChip p53 Probe Array, each probe cells contains approximately 10^7 copies of a given probe

Probe arrays are manufactured in a series of cycles. The glass substrate is coated with linkers, containing photolabile protecting groups. Then, a mask is applied to expose selected portions of the probe array. Illumination removes photolabile protecting groups, thereby enabling the addition of selective nucleoside phosphoramidite only at previously exposed sites. Next, a different mask is applied and the cycle of illumination and chemical coupling is performed again. By repeating this cycle, a specific set of oligonucleotide probe is synthesized, each probe type in a known location. The completed probe arrays are packaged into cartridges (See Figure 9 and Figure 15) [69].

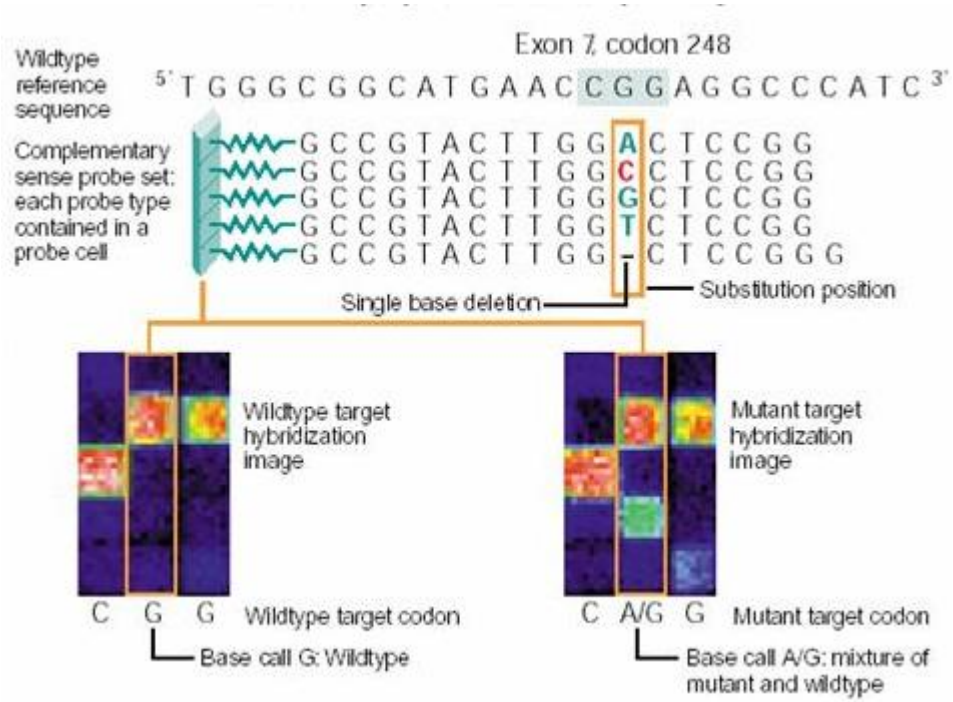


Figure 9 – GeneChip p53 Probe Array Design.

Probes in the array are arranged in sets of five. Each probe within the set is complementary of the reference sequence, except for a mismatch position, called the "substitution position." At this site, each of the four possible nucleotides (A, C, G, T) and a single-base deletion are represented in the probe set. Assay conditions optimize hybridization of the fluorescent-labeled DNA target to the probe that best matches its sequence. This hybrid yields higher fluorescence intensity relative to the other four target-probe hybrids in the set. There are probe sets complementary to every base in the target gene, so each base along the gene is examined for the presence of a mutant sequence.

During laboratory experiments, fluorescent-labeled DNA fragments (the "targets") are hybridized to the probe array on the GeneChip Fluidics Station. Then, the hybridized probe array is scanned in the GeneArray Scanner with a laser beam, which excites the fluorescent label. The intensity of emitted light is proportional to the amount of bound target at each location on the probe array. The amount of bound target is used by the Affymetrix Microarray Suite to compare the individual bases of the target being analyzed to those of a reference sequence (Figure 10) [[69]].

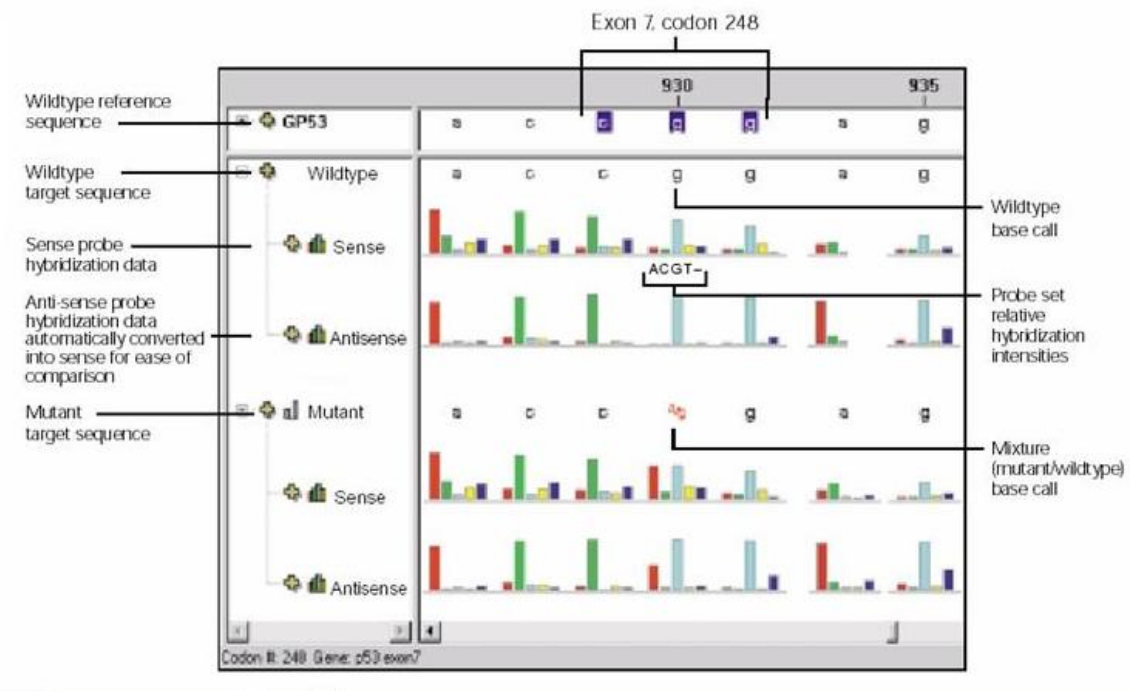


Figure 10 – Microarray Suite Software for Instrument Control and Sequence Analysis.

Immunohistochemistry

Immunohistochemistry (IHC) method involves localization of antigens in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element, or colloidal gold. As IHC is based on a specific antigen-antibody reaction, it has an apparent advantage over the traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, IHC has become an indispensable technique widely used by many medical research laboratories as well as in clinical diagnostics. There are numerous IHC methods for localizing antigens. The selection of a suitable method should be based on relevant parameters such as the type of the specimen investigated and the degree of sensitivity required [[70]].

IHC for p53 protein expression shows a strong, but not perfect correlation with the status of the p53 gene. In general, the presence of immunoreactive p53 correlates with the presence of p53 mutation, especially when the proportion of immunoreactive cells exceeds 10-20%. This is because transcription of the mutated p53 gene usually

results in the production of a protein that is more stable than the wild type – therefore, it accumulates in the nucleus, and this can be detected by IHC [71].

IHC analysis of primary gastric cancer appears to be an accurate and simple method of screening for p53 expression [72].

The majority of mutant p53 proteins is characterized by a prolonged half-life, accumulation in tissues, and can be directly detected by IHC [73]. Therefore, p53 protein accumulation is commonly interpreted as an indicator of p53 mutations. The prognostic value of p53 mutations or p53 protein accumulation has not been consistently demonstrated in a variety of human neoplasms, including gastric cancer [74] [75]. Both positive and negative findings have been reported from studies with various sample sizes, and this indicates that the discrepancy is not explained entirely by insufficient statistical power alone, as suggested by an early observation [76].

It has been shown that p53 mutations vary in their biological effects [77]. Some mutated p53 proteins behave like their wild-type counterpart, whereas others are correlated with tumor aggressiveness. In addition, accumulation of the p53 protein may not necessarily indicate the presence of a p53 mutation, because not all mutated p53 proteins yield positive IHC staining, and wild-type protein can also accumulate in response to stress stimulants such as DNA damage [78]. Because of the biological variability of p53 mutations and the diverse causes of p53 protein accumulation, either parameter alone may fail to predict the prognosis consistently [79].

Despite mounting evidence that IHC detection of p53 protein accumulation does not necessarily confirm the presence of missense p53 gene mutation, p53 protein accumulation has been shown to be strongly associated with missense mutations in a highly conserved region (exons 5-8) of the p53 gene [80]. While the half-life of wild-type p53 protein is very short, mutant proteins have an extended half-life, and this makes them readily detectable by IHC analysis. According to reports, accumulation of the p53 protein does not occur in chronic active gastritis and in various IM subtypes [81] [82]. This finding does not necessarily deny the premalignant potential of either chronic active gastritis or IM (of type III in particular), but rather, it suggests that p53 protein accumulation occurs beyond the stage of chronic active gastritis and IM formation.

AIMS

The objectives were as follows:

- To examine the effect of T-251A polymorphism of the IL-8 gene and G-308A polymorphism of the TNF-α gene in different histological alterations of the stomach, including gastritis, IM, atrophy, and in macroscopic diseases such as gastric erosions.
- To determine whether these two polymorphisms can influence the occurrence of *H. pylori* infection in the examined population.
- To describe genetic alterations of the p53 gene in IM.
- To compare possible sequence aberrations between samples obtained from different sites (DNA extracted from peripheral blood, from biopsy samples of the corpus and the antrum of the stomach) within the same patient.
- To examine wild-type p53 protein expression in IM and to test if there is a relationship between the polymorphisms and the expression of this protein.
- To compare and evaluate the capacity of GeneChip p53 Probe Array and the CS method in detecting polymorphisms and mutations.

