UNIVERSITY OF MEDICINE AND PHARMACY

CRAIOVA

FACULTY OF MEDICINE

GENETIC POLYMORPHISMS ASOCIATED TO NOD2 AND ATG5 GENES AND THE RISK OF GASTRIC CANCER

PhD thesis summary

SCIENTIFIC ADVISOR:

PROF. UNIV. DR. TUDOREL CIUREA

PhD student:

FLORESCU DAN NICOLAE

INTRODUCTION

Gastric cancer is a disease with onset and evolution oftenly insidious which involve a delay in diagnostics that is associated with a poor prognosis. Gastric cancer affects millions of people worldwide, with decreasing incidence especially among countries that have substantial national screening programs for gastric cancer.

Countries that do not benefit from more than opportunistic screening still report higher incidences. However, recent developments in the technologies used for endoscopic devices using endoscopes with high resolution and detail techniques allow visualization of mucosal and vascular patterns increase the rate of early gastric cancer diagnosis.

The complex etiopathogenesis of gastric cancer includes interactions between persistent risk factors include untreated infection with Helicobacter pylori, smoking, food as well as environmental factors and genetic susceptibility of each individual.

Treatment of Helicobacter pylori eradication and monitoring of precancerous lesions play a key role in stopping the process of gastric carcinogenesis.

Continuous progress of oncology treatment and surgery have failed to significantly improve the prognosis of patients and research attention has centered on the study of molecular mechanisms of carcinogenesis.

Autophagy is a intracellular biological process with highly debated role in the emergence and development of cancers hence the degradation of intracellular organelles may provide enough energy for cancer cells that undergo metabolic stress. However, autophagy is also involved in the induction of apoptosis and cell differentiation for recognition and processing of viral or bacterial antigens during the activation of the innate and acquired immune response, therefor granting a role in stopping tumor growth.

Mutations of genes involved in autophagy may alter the effectiveness of autophagy and thus promote the process of carcinogenesis. Polymorphisms of genes involved in autophagy have not been fully studied until now. Evaluation of these polymorphisms could bring important benefits for discerning the contribution of genetic susceptibility in interaction with other risk factors for developing cancer.

CURRENT STATE OF KNOWLEDGE

EPIDEMIOLOGY

Gastric cancer is a type of malignant disease which oftenly progresses asymptomatic that determines a delay of early diagnosis. Although methods of diagnosis and treatment have been developed in recent decades, gastric cancer is the fourth most common malignancy and the third leading cause of death in cancer patients after liver cancer and lung cancer, despite its' declining incidence. Ferlay and his collaborators published in 2014 data regarding the incidence of cancers by gender and geographical location.

In Romania, gastric cancer incidence is around 16.66 / 100,000 inhabitants with a male / female ratio of 2 to 1. Every year in our country 3000-4000 cases of gastric cancers are diagnosed. The annual death rate lies around 5,9% in women and 7.7% for men. In Romania, the incidence of gastric cancer ranks fourth in men and fifth among woman for all types of malignancies and is the second cause of death due to cancer. Between 2000 and 2004, the average mortality of gastric cancer was 16.75 / 100,000 in men and 6.27 / 100,000 in women, a rate slightly decreasing during 2005-2009 in both genders (14.68 / 100,000 in men and 5.31 / 100,000 women).

GASTRIC CANCER PATHOGENESIS

Intestinal type gastric cancer development takes place in a gradual process of transformation concerning the normal mucosa by epithelial proliferation, followed by adenomatous transition and then by the appearance of carcinoma.

The pathogenesis is supported by the observation of chronic atrophic gastritis and intestinal metaplasia in patients with gastric cancer. This training model of intestinal type gastric carcinomas was described by Correa and his colleagues that have postulated the temporal sequence of neoplastic changes that subsequently lead to cancer.

An important aspect of intestinal type gastric carcinogenesis is given by the existence of chronic inflammation. In this regard, the involvement of Helicobacter pylori infection has been extensively studied because the persistence of infection leads to chronic gastritis atrophic, with loss of glandular tissue, followed by progression to intestinal metaplasia, dysplasia and finally to advanced gastric cancer. Studies in animal models of any grade of dysplasia state that dysplasia is reversible, though studies are not clear about the reversibility of high grade dysplasia.

