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

PhD thesis

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INTRODUCTION

Gastric cancer and the preceding gastric diseases are very frequent morbidity. They are in the focus of various studies, many mutations are just known as genetic influential factors in the development of gastric cancer. *Helicobacter pylori* (*H. pylori*) infection is a risk factor for the development of gastric cancer, via developing atrophy, intestinal metaplasia (IM) and dysplasia in chronic infection. Although bacterial factors have an important role in disease pathogenesis, most of the evidences suggest that host factors are paramount in determining progression to gastric cancer.

The IL-8 T-251A is a functional polymorphism that seems to be correlated with the pathogenesis of *H. pylori* related diseases. The G-308A polymorphism causes elevated expression of the TNF-α protein. This variation in the TNF-α promoter region was found to be associated with susceptibility to several infectious diseases. The development and progression of gastric cancers is generally driven by an accumulation of genetic alterations that have also been detected in precancerous lesions, such as IM adjacent to gastric carcinoma. From the genetic alterations, mutations and polymorphism of the p53 tumor suppressor gene seem to be the key factors in the development of gastric cancer.

Genetic alterations could appear in each cell of the organism or just in one organ, which is affected by neoplasm. The local mutations can be tested on the level of the DNA with mutation and polymorphism detection or on the level of proteins with e.g. immunohistochemistry.

Various methods for polymorphism detection are able to determine the sequence alterations. The most reliable method for mutation detection and polymorphism screening is the capillary DNA sequencing (CS). The chip technology should be a novel, alternative method of CS, which could be very useful for screening.

According to these in this thesis focuses on the influence of the T-251A polymorphism of IL-8 gene, the G-308A polymorphism of TNF-α gene and various polymorphisms of the p53 gene and the possible influence of the *H. pylori* infection on the development of gastric cancer preceding diseases. Furthermore, the local alterations of DNA and alterations on protein level and the application of different genetic diagnostic methods like CS and chip methods were examined.

AIMS

The objectives were:

- To examine the effect of T-251A polymorphism of IL-8 gene and G-308A polymorphism of TNF-α gene in histological alterations of the stomach like gastritis, IM, atrophy and in macroscopic diseases, like gastric erosion.
- To examine, whether these polymorphisms can influence the incidence of *H. pylori* infection in the examined population.
- To describe genetic alterations of p53 gene in IM.
- To compare the possible sequence aberrations between the samples of different origin (DNA extracted from peripheral blood, from biopsy samples of the corpus and the antrum of the stomach) of the same patient.
- To examine the wild type p53 protein expression in IM, and to verify if there is a relation between the polymorphisms and the protein expression.
- To compare and evaluate the capacity of GeneChip p53 Probe Array and CS method to detect polymorphisms and mutations.

METHODS

I. Patients and samples

Gastric biopsy samples were taken from a total of 218 patients with gastritis (n=86, *H. pylori* positive: n=41), IM (n=43, *H. pylori* positive: n=22), atrophy (n=32, *H. pylori* positive: n=13) and histologically negative (Hist Negative) patients (n=57) during routine gastric endoscopy. The cases were clustered also by macroscopic diagnosis as complete erosion (n=102, *H. pylori* positive: n=41) and macroscopically negative (Macr Negative) (n=88, *H. pylori* positive: n=21) groups. Gastric biopsy samples from the antrum were taken from 50 patients with IM (*H. pylori* positive: n=27) and 51 histologically negative patients (Hist Negative) (all were *H. pylori* negative) served as control. From 7 of the 50 IM patients, gastric biopsy specimens were taken not just from the pathological (antrum) but also from the healthy (corpus) part of the stomach. Also peripheral blood samples were collected to

compare the source of the detected local genetic alterations. The biopsy samples were stored in RNAlater (Qiagen, Düsseldorf, Germany) at -80 ^{o}C until isolation, than genomic DNA was isolated.

II. PCRs, RFLPs and capillary sequencing

The T-251A polymorphism of the IL-8 gene was examined in the samples using Amplification Refractory Mutation System method, the G-308A polymorphism of the TNF-α gene was determined by PCR-RFLP method. The genotypes of the patients were determined by gel-electrophoresis.

The 43 IM patients and the 51 controls were tested only for the exon 4 of p53with CS. In the case of the 7 IM patients 21 samples in total were taken (antrum, corpus and blood samples of each patient) and six exons (4, 5, 6, 7, 8 and 10) of the p53 gene were examined with CS. The CS of exon 4, 5, 6, 7, 8 and 10 of p53 gene was performed using Big Dye Terminator Kit and ABI310 genetic analyzer (Applied Biosystems, Foster City CA, USA) in the case of the 50 IM and the 51 histologically negative patients. The results were analyzed by Chromas vs. 2.3 software and with Sequence Scanner vs1 (Applied Biosystems, Foster City CA, USA).

III. DNA resequencing microarray

The GeneChip p53 Assay (Affymetrix, Santa Clara CA, USA) was used to detect p53 sequences on exons of 2-11 of the 21 samples of the 7 IM patients. The DNAs of the patients and the reference DNA were amplified in a multiplex PCR, than fragmented, labeled and hybridized to the p53 microarrays, finally washed and scanned as recommended by the manufacturer. Data analysis was performed with the Affymetrix Microarray Analysis Suite 5.1 software according to the manufacturer's protocol.