METHODS

I. SELECTION OF PATIENTS, COLLECTION AND STORAGE OF SAMPLES

After informed consent, gastric biopsy samples were obtained from 218 consecutive patients with gastritis (n=86, *H. pylori* positive: n=41) (Gastritis), intestinal metaplasia (n=43, *H. pylori* positive: n=22) (IM), atrophy (n=32, *H. pylori* positive: n=13) (Atrophy) and histologically negative patients (n=57) (Hist Negative) during routine gastric endoscopy. The subjects were grouped also by macroscopic diagnosis into subsets with complete erosion (n=102, *H. pylori* positive: n=41) (Erosion) or with no macroscopic abnormality (n=88, *H. pylori* positive: n=21) (Macr Negative). The biopsy samples were stored in RNAlater (Qiagen, Düsseldorf, Germany) at -80 ^{o}C until isolation.

After informed consent, gastric biopsy samples of the antrum were obtained from 50 patients with IM (*H. pylori* positive: n=27) (IM) and from 51 histologically negative patients (all were *H. pylori* negative) (Controls). In 7 of the 50 IM patients, biopsy specimens were obtained not only from the diseased part (antrum), but also from a healthy region (corpus) of the stomach. Additionally, peripheral blood was drawn to compare local genetic alterations identified in samples from different sources. The biopsy samples were stored in RNAlater at -80 ^{o}C until isolation.

Endoscopy was warranted by gastric signs and symptoms, both in patients and in the controls. Gastritis and atrophy scores were equal in *H. pylori* positive and negative groups.

All the patients were white Caucasians of Hungarian origin; no ethnic subgroups were involved in the studies.

II. DNA ISOLATION, DESIGN AND PERFORMANCE OF PCR AND RFLP TESTS. EVALUATION OF DATA

DNA isolation

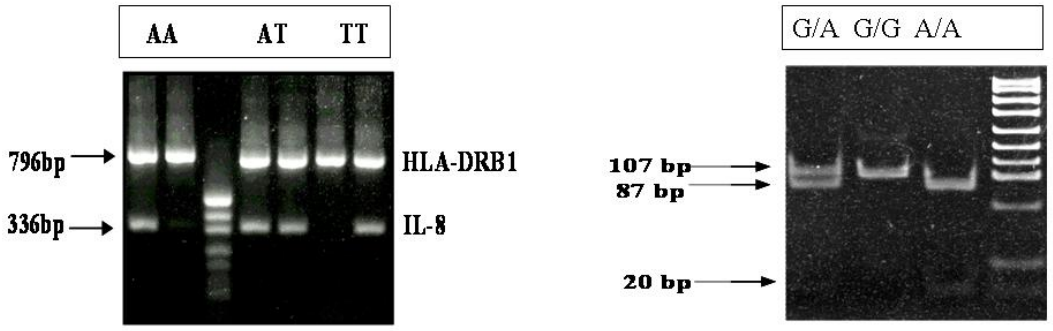
After overnight digestion with proteinase K and Rnase, genomic DNA was isolated from all gastric biopsy samples using the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) in compliance with the manufacturer's protocol.

In the case of the 7 IM patients, 21 genomic DNA samples were extracted not only from biopsy samples of the antrum and corpus, but also from peripheral blood. Genomic DNA from peripheral blood was isolated immediately after sampling, with the same kit and in accordance with the manufacturer's instructions.

Design and performance of PCR and RFLP; evaluation of results

T-251A polymorphism of the IL-8 gene was examined in the samples using the Amplification Refractory Mutation System (ARMS) method. Two specific and a consensus primer were used for the PCR reactions. The two specific primers – designed for the wild type or for the mutant allele – differed only in the 3' end. Two parallel PCR reactions were performed with a HLA-DR internal control [[83]]. The three different genotypes were determined by agarose gel-electrophoresis (Figure 12).

G-308A polymorphism of the TNF-α gene was determined with the PCR-RFLP method, according to the protocol of Wilson [[84]]. The A allele abolishes a restriction site for the *Nco*I restriction enzyme. Using the same primers of Wilson, we amplified a 107-bp-long PCR product. If the polymorphism is present, the enzyme cannot digest the 107-bp-long fragment. If, however, the polymorphism is present, digestion with *Nco*I yields two smaller, 87- and 20-bp-long fragments, which can be detected with agarose gel-electrophoresis (Figure 12).



T-251A polymorphism of IL-8

A/A=homozygote wild type
A/T=heterozygote
T/T=homozygote mutant
HLA-DRB1=internal control

G-308A polymorphism of TNF-α

G/G=homozygote wild type
G/A=heterozygote
A/A=homozygote mutant

Figure 12 – Electrophoretic image of A-215T polymorphism of IL-8 and G-308A polymorphism of TNF-α.

The 43 IM patients and the 51 controls were examined only for exon 4 of p53. In the case of the 7 additional IM patients, 21 samples (antrum, corpus and blood samples of every patient) were obtained and six exons (4, 5, 6, 7, 8 and 10) of the p53 gene were examined using the same primer pairs and the same protocol as in the GeneChip p53 Assay protocol (Affymetrix, Santa Clara, CA, USA) – see below, in chapter IV (Table 1.). The products of PCR reactions were cleaned (High Pure PCR Product Purification Kit, Roche) to remove the redundant bases, and to make PCR products suitable for sequencing.

III. CAPILLARY SEQUENCING, INTERPRETATION OF RESULTS, VERIFICATION OF GENECHIP P53 ASSAY-BASED RESULTS

Capillary sequencing, using Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA), of exons 4, 5, 6, 7, 8 and 10 of the p53 gene was performed in

the 50 IM patients and in the 51 histologically negative subjects. PCRs were performed in a Mastercycler epgradientS thermal cycler (Eppendorf AG, Hamburg, Germany) and each reaction contained 250 ng genomic DNA, 5-5 µl forward and reverse primers (See Table 1), 10 units of AmpliTaq Gold (Roche, Basel, Switzerland), 10x PCR Buffer, 2,5 mM $MgCl_2$ and 0,2 mM deoxynucleotide triphosphate in a final volume of 50 µl. PCR was carried out under the following conditions: denaturation at 95 °C for 10 min, then 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 45 sec, followed by a final extension cycle of 72 °C for 10 min and 4 °C for 1 hour. A 10-µl aliquot was visualized on a 2% agarose gel to confirm correct size (See Table 1) of the PCR products, which were then cleaned (High Pure PCR Product Purification Kit, Roche, Basel, Switzerland). Sequencing PCR reactions were carried out under the following conditions: 96 °C for 1 min, then 25 cycles of 96 °C for 20 sec, 50 °C for 5 sec and 60 °C for 4 min, then 4 °C for 1 hour. After the cycles, the sequencing product was cleaned with the ethanol precipitation technique, and the pellet was resuspended in 20 µl TSR (Applied Biosystems, Foster City, CA, USA). Capillary sequencing was carried out with an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

The results were analyzed using Chromas v2.3 software and Sequence Scanner version 1 (Applied Biosystems, Foster City, CA, USA) (Figure 13). To search for the nucleotide alterations we used the Blast 2 sequences program of NCBI homepage (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and compared the data obtained from the sequence-managing software with the reference sequence (X54156).

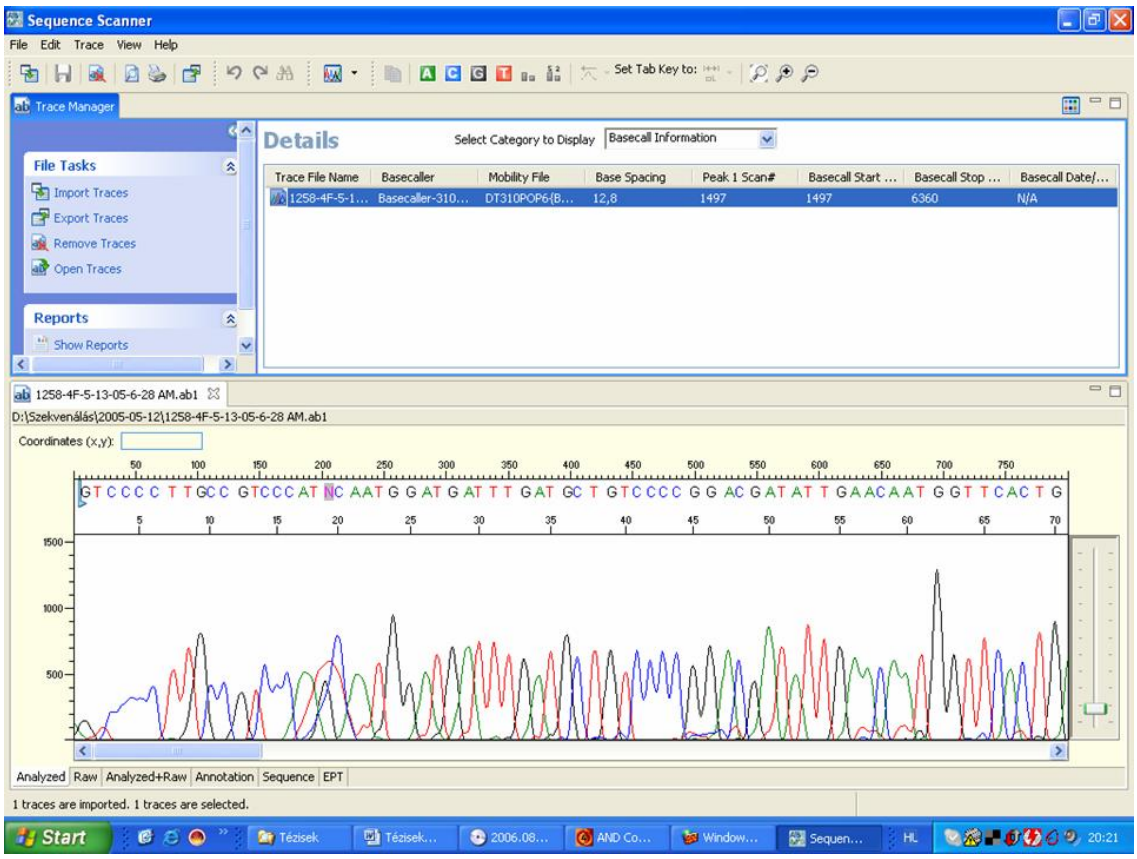


Figure 13 – Analysis of sequencing results (exon 4) with Sequence Scanner version 1 (Applied Biosystems Foster City, CA, USA).

IV. PERFORMING THE DNA RESEQUENCING MICROARRAY AND EVALUATION OF RESULTS

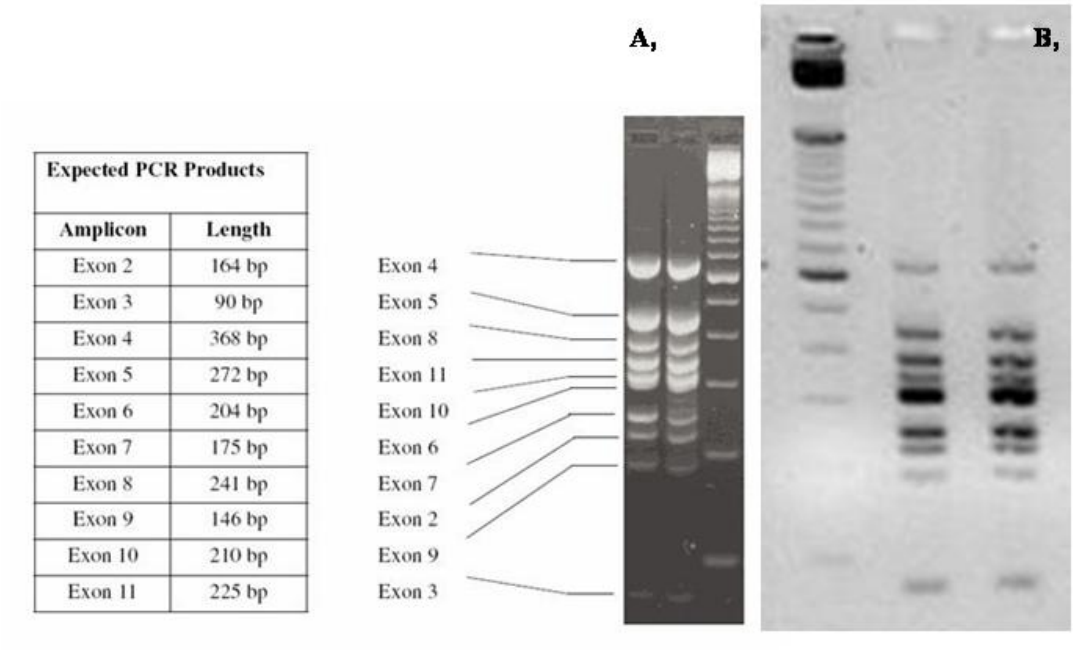
GeneChip p53 Assay

The GeneChip p53 Assay (Affymetrix, Santa Clara, CA, USA) was used to detect p53 sequences on exons 2 through 11 in 21 samples from the 7 IM patients. The DNAs of patients and the normal reference DNA were amplified in a multiplex PCR, as recommended by the manufacturer. The PCRs were performed in a PE 9600 thermal cycler (Perkin Elmer, Waltham, Massachusetts, USA) and each reaction contained 250 ng genomic DNA, 5 µl primer set (Affymetrix, Santa Clara, CA, USA) (See Table 1), 10 units of AmpliTaq Gold (Roche, Basel, Switzerland), 10x PCR Buffer, 2,5 mM $MgCl_2$ and 0,2 mM of each deoxynucleotide triphosphate in a final volume of 100 µl.

Table 1 – PCR primers used for p53 GeneChip p53 Assay analysis.

Exon	Primers (5'-3')	PCR product size (bp)
2	tcatgctggatccccacttttcctcttg tggcctgcccttccaatggatccactca	162
3	aattcatgggactgactttctgctcttgtc tccaggtcccagcccaacccttgtcc	88
4	gtcctctgactgctcttttcacccatctac gggatacggccaggcattgaagtctc	366
5	cttgtgccctgactttcaactctgtctc tgggcaaccagccctgtcgtctctcca	270
6	ccaggcctctgattcctcactgattgctc gccactgacaaccacccttaacccctc	202
7	gcctcatcttgggcctgtgttatctcc ggccagtgtgcagggtggcaagtggctc	173
8	gtaggacctgatttccttactgcctcttgc ataactgcacccttggtctcctccaccgc	239
9	cacttttatcacctttccttgcctctttcc aactttccacttgataagaggtcccaagac	144
10	acttacttctccccctcctctgttgctgc atggaatcctatggctttccaacctaggaag	208
11	catctctcctccctgcttctgtctcctac ctgacgcacacctattgcaagcaagggttc	223

PCRs were carried out under the following conditions: 95 ^{o}C denaturation for 10 min, then 35 cycles of 95 ^{o}C for 30 sec, 60 ^{o}C for 30 sec and 72 ^{o}C for 45 sec, followed by final extension at 72 ^{o}C for 10 min and at 4 ^{o}C for forever. With this protocol, the coding region of the p53 gene was amplified as 10 separate amplicons in a single reaction. A 5-µl aliquot of the multiplex PCR was visualized on 4% agarose gel (see Figure 14) to confirm correct size of the 10 PCR products [85].



Expected PCR Products	
Amplicon	**Length**
Exon 2	164 bp
Exon 3	90 bp
Exon 4	368 bp
Exon 5	272 bp
Exon 6	204 bp
Exon 7	175 bp
Exon 8	241 bp
Exon 9	146 bp
Exon 10	210 bp
Exon 11	225 bp

Figure 14 – Multiplex p53 PCR.

A: Theoretical image provided by the manufacturer (Affymetrix, Santa Clara, CA, USA). PCR products are visualized by ethidium-bromide in agarose gel-electrophoresis; correct sizes could be estimated with the help of the 50-bp DNA ladder.

B: Gel-image of two samples performed in my laboratory.

Then, 45 µl of the DNA amplicons was fragmented into approximately 50 bp fragments with 0,25 units of fragmentation reagents (Affymetix, Santa Clara, CA, USA), at 25 ^{o}C for 20 min in 20 mM EDTA, 2,5 units of calf intestine alkaline phosphatase and 10 mM Tris-acetate, followed by heat inactivation at 95 ^{o}C for 10 min. The fragmented amplicons were labeled at the 3' end with a fluoresceinated dideoxynucleotide, using the terminal transferase enzyme. Each terminal labeling reaction contained 50 µl fragmented PCR product, 5x reaction buffer, $CoCl_2$, fluorescein-ddCTP, and terminal deoxynucleotide transferase (Enzo Biochem, New York, USA). The reaction was incubated at 37 ^{o}C for 15 min and stopped with 5 µl of 0.2 M EDTA. The fluorescent-labeled fragments were hybridized in a reaction containing 6xSSPE, 0,05% Triton X-100, 2 mg/ml acetylated BSA and 2 nM control

oligonucleotide F1 (Affymetrix, Santa Clara, CA, USA). This hybridization mixture was hybridized to p53 microarrays, washed, and scanned (Figure 15).

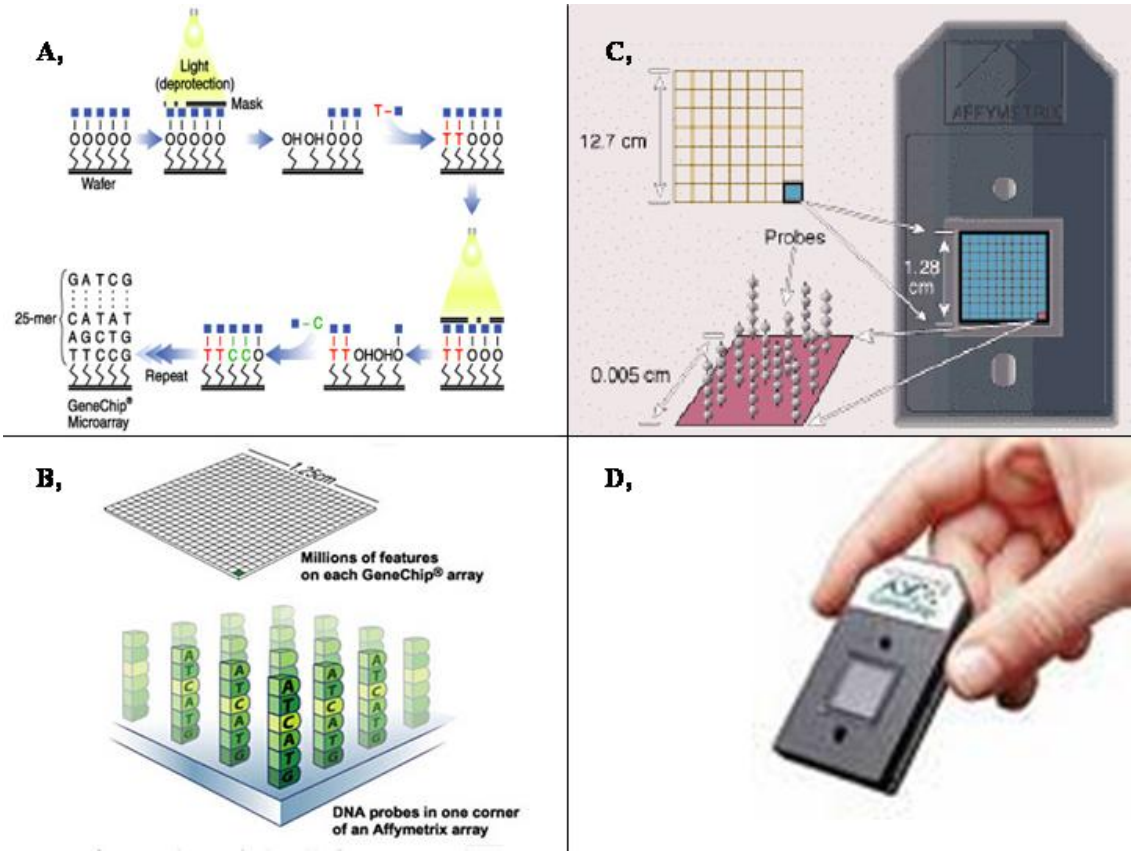


Figure 15 – The GeneChip p53 Assay.