IV. Wild type p53 (DO-7) immunohistochemistry

It has been reported that DO-7 antibody did not react with normal human tissues including stomach, although in 22-76% of malignancies such as gastric cancer, an overexpression was demonstrated. Immunohistochemistry was performed in the tissue sections of the antrum of 19 IM patients, the sections were searched only for the IM glands. Labeling index (LI), defined as the percent of the DO-7 anti-p53 antibody positive cells of the

IM glands that was calculated as the number of brown nuclei (DO-7 positive) divided by the total number of nuclei.

V. Statistical analysis

In the case of IL8 and TNF-α gene polymorphisms for genotype evaluation 2x2 contingency table with Fischer's exact test was used and Odds Ratio (OR) with 95% Confidence Interval (CI) were calculated. In the analysis of p53 gene polymorphisms in IM patients logistic regression was used to quantify the association between the dependent and independent variables. The Odds Ratios (ORs) were calculated as the exponent of the coefficients. The p-value < 0.05 was accepted as statistically significant. For statistical evaluations we used the R environment.

RESULTS AND DISCUSSION

I. Gastric diseases and the T-251A polymorphism of the IL8 gene

The association between the T-251A polymorphism of IL8 gene and the gastric diseases is not clear. In the case of the T-251A polymorphism of IL8 the T/T and the T/A genotypes were significantly different in Hist Negative and IM groups (p=0.038, CI=1.07-Inf, OR=2.96, if the alternative hypothesis is true OR>1).

The A/A genotype was significantly more frequent in the *H. pylori* negative Gastritis group (p=0.049, CI=0.93-8.24, OR=2.73, if true OR≠1) as compared to the control. Interestingly the *H. pylori* negative Atrophy subgroup showed very similar results to the control group and was significantly different from the *H. pylori* negative Gastritis group (p=0.033, CI=0-0.86, OR=0.12, if true OR<1).

Concerning the macroscopic erosions and *H. pylori* infection, the IL8 T-251A polymorphism showed no difference between the observed groups.

II. Gastric diseases and the G-308A polymorphism of the TNF-A gene

In the case of the TNF-α G-308A polymorphism the genotype distributions showed no differences between the different histological groups and no differences in relation to the presence of *H. pylori* infection.

In the case of G-308A polymorphism of the TNF-α gene, the genotype distributions showed statistical significance only if the samples were clustered by macroscopic diagnosis. The G/G and the heterozygote (G/A) genotypes were statistically different in Erosion and Macr Negative groups (p=0.035, CI=1.0055-4.72, OR=2.15 if OR≠1), and in *H. pylori* positive Erosion and *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 elevated in both groups compared to the control group.

III. IM and the polymorphisms of the p53 gene

On comparition *H. pylori* positive IM group to control the chance of IM significantly reduced with RR genotype (p=0.0087). Between the *H. pylori* negative IM and *H. pylori* positive IM groups no statistically significant relationship was found.

Our results suggest that the RR genotype and the presence of the R allele decrease the incidence of IM. This correlates with the finding that R72 variant induces apoptosis markedly better than the P72 variant because of its preferential mitochondrial localization and its greater ubiquination by the E3 ubiquitin ligase MDM2. There were no sequence aberrations found between the samples of different origin (DNA extracted from peripheral blood, from biopsy samples of the corpus and the antrum of the stomach) of the same patient which suggest that there are no local alterations in the p53 gene in IM.

IV. Examination of the genetic alterations of the different origin samples

No different genotypes were detected in the samples of different origin of the same patient. All the polymorphisms were found with the same genotype in the DNA isolated from peripheral blood, from the normal (corpus) and the altered part (antrum) of the stomach.

V. Examination of protein expression by DO7-p53 immunohistochemistry

Analyzing the LI results of the sections, in case of RR genotype samples LI was significantly higher (p=0.004, the difference is 23.52) in the *H. pylori* positive sections than in

the *H. pylori* negative ones and that LI was significantly higher in the RP genotype sections (p=0.012, the difference is 23.31), than in the RR genotype ones in *H. pylori* negative cases. LI was significantly higher in the PP genotype sections (p=0.0027, the difference was 26.99) than in the RR genotype ones in *H. pylori* positive cases.

The R-P change in codon 72 did not affect the binding capacity of the DO-7 antibody, and the protein gave positive sign in the case of staining with this antibody. It seems that the presence of the P allele elevates the presence of the p53 protein in the IM cells. Our results suggest that the *H. pylori* infection also elevates the expression of the p53 protein.

VI. Comparison of microarray based sequencing to CS

All the 21 samples from 7 IM patients were analyzed with GeneChip p53 Array method for 10 exons and with CS for six exons. Each of the mutations detected by GeneChip was controlled by CS. With CS the R72P polymorphism was found in all sample of each patient, while the GeneChip identified only three of these twenty-one R72P polymorphisms, and identified them to RP instead of PP. With GeneChip 16 missense mutations were detected with amino-acid change and 2 without amino-acid change, which was not confirm with CS.

It seems that the GeneChip p53 Assay (Affymetrix) could not be used as a stand-alone test for mutational analysis of the p53 gene, as the results of the microarray were not reproducible with CS method. In the background of the observed discrepancies, the difficulties of multiplex PCR used in the microarray method.

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.

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