A: Principle of the synthesis of oligonucleotides on GeneChip

The synthesis of these oligonucleotides on GeneChip microarrays is based on the principle of photolithography. A chip with initial starting strands (for building DNA) is exposed to light through a mask. The mask has specific, tiny openings that allow the light to contact the wafer at specific sites only (in this example, there are 5 probes only and each could represent a different feature). At exposed sites, the light removes a "protective" group from the strands. The chip is flushed with free nucleotides (the red T in the figure), which combine with any strand that had lost its protective group during the previous step. This cycle (exposure to light through a mask, removal of protecting groups from the strands, addition of free nucleotides) is then repeated numerous times, until the length of each strand under construction reaches 25 base pairs.

(www.dkfz.de/gpcf/24.html)

B, C: Arrangement of the microarrays.

D: Size and appearance of the chips.

Data analysis was performed with the Affymetrix Microarray Analysis Suite v5.1 software, according to the manufacturer's protocol. Hybridization pattern and intensity were analyzed with a mixture-detection algorithm. When a mutation occurred, the software named the mutation according to the codon in which the mutation existed. The algorithm also assigned a confidence score to each site containing a mutation or a single-base deletion. The higher the score for a given position, the greater is the possibility that the site contains a mutant base [[86]] (Figure 16).

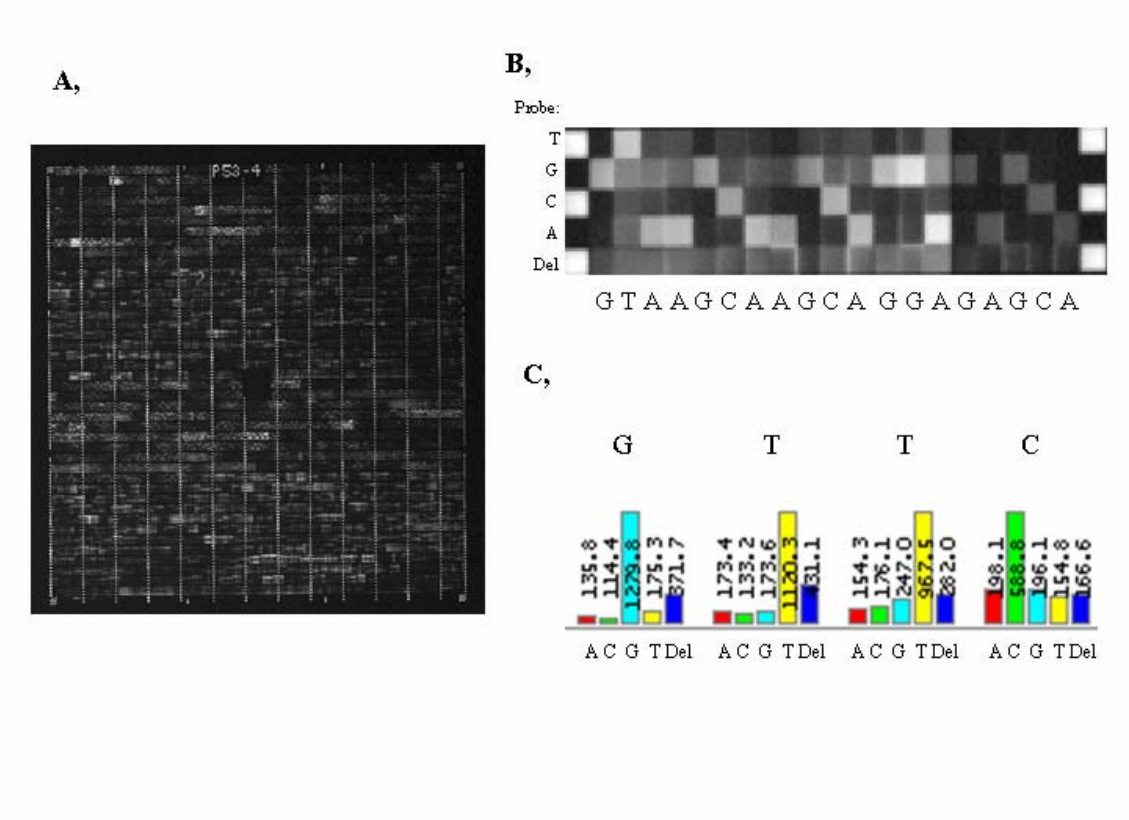


Figure 16 – Hybridization of the GeneChip p53 Array.

A: The p53 chip architecture. Image of a microarray after hybridization with labeled PCR products.

B: An enlarged region of a chip. Each column corresponds to a base in the sequence, whereas the five rows correspond to each of the four bases and to a single deletion. The bright white squares form the alignment controls.

C: Hybridization signal intensity measurements represented in each position as five columns, corresponding to the four bases and a single base deletion.

V. IMMUNOHISTOCHEMISTRY, INTERPRETATION OF RESULTS

Wild-type p53 (DO-7) IHC

The DO-7 anti-p53 protein (Invitrogen, Carlsbad, CA, USA) recognizes an epitope located within amino acids 35-45 of the N-terminus of wild and mutant types of the p53 protein. It has been reported that DO-7 does not react with normal human tissues (including stomach); however, its overexpression has been demonstrated in 22-76% of malignancies such as cancer of the stomach. The cellular staining pattern for DO-7 is nuclear.

The 4-μm thick tissue sections were de-waxed and re-hydrated. Antigen unmasking was carried out by microwave heat treatment (samples in plastic jars containing citrate buffer pH 6.0 were put into a preheated (95-99^{o}C) plastic water bath and were heated with 500W for 15 min). Then, the samples were cooled in PBS for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 30 minutes at room temperature. After washing 3 times in PBS for 3 minutes, the slides were incubated with ready-to-use, optimally diluted p53 antibody at 37 ^{o}C for 37 minutes, in a humidified chamber. After washing the slides 3 times in PBS, signal conversion was carried out with the ready-to-use LSAB2 system (DAKO, Glostrup, Denmark) as described in the manual. In brief, samples were incubated in Biotinylated Link solution for 10 mins at room temperature. After rinsing with PBS, samples were incubated in Streptavidin-HRP solution for 10 mins at room temperature. After the final rinsing with PBS, ready-to-use Amino-Ethyl-Carbasole Substrate-Chromogen Solution was applied for 10 minutes at room temperature. Hematoxylin co-staining was done.

Microscopic analysis

IHC was performed in tissue sections of the antrum from 19 IM patients (see Table 2). In each case, many sections were examined with three different magnifications (20x, 40x, 100x), and searched for the IM glands only. Labeling index (LI), defined as the percentage of the cells in the IM glands that stain positive with DO-7 anti-p53 antibody, was calculated as the number of brown-colored (DO-7 positive) nuclei, divided by the number of total nuclei.

Table 2 – Number of patients analyzed by immunohistochemistry.

	RR genotype	RP genotype	PP genotype
***H. pylori* positive**	4	3	2
***H. pylori* negative**	5	5	0

VI. IDENTIFICATION OF *H. PYLORI*

Histological examination is considered the reference standard for *H. pylori* detection. Although *H. pylori* may be recognized in hematoxylin-eosin stained sections, special supplementary staining (e.g. with Giemsa, Gimenez or silver) is always necessary to detect low-grade infection and to identify the characteristic morphology of *H. pylori.* Histology is also suitable as a historical record: sections (or additional sections) can always be (re-)examined and atrophy or IM assessed. Additional biopsies from other parts of the stomach can be preserved in formalin for processing if antral histology is inconclusive [87].

Biopsy specimens obtained from the corpus and antrum of the stomach for histopathological examinations were fixed in buffered 4% formalin overnight and then, embedded in paraffin. Two 4-μm sections were stained with hematoxylin-eosin and an additional section was stained by the modified Giemsa procedure and examined under a microscope.

VII. STATISTICAL ANALYSIS

All the examined groups were tested for Hardy-Weinberg equilibrium to check for Mendelian inheritance.

In the case of T-251A polymorphism of the IL-8 gene and G-308A polymorphism of the TNF-α gene, a 2x2 contingency table with Fischer's exact test was used for genotype evaluation and Odds Ratio (OR) with 95% Confidence Interval (CI) was calculated. A p-value of < 0.05 was considered statistically significant.

The analysis of polymorphisms of the p53 gene in IM patients and histologically negative patients was undertaken with logistic regression, used for the quantification of

association among the dependent and independent variables. Odds Ratios (ORs) were calculated as the exponent of the coefficients [88]. A p-value of < 0.05 was regarded as statistically significant. For the statistical evaluations the R environment was used [89].

For the evaluation of the IHC data, a linear model was used to analyze the differences among the groups defined by disease status and genotypes.

RESULTS

I. GASTRIC DISEASES AND T-251A POLYMORPHISM OF THE IL8 GENE

All the genotype distributions assessed were in Hardy-Weinberg Equilibrium. In order to investigate the differences in genotype, Fisher's exact test was applied to 2x2 contingency tables (see all the data in Tables 3, 4, 5 and 6).

In T-251A polymorphism of IL8, the incidence of T/T and T/A genotypes was significantly different between Hist Negative and IM groups (p=0.038, CI=1.07-Inf, OR=2.96 if the alternative hypothesis is true OR>1) (Table 3).

Table 3 – Numbers and proportions of patients with different genotypes of T-251A polymorphism of IL-8 within the disease groups evaluated.

	HISTOLOGICAL DIAGNOSIS							
	Hist Negative		**Gastritis**		**Atrophy**		**IM**	
n	57		82		29		35	
T/T	9	(15.8%)	21	(25.6%)	8	(27.6%)	11	(31.4%)
T/A	37	(64.9%)	40	(48.4%)	16	(55.2%)	15	(42.9%)
A/A	11	(19.3%)	21	(25.6%)	5	(17.2%)	9	(25.7%)
T	0.48		0.5		0.55		0.53	
A	0.52		0.5		0.45		0.47	
HW	0.08		0.98		0.82		0.71	

Hist Negative *versus* IM group: p=0.038, CI=1.07-Inf, OR=2.96

Abbreviations: T/T=homozygote for the T allele; T/A=heterozygote; A/A=homozygote for the A allele; T and A show the allele frequencies; HW=p value for Hardy-Weinberg Equilibrium

The A/A genotype was significantly more common in the *H. pylori* negative Gastritis group (p=0.049, CI=0.93-8.24, OR=2.73 if true OR≠1), than in the uninfected, *H. pylori* negative Hist Negative group. Interestingly, the results of the *H. pylori* negative Atrophy subgroup were very similar to those of the Hist Negative group, and were significantly different from those of the *H. pylori* negative Gastritis group (p=0.033, CI=0-0.86, OR=0.12 if true OR<1) (Table 4).

Table 4 – Number of patients in the different disease groups, sorted by histological diagnosis and by *H. pylori* status

	HISTOLOGICAL DIAGNOSIS									
	Hist Negative		**Gastritis**		**Atrophy**		**IM**		**ALL**	
	H. pylori		***H. pylori***		***H. pylori***		***H. pylori***		***H. pylori***	
	pos	**neg**	**pos**	**neg**	**pos**	**neg**	**pos**	**neg**	**pos**	**neg**
n	-	57	41	41	13	16	22	13	76	124
T/T	-	9 (15.8%)	11 (26.8%)	10 (24.4%)	3 (23.1%)	5 (31.3%)	7 (31.8%)	4 (30.7%)	21 (27.6%)	28 (22.1%)
T/A	-	37 (64.9%)	23 (56.1%)	17 (41.5%)	6 (46.2%)	10 (62.5%)	10 (45.5%)	5 (38.6%)	39 (51.3%)	69 (54.3%)
A/A	-	11 (19.3%)	7 (17.1%)	14 (34.1%)	4 (30.7%)	1 (6.25%)	5 (22.7%)	4 (30.7%)	16 (21.1%)	30 (23.6%)
T	-	0.48	0.55	0.45	0.46	0.625	0.55	0.5	0.53	0.49
A	-	0.52	0.45	0.55	0.54	0.375	0.45	0.5	0.47	0.51
HW	-	0.08	0.69	0.58	0.96	0.41	0.93	0.71	0.96	0.62

H. pylori negative Hist Negative *versus H. pylori* negative Gastritis group: p=0.049, CI=0.93-8.24, OR=2.73

H. pylori negative Gastritis *versus H. pylori* negative Atrophy group: p=0.033, CI=0-0.86, OR=0.12

The abbreviations are the same as in Table 3.

As regards macroscopic erosions and *H. pylori* infection, T-251A polymorphism of IL8 was not different between the studied groups (Table 5 and 6).

Table 5 – Number of patients in the different disease groups, sorted by macroscopic diagnosis.

	MACR NEGATIVE		**EROSION**	
n	84		94	
T/T	18	(21.4%)	22	(23.4%)
A/T	47	(56%)	51	(54.3%)
A/A	19	(22.6%)	21	(22.3%)
T	0.49		0.51	
A	0.51		0.49	
HW	0.5506		0.7108	

The abbreviations are the same as in Table 3.

Table 6 – Number of patients in the different disease groups, sorted by macroscopic diagnosis and *H. pylori* status.

	MACR NEGATIVE				EROSION			
	***H. pylori* positive**		***H. pylori* negative**		***H. pylori* positive**		***H. pylori* negative**	
n	21		63		41		53	
T/T	6	(28.8%)	12	(19%)	8	(19.5%)	14	(26.4%)
A/T	10	(47.6%)	37	(58.8%)	25	(61%)	26	(49.1%)
A/A	5	(23.8%)	14	(22.2%)	8	(19.5%)	13	(24.5%)
T	0.52		0.48		0.5		0.51	
A	0.48		0.52		0.5		0.49	
HW	0.98		0.38		0.37		0.99	

The abbreviations are the same as in Table 3.

II. GASTRIC DISEASES AND G-308A POLYMORPHISM OF THE TNF-α GENE

All the genotype distributions assessed were in Hardy-Weinberg Equilibrium. In order to investigate differences in genotype, Fisher's exact test was applied to 2x2 contingency tables (see all the data in Tables 7, 8, 9 and 10).

In the case of the TNF-α G-308A polymorphism, genotype distributions were not different between the individual histological groups and no differences were found as regards the presence of *H. pylori* infection (Table 7 and 8).

Table 7 – Number of patients in the different disease groups, sorted by the genotypes of G-308A polymorphism of TNF-α.

	HISTOLOGICAL DIAGNOSIS							
	Hist Negative		Gastritis		Atrophy		IM	
n	57		86		32		43	
G/G	42	(73.7%)	67	(77.9%)	27	(84.4%)	29	(67.4%)
G/A	13	(22.8%)	17	(19.8%)	5	(15.6%)	12	(27.9%)
A/A	2	(3.5%)	2	(2.3%)	0	(0.0%)	2	(4.7%)
G	0.85		0.88		0.92		0.81	
A	0.15		0.12		0.08		0.19	
HW	0.75		0.99		0.89		0.88	

Abbreviations: G/G=homozygote for the G allele; G/A=heterozygote; A/A=homozygote for the A allele; G and A show the allele frequencies; HW= p value for Hardy-Weinberg Equilibrium

Table 8 – Number of patients in the different disease groups, sorted by histological diagnosis and *H. pylori* status.

	HISTOLOGICAL DIAGNOSIS									
	Hist Negative		Gastritis		Atrophy		IM		ALL	
	H. pylori		*H. pylori*		*H. pylori*		*H. pylori*		*H. pylori*	
	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
n	-	57	41	45	13	19	21	22	75	143
G/G	-	42 (73.7%)	34 (82.9%)	33 (73.3%)	12 (92.3%)	15 (78.9%)	13 (61.9%)	16 (72.2%)	59 (78.7%)	106 (74.2%)
G/A	-	13 (22.8%)	7 (17.1%)	10 (22.2%)	1 (7.7%)	4 (21.1%)	7 (33.3%)	5 (22.7%)	16 (20%)	32 (22.4%)
A/A	-	3 (3.5%)	0 (0%)	2 (3.5%)	0 (0%)	0 (0%)	1 (4.8%)	1 (3.4%)	1 (1.3%)	5 (3.4%)
G	-	0.85	0.91	0.84	0.96	0.89	0.79	0.84	0.89	0.85
A	-	0.15	0.09	0.16	0.04	0.11	0.21	0.16	0.11	0.15
HW	-	0.75	0.84	0.59	0.99	0.88	0.99	0.78	0.99	0.44

The abbreviations are the same as in Table 7.

The G/G and the heterozygote (G/A) genotypes were found to be statistically different between the Erosion and the Macr Negative groups (p=0.035, CI=1.0055-4.72, OR=2.15 if OR≠1), as well as between the *H. pylori* positive Erosion and the *H. pylori* positive Macr Negative subgroups (p=0.027, CI=1.105-28.84, OR=5.22 if

OR≠1). The G/G homozygote genotype was significantly more common in both groups, than in the normal group (see table 9 and 10).

Table 9 – Number of patients in the different disease groups, sorted by macroscopic diagnosis.

	MACR NEGATIVE		EROSION	
n	88		102	
G/G	60	(68.2%)	83	(81.4%)
G/A	26	(29.5%)	16	(15.7%)
A/A	2	(2.3%)	3	(2.9%)
G	0.83		0.89	
A	0.17		0.11	
HW	0.9157		0.1752	

Macr Negative *versus* Erosion group: p=0.035, CI=1.0055-4.72, OR=2.15

The abbreviations are the same as in Table 7.

Table 10 – Number of patients in the different disease groups, sorted by macroscopic diagnosis and *H. pylori* status.

	MACR NEGATIVE				EROSION			
	***H. pylori* positive**		***H. pylori* negative**		***H. pylori* positive**		***H. pylori* negative**	
n	20		67		41		61	
G/G	12	(60%)	48	(71.6%)	37	(90.2%)	46	(75.4%)
G/A	7	(35%)	18	(26.9%)	4	(9.8%)	12	(19.7%)
A/A	1	(5%)	1	(1.5%)	0	(0%)	3	(4.9%)
G	0.775		0.85		0.95		0.85	
A	0.225		0.15		0.05		0.15	
HWp	0.99		0.89		0.95		0.23	

H. pylori positive Macr Negative *versus H. pylori* positive Erosion group: p=0.027, CI=1.105-28.84, OR=5.22

The abbreviations are the same as in Table 7.

III. IM AND POLYMORPHISMS OF THE P53 GENE

Capillary sequencing of exon 4 of the p53 gene in *H. pylori* positive or negative IM patients and in *H. pylori* negative controls

All the genotype distributions assessed were in Hardy-Weinberg Equilibrium.

No subject was found either with codon 34 or with codon 47 polymorphisms. Codon 36 polymorphism was ignored during the statistical analysis, because it was found only in three cases (2.98%) and in heterozygote form. RR genotype of codon 72 was found in 25 IM patients and in 28 controls; RP in 22 IM patients and in 17 controls; and PP in 3 IM patients and in 6 controls (see Table 11).

Table 11 – Genotype and allele frequencies of codon 72 (exon 4) of the p53 gene in the IM and in the Control group.

	n	Codon 72 genotype (%)			Allele frequency		HW
		R/R (n)	R/P (n)	P/P (n)	R	P	
***H. pylori* positive IM patients**	27	40.7 (11)	51.9 (14)	7.4 (2)	0.67	0.34	0.6873
***H. pylori* negative IM patients**	23	60.9 (14)	34.8 (8)	4.3 (1)	0.78	0.22	0.9943
All patients with IM	50	50 (25)	44 (22)	6 (3)	0.72	0.28	0.812
***H. pylori* negative controls**	51	55 (28)	33.3 (17)	11.7 (6)	0.72	0.28	0.4340
IM patients and controls	101	52.5 (53)	38.9 (39)	8.8 (9)	0.72	0.28	0.8952

H. pylori positive IM patients *versus H. pylori* negative controls: p=0.0087

Abbreviations: R/R=homozygote for Arginine (R); R/P=heterozygote; P/P=homozygote for Proline (P); R and P show the allele frequencies; HW= p value for Hardy-Weinberg Equilibrium

The IM group was divided into two subgroups according to *H. pylori* status. In search of a relation between the development of IM, codon 72 genotypes and *H. pylori* infection, the following groups and subgroups were compared: the IM *versus* the

Control group, the *H. pylori* positive IM *versus* the Control group, and the *H. pylori* negative *versus* the Control group. Comparison of the *H. pylori* positive IM group to the Control group revealed that RR genotype significantly reduces the chance of developing IM (p=0.0087). To find an association between codon 72 genotype and *H. pylori* status in IM patients, the *H. pylori* negative IM group was compared to the *H. pylori* positive IM group, but no statistically significant relationship could be found.

IV. EVALUATION OF P53 POLYMORPHISMS IN DNA SAMPLES ISOLATED FROM THE GATRIC ANTRUM AND CORPUS, AS WELL AS FROM PERIPHERAL BLOOD

Diverse genotypes in the samples obtained from different sites within the same patient could not be found. All the polymorphisms identified were of the same genotype in the DNA isolated from peripheral blood, from the normal (corpus) and diseased part (antrum) of the stomach.

V. EXAMINATION OF PROTEIN EXPRESSION BY DO7-P53 IHC

Analyzing the LI values of sections revealed that in the case of the RR genotype, LI was significantly higher (p=0.004, difference: 23.52) in *H. pylori* positive sections, than in *H. pylori* negative ones (Table 12).

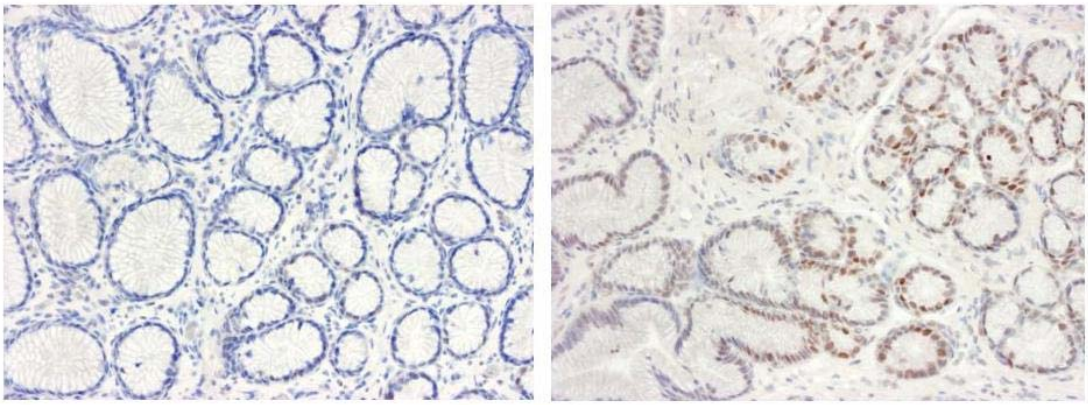
Table 12 – Distribution of p53 Labeling Indexes (LI) in different allele groups, in relation to *H. pylori* infection.

	***H. PYLORI* POSITIVE**				***H. PYLORI* NEGATIVE**		
Genotype	**Sample n**	**Mean LI**	**Standard deviation**		**Sample n**	**Mean LI**	**Standard deviation**
RR	14	38,21	19,66	↔ p=0.004	13	14,69	14,83
	p=0.358↕				p=0.012↕		
RP	12	25,75	12,69	↔ p=0.473	10	38	18,89
	p=0.316↕						
PP	9	11,22	8,87				

H. pylori positive RR *versus H. pylori* positive PP: p=0.0027

The abbreviations are the same as in Table 11.

The LI was significantly higher in RP genotype sections (p=0.012, difference: 23.31), than in RR genotype sections from *H. pylori* negative cases (Figure 17).



A, **B,**

Figure 17 – p53-DO7 immunohistochemistry staining of IM glands.

A: IHC of a tissue section obtained from the antrum of an RR genotype, *H. pylori* negative patient. Nuclear overexpression of the p53 protein is not visible in IM glands. The LI is 0%.

B: IHC of a tissue section from the antrum of an RP genotype, *H. pylori* negative patient. Nuclear overexpression of the p53 protein (staining in brown) is seen in IM glands. The LI is 36%.

In *H. pylori* positive subjects, an opposite difference was found, but it was not statistically significant. However, LI was significantly higher in the PP genotype sections (p=0.0027, difference: 26.99) than in those of the RR genotype, in *H. pylori* positive cases (Figure 18).

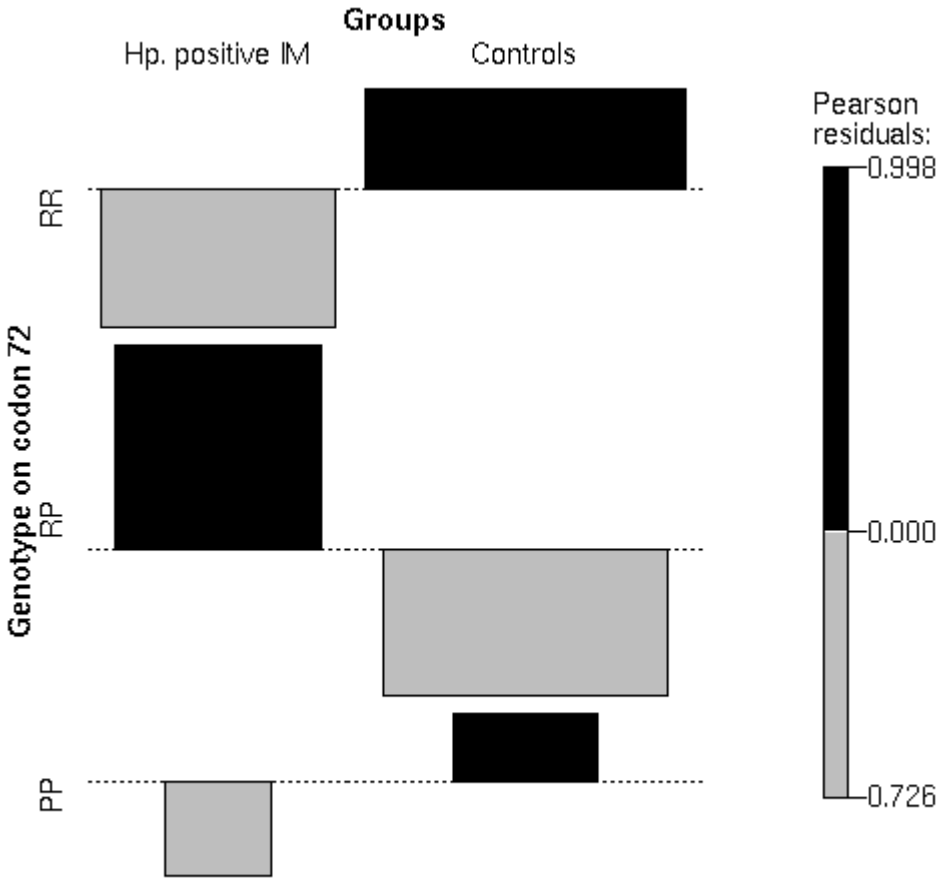


Figure 18 – Visualization of the independence between the *H. pylori* positive IM and control groups and the genotypes on codon 72 using association plots.
In this diagram, each cell of a contingency table is represented by a rectangle that has a (signed) height proportional to Pearson's residual, as well as a width proportional to the square root of the expected frequency. The rectangles in each row are positioned relative to a baseline indicating independence. If the observed frequency of a cell is greater than expected, the box rises above the baseline, and sinks below otherwise (Meyer et al. 2006).

VI. COMPARISON OF MICROARRAY-BASED SEQUENCING TO CAPILLARY SEQUENCING

The 21 samples of the 7 IM patients were analyzed with GeneChip p53 Array (Affymetix, Santa Clara, CA, USA) method for 10 exons and with CS for six exons. Every mutation detected by GeneChip was verified by CS.

Using the chip method, only 6 samples (of 5 patients) could be found without alteration.

With CS, R72P polymorphism was found in all samples of all patients, whereas the chip method detected only 3 of these 21 R72P polymorphisms, and misidentified them as RP, instead of PP.

GeneChip detected 16 missense mutations with and 2 without amino-acid change, but these could not be confirmed with CS (Table 13).

Table 13 – Comparison of the results obtained by GeneChip and CS methods.

ID of the patient/sample source	p53 resequencing chip	Capillary sequencing
1/antrum	No alteration	R72P, cgc-ccc/cgc
1/corpus	E286E, gaa-gag/gaa	R72P, cgc-ccc/cgc
1/blood	C135E, tgc-ggc/tgc	R72P, cgc-ccc/cgc
2/antrum	R72P, cgg-ccc/cgg	R72P, cgc-ccc
2/corpus	R72P, cgg-ccc/cgg	R72P, cgc-ccc
2/blood	S127C, tcc-tgc/tcc E286E, gaa-gag/gaa	R72P, cgc-ccc
3/antrum	No alteration	R72P, cgc-ccc
3/corpus	R72P, cgg-ccc/cgg	R72P, cgc-ccc
3/blood	No alteration	R72P, cgc-ccc
4/antrum	R196P, cga-ccg/cga	R72P, cgc-ccc/cgc
4/corpus	No alteration	R72P, cgc-ccc/cgc
4/blood	S240I, agt-att/agt	R72P, cgc-ccc/cgc
5/antrum	R209K, aga-aaa/aga	R72P, cgc-ccc/cgc
5/corpus	R196P, cga-ccg/cga	R72P, cgc-ccc/cgc
5/blood	No alteration	R72P, cgc-ccc/cgc
6/antrum	Il0S, att-agt/att E51D, gaa-gat/gaa	R72P, cgc-ccc/cgc
6/corpus	P359P, cca-cct/cca	R72P, cgc-ccc/cgc
6/blood	No alteration	R72P, cgc-ccc/cgc
7/antrum	C135G, tgc-ggc/tgc H179Q, cat-caa/cat	R72P, cgc-ccc/cgc P36P, ccg-cca/ccg
7/corpus	R196P, cga-cca/cga I232S, atc-agc/atc I254T, atc-acc/atc I255L, atc-ctc/atc	R72P, cgc-ccc/cgc P36P, ccg-cca/ccg
7/blood	R196P, cga-cca/cga R209L, aga-aaa/aga	R72P, cgc-ccc/cgc P36P, ccg-cca/ccg

DISCUSSION

I. GASTRIC DISEASES AND T-251A POLYMORPHISM OF IL8 AND G-308A POLYMORPHISM OF TNF-α

The association between the examined polymorphism of the IL8 gene and gastric diseases is not clear. Smith et al. proposed that although carriage of the A allele in T-251A polymorphism of the IL8 gene is associated with a more intense mucosal inflammatory reaction in *H. pylori* infection; this does not alter the risk of developing gastric cancer [39]. At the same time in Japan, this polymorphism was found to be associated with the risk of gastric cancer – as well as that of antral atrophy and metaplasia, but only in patients younger than 49 years [40].

In my study, the relative frequency of the T/A genotype – in comparison to that of the T/T genotype – was significantly higher among Hist Negative patients, than in IM patients. This tendency was also evident in the Gastritis and in Atrophy groups – T/A is more frequent in these, than T/T; however, these differences are not statistically significant. In Hist Negative patients, the frequency of the A allele was higher and the A/A genotype was more common, than in the disease groups. Taking also the *H. pylori* status into consideration, A allele frequency was slightly higher in the *H. pylori* negative Gastritis subgroup, than in the Hist Negative group. Nevertheless – *H. pylori* infection and the macroscopic erosions notwithstanding – T-251A polymorphism of IL-8 does not seem to influence the outcome.

As regards G-308A polymorphism of the TNF-α gene, a statistically significant difference in genotype distributions could be demonstrated only if the samples were grouped by macroscopic diagnosis. Specifically, the heterozygote genotype (G/A) was twice as common in the Macr Negative group, than in the Erosion group.

Yea et al. found in Korea that G-308A polymorphism is closely related to infection by *H. pylori,* – in contrast to uninfected patients with gastric disease [90]. My results are in disagreement with this finding. Possible reason for this discrepancy might include variable normal allele frequency of the polymorphism in different regions and races. In my study conducted on healthy Hungarian (white Caucasian) subjects, the frequency of the A allele was 0.15, whereas it was 0.03 in Yea's Korean study population – and this is a substantial difference. An alternative explanation could be

that in our study population, the correlation between genotype and the occurrence of gastric erosion is stronger than that between genotype and the presence of *H. pylori* infection.

II. IM AND R72P POLYMORPHISMS OF P53 THE GENE

Sharp ethnic differences in codon 72 allele frequencies have been observed. In the Northern hemisphere, the P72 allele shows a North-to-South gradient, from 0.17 in Swedish Saamis to 0.63 in African Blacks (Nigerians) [50]. In Western Europe, in North, Central and South America and in Japan, the most common allele is R72 with frequencies ranging from 0.60 to 0.83. However, P72 frequency above 0.40 has been observed in African-Americans and in a Chinese population [91]. This statistically significant, linear correlation between the frequency of the allele encoding P72 and latitude suggests that the two alleles may produce functionally distinct proteins and that the allele encoding P72 might be selected for in environments subjected to high levels of ultraviolet light [50]. According to the literature, the incidence of the R72 allele is most probably below 0.5 in Hungary [92].

Only a few studies have examined the role of p53 codon 72 polymorphisms in gastric cancer [93]. In a Japanese study, 117 cases of gastric cancer with *H. pylori* positive chronic gastritis were compared with 116 cancer-free controls with *H. pylori* positive chronic gastritis. It was found that the P/P allele was associated with an increased risk of developing diffuse-type gastric cancer, compared to the R/R allele (OR 2.98 CI: 1.07–8.32) [94]. In another study by Zhang et al. [95], 120 cancer patients were compared with 277 cancer-free controls. After adjustment for age and gender, logistic regression analysis showed that the risk for cancer of the cardia is 3.1 times (95% CI: 1.4–7.3) higher in homozygous R/R patients, than in P/P homozygous or R/P heterozygous subjects [95]. A Chinese meta-analysis included 12 case-control studies with 1665 gastric cancer cases and 2358 controls. Its results suggest that p53 codon 72 polymorphism may be associated with gastric cancer among Asians, as well as that difference in genotype distribution may be related to the location, stage, and histological differentiation of gastric cancer [96].

The number of the studies conducted to explore the relationship between cancer preceding IM and R72P polymorphism of the p53 gene is yet limited. In the study of Perez-Perez et al. [55] genotype frequencies were 32% (R/R), 48% (R/P) and 20%

(P/P) in IM patients, and 25% (R/R), 50% (R/P) and 25% (P/P) in normal subjects. In comparison with this study, genotype distributions were dissimilar in Hungary, that is, a statistically significant difference in genotype frequencies could be demonstrated between the control and IM groups.

Codon 72 polymorphism was examined in a Costa Rican population exposed to a high risk of gastric cancer and pre-malignant lesions. No association was found for this polymorphism with either the risk of gastric cancer or that of less severe gastric lesions [97]. Similar results were reported from the UK. Zhang et al. studied 391 white Caucasian patients with chronic gastritis, gastric or duodenal ulcer, with and without IM [98]. Different genotype distributions were found in the different alterations, but none of these reached statistical significance.

As reported previously, the apoptotic index is lower in the mucosa exhibiting IM – in both *H. pylori* positive and negative cases –, than in the normal epithelium [99]. My results suggest that the RR genotype and the presence of the R allele reduce the incidence of IM. These are in agreement with the finding that R72 variant induces apoptosis markedly better than the P72 variant, because of its preferential localization in mitochondria and greater ubiquination by the E3 ubiquitin ligase MDM2 [54].

III. THE EFFECT OF IL-8, TNF-α AND P53 POLYMORPHISMS ON *H. PYLORI* INFECTION

Hamajima et al. suggested that individuals with the IL-8–251TT and IL-10–819TT genotypes stand a greater chance of chronic *H. pylori* infection, especially among current smokers [100]. The risk of gastric cancer associated with this polymorphism is increases slightly further in subjects with *H. pylori* infection, but no evidence of an interaction could be found between IL-8 polymorphism and *H. pylori* infection [101].

Although G308A polymorphism of TNF-α in patients with *H. pylori* infection was not significantly different from that in patients not infected by *H. pylori*, it seems that the A allele is significantly related to an increased risk for infection, but only in the presence of *H. pylori* cagA subtype, and in Korean patients with gastric disease [90]. An association between TNF-α G308A gene polymorphisms and *H. pylori* infection was not found either in a Chinese population with gastroduodenal disease [102].

A non-significant relationship was found between the presence of *H. pylori* and genotypes P/P and P/R of the codon 72 of the p53 gene in a Brazilian study. In these populations, an association between the presence of *H. pylori* and gastric adenocarcinoma was also observed [103].

Based on my results, it seems that regardless of the microscopic and macroscopic alterations of the stomach, neither T-251A polymorphism of IL-8, nor G-308A polymorphism of TNF-α, or R72P polymorphism of the p53 gene influences *H. pylori* infection.

On the other hand, my results suggest that *H. pylori* infection enhances the expression of the p53 protein in IM cells.

IV. POSSIBLE GENETIC ALTERATIONS OF THE P53 GENE IN THE SAMPLES FROM DIFFERENT SOURCES

Although inherited p53 mutations exist in all somatic cells, malignant transformation is limited to certain organs and target cells. The study of Kleihues [104] analyzed 475 tumors in 91 families with p53 germline mutations. The sporadic counterparts of these tumors also carry a high incidence of p53 mutations, suggesting that in the examined tissues, p53 mutations are capable of initiating the process of malignant transformation. The location of mutations within the p53 gene was similar to that of somatic mutations in sporadic tumors. There was no evidence of any organ- or target cell-specificity of p53 germline mutations; the occasional, familial clustering of certain tumor types is more likely to reflect the genetic background of the studied kindred or the additional influence of environmental and non-genetic host factors.

On the other hand, several p53 mutations were found in DNA isolated from the tumor tissue and only some of these appeared also in peripheral blood (leukocyte DNA used as control) of gastric cancer patients of an Italian study [105].

The question of local mutations seems unclear. For this reason, my research focused on the genetic pattern of samples obtained from different sites within the same patient. The genotypes of all the 21 examined samples of the 7 IM patients (3 samples were obtained from different sites from each patient: DNA was extracted from peripheral blood, from biopsy samples of the corpus and the antrum of the stomach) were determined, but no sequence differences were found between DNA samples isolated from peripheral blood and from biopsy samples taken from the healthy and

diseased regions of the stomach. These results suggest that no local alterations of the p53 gene occur in IM.

V. WILD-TYPE P53 PROTEIN EXPRESSION IN IM, RELATIONSHIP BETWEEN POLYMORPHISMS, *H. PYLORI* INFECTION AND PROTEIN EXPRESSION

IHC examination of p53 protein expression showed a low-level expression in some of the normal tissue adjacent to gastric tumor in the study of Zhang [95], while it was high in cancer tissue. There was no significant correlation between codon 72 genotypes, age, gender, or tumor site, and p53 protein expression in tumor tissue or in the adjacent, normal gastric mucosa. The p53 antibody used recognizes both mutant and wild-type p53.

It seems that a significant increase of p53 expression occurs in *H. pylori*-infected – as compared to normal – gastric mucosa, but successful eradication of the bacteria can dramatically reduce p53 level [[106]].

In another study, p53-positive IHC staining was detected in 13.3% of IM samples and in 43.5% of gastric carcinoma samples. Subdivision of the latter into *H. pylori*-positive and -negative subsets revealed a significantly (p=0.013) higher p53 levels in *H. pylori*-positive samples [[107]].

The R-P change in codon 72 does not affect the binding capacity of the DO-7 antibody used in my study, and the protein exhibits positive staining when reacting with this antibody. In my study, LIs were different when the genotype of codon 72 and *H. pylori* status of the patients were considered. In *H. pylori* negative cases the RP genotype, whereas in *H. pylori* positive cases the PP genotype increases the p53 protein content of IM cells, and accordingly, it seems that the presence of the P allele is associated with accumulation of the p53 protein in IM cells. My results suggest – in accord with other studies – that *H. pylori* infection also enhances the expression of the p53 protein.

The DNA isolated from the whole biopsy specimen was analyzed, which made the measurement less sensitive to differences between individual cells. For a better understanding of the relationship between IHC results and the genetic pattern, DNA should be isolated directly from positively staining IM gland cells. Separation of these cells is most expediently done by laser-microdissection.

VI. COMPARISON OF THE POLYMORPHISM DETECTION CAPABILITY OF GENECHIP P53 PROBE ARRAY AND CS

With GeneChip p53 Assay (Affymetrix, Santa Clara, CA, USA) sequence analysis of the entire coding region of the p53 gene is possible in just a single microarray experiment. This array performs a sense and anti-sense analysis during a single hybridization reaction. It is recommended by the manufacturer for identification of missense mutations and single base deletions. The detection of mutant p53 happens in a background of wild-type p53 [[108]]. The advantage of the microarray over CS should be that this method is faster and less labor-intensive. In the case of CS, forward and reverse strand sequencing is generally required, especially if it is used for screening. The evaluation of the sequences sometimes is dependent on human decision to separate the sign from noise. Notwithstanding this, CS is the safest and most reliable method for sequencing.

I agree with Allen et al. that GeneChip p53 Assay should not be used as a stand-alone test for mutation analysis of the p53 gene [[109]]. As shown by previous results, the GeneChip provides a rapid screen, because it detects most (>80%) of the mutations with a very low false-positive rate [[110]] [[111]] [[112]]. By contrast, Cooper et al. found that this microarray platform could not be used to detect exon 4 polymorphisms (codon 72) in formaline-fixed, paraffin-embedded breast cancer tissue [[113]].

In my study, the results of the GeneChip p53 Assay were not reproducible with the CS method. Possible reasons for the observed discrepancies include the difficulties of multiplex PCR used in the chip method. Although all the circumstances recommended by the manufacturer were maintained during the preparation of the arrays, the quality of the multiplex PCRs was poor. The 10 exons of the p53 gene were amplified in the same reaction, in the same tube, and the quality and the quantity of the PCR products were estimated by agarose gel-electrophoresis. Unfortunately, surplus bands were observed and additional manipulations were necessary – in observance of the manufacturer's recommendations. Using an appropriate DNA ladder, the bands corresponding to the 10 exons of p53 were cut out from the gel, collected in a tube, and then, cleaned from the gel. Eventually, all the cleaned PCR products were concentrated and re-controlled by agarose gel-electrophoresis. After these interventions, the mixture of PCR products seemed appropriate for further processing (see Figure 13).

As the newer versions of the Affymetrix Hybridization Oven and Scanner available in my laboratory were not appropriate for processing the GeneChip p53 Assay, the samples and the arrays were dispatched (after preparation of the hybridization mixture) to the headquarters of Affymetrix in the UK. Thus, hybridization, scanning, and data analysis were undertaken by Affymetrix.

During my next project, I worked with the GeneChip CustomSeq Resequencing Array (Affymetrix, Santa Clara, CA, USA) and concluded that this method, which is very similar to the GeneChip p53 Assay, was quite robust when good quality PCR products were used [114].

Affymetrix no longer sells the GeneChip p53 Assay; this product has been purchased and perhaps improved by Roche (Basel, Switzerland); currently, it is available as the AmpliChip p53 Test.

CONCLUSIONS

- **IL-8-, TNF-α-, p53 polymorphisms and gastric diseases**

The effect of T-251A polymorphism of IL-8 seems to play a role in the pathogenesis of histological gastritis and IM, whereas the sequels of G-308A polymorphism of TNF-α are relevant in the etiology of macroscopic erosive gastritis.

Apparently, R72P polymorphism of the p53 gene is related to IM – that is, the R allele is associated with a lower incidence of IM. The tentative mechanism behind this could be apoptosis, as the R72 allele is a more potent inducer of apoptosis, than P72.

- **The effect of IL-8, TNF-α and p53 polymorphisms on *H. pylori* infection**

Taking *H. pylori* infection, as well as the microscopic and macroscopic alterations of the stomach into consideration, neither T-251A polymorphism of IL-8 nor G-308A polymorphism of TNF-α seems to influence the outcome.

According to my results, *H pylori* infection enhances expression of the p53 protein in IM cells.

- **Possible genetic diversity of samples from different sources**

No sequence aberrations of the p53 gene were found in the samples obtained from different locations (i.e. DNA extracted from peripheral blood, biopsy specimens from the corpus and the antrum of the stomach) within the same patient.

- **Wild-type p53 protein expression in IM, and the relationship between polymorphisms and protein expression**

In IM, p53 protein expression of gland cells is related to the genotype of codon 72 of the p53 gene and is dependent also on the presence or absence of *H. pylori* infection.

- **Evaluation of the capability of the GeneChip p53 Probe Array to detect polymorphisms and mutations, in comparison with the CS method**

This microarray method was not sensitive and reliable enough for genetic screening of p53 gene, undertaken for diagnostic purposes.

SUMMARY

Certain premalignant conditions of gastric cancer are related to particular genetic alterations. Polymorphisms of the IL-8, TNF-α, and p53 genes – which play a significant role – can be identified in patients suffering from gastritis and IM. *H. pylori* infection is another important factor, which influences the effects of the investigated genes and polymorphisms.

With the application of high-capacity screening methods such as resequencing arrays also offering the additional advantage of an expedited diagnosis, further genetic factors can be identified. Various methods for polymorphism detection are available for identifying sequence alterations. Unfortunately, the GeneChip p53 Probe Array is apparently not reliable enough for use as a stand-alone DNA resequencing method. Based on my research results certain types of resequencing microarray may prove a sensitive and reliable method for genetic screening for diagnostic purposes. Nevertheless, CS is always recommended for verification.

For a better understanding of the role of polymorphisms or mutations in the etiology of gastric disease, laser-microdissection of the affected cells, identified by IHC, is proposed. This could afford early recognition of patients at risk of developing micro- or macroscopic, pathological lesions as well as the introduction of preventive measures.

ÖSSZEFOGLALÁS

Egyes gyomor rák-megelőző állapotok bizonyos genetikai eltérésekhez köthetőek. Gasztrítiszben és intesztinális metapláziában szenvedő betegekben az IL-8, TNF-α és a p53 gének olyan polimorfizmusait lehetett azonosítani, amelyek fontos szerepet játszanak a betegség kialakulásában. A *H. pylori* fertőzés is fontos faktor, amely befolyásolja a vizsgált gének és polimorfizmusok hatását.

A nagy teljesítményű szűrőmódszerek, mint pl. reszekvenáló microarray-ek alkalmazása a diagnózis megszületésének felgyorsulását és további, befolyásoló genetikai faktorok felismerését segítik elő. Mint ismeretes, számos módszer áll már rendelkezésünkre polimorfizmusok azonosítására. Sajnos úgy tűnik, hogy a GeneChip p53 Probe Array önmagában nem elég megbízható módszer a DNS reszekvenáláshoz. Kutatási eredményeim alapján azonban elmondható, hogy bizonyos, egyéb típusú reszekvenáló microarray-ek elég érzékenyek és megbízhatóak ahhoz, hogy diagnosztikai célú genetikai szűrésre alkalmasak legyenek. Mindazonáltal az eredmények megerősítéséhez kapilláris szekvenálás használata mindig ajánlott.

A polimorfizmusok vagy mutációk gyomorbetegségek etiológiájában játszott szerepének jobb megismeréséhez az érintett sejtek immunhisztokémiai módszerrel való azonosítása és lézer-mikrodisszekcióval történő izolálása ajánlott. Ez az eljárás lehetővé teszi a betegekben mikor- vagy makroszkópos kóros elváltozások kifejlődésének korai felismerését, illetve lehetőséget ad a megelőző beavatkozásokra.

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