RISK FACTORS

Gastric cancer is a disease that includes the interaction of many factors and is one of the few malignant diseases whose etiology includes besides environmental factors also dietary or genetic factors as well as infectious agents (Helicobacter pylori).

Current studies take into account the risk factors for gastric cancer and occupational environment, such as working in the toxic copper mines or the steel industry.

The risk factors involved in gastric carcinogenesis can be divided into:

- ✓ KNOWN RISK FACTORS
    - ○ Helicobacter pylori infection,
    - ○ chronic atrophic gastritis,
    - ○ intestinal metaplasia,
    - ○ dysplasia,
    - ○ gastric adenomatous polyps,
    - ○ smoking
    - ○ diet rich in nitric oxide
    - ○ stomach surgery (gastric resection especially Billroth II)
    - ○ genetic,

- family history of gastric cancer (first degree relatives)
- adenomatous polyposis family,
- Peutz-Jeghers syndrome.

✓ LIKELY RISK FACTORS

- obesity
- pernicious anemia,
- high consumption of salt.

✓ POSSIBLE RISK FACTORS

- Menetrier disease,
- low socioeconomic level.

GENETIC FACTORS

As in most malignancies, genetic factors and the interaction between environmental causes leads to progression of gastric cancers. About 10% of gastric adenocarcinoma patients have a family history of cancer who acts independently of the control of HP eradication gastric infection or other environmental factors.

AUTOPHAGY

A special role during the carcinogenesis process seems to be attributed to autophagy. The role of autophagy in carcinogenesis is controversial. Autophagy activation mechanisms may promote tumor as malignant cells can use autophagy to survive the metabolic stress. A potential mechanism may be represented by digesting their cell components (self-digestion) and getting amino acids as an alternative source of energy. In contrast reduced autophagy contributes to tumorigenesis and tumor growth by inhibiting type II cell death.

Studied genes involved in autophagy with described role in gastric cancer are ULK1, Beclin1, ATG5, ATG9, ATG10, ATG12, LC3B who have proven prognostic value as key regulators of the entire building process of the autophagosome.

ATG5 gene is located on chromosome 6q21 and is designed to encode protein ATG5 and together with ATG12 and ATG16L1 forms a multimeric protein complex necessary for the establishment of the autophagosome, essential in the development process of autophagy. ATG5 protein is involved in the molecular mechanism that prevents the death of tumor cells during chemotherapy and some gene polymorphisms such rs2245214 have been associated with increased susceptibility to cancer, especially thyroid cancer.

NOD2 gene is localized on the long arm of chromosome 16 at position 21 (16q21) and is involved in the immune response by activating autophagy or exogenous proteins, but it has no role in the autophagosome creation. This gene provides instructions for the synthesis of proteins involved in immune function such macrophages or monocytes.

ORIGINAL CONTRIBUTIONS

OBJECTIVES OF THE PhD THESIS

The main purpose of the published studies was to evaluate the association between the main polymorphisms located in genes involved in autophagy and susceptibility to gastric cancer. These genetic variants have an unclear role or were not been investigated so far.

Evaluation of selected polymorphisms could bring an important benefit in understanding the role of autophagy in gastric carcinogenesis and explain the complex mechanisms of interaction between genetic susceptibility of the host and other risk factors.

OBJECTIVES

The analysis of the frequency of NOD2 polymorphism rs2066844C in a group of gastric cancer patients in comparisson to healthy subjects in order to appreciate the risk of gastric cancer in people who exhibit variations in the gene NOD2 gene.

The study of gene polymorphism frequency key role in autophagy (ATG5 rs2245214 C / G) on a group of Romanian volunteers due to the fact that up until this time there were no data regarding the frequency of this variant gene in Eastern Europe.

In addition the frequency of ATG5 rs2245214 polymorphism was studied on a group of patients with gastric cancer to check a possible susceptibility to gastric cancer in these patients.

MATERIAL AND METHOD

We analyzed the frequency of NOD2 and ATG5 gene polymorphisms on separate batches of gastric cancer patients compared to healthy subjects groups in order appreciate the risk of gastric cancer for people who have the gene NOD2 gene variations.

To complete the research we followed several steps:

- Forming the study groups by establishing clinical-morphological parameters to be investigated as well as rigorous criteria for inclusion or exclusion from the study:
    - control group,
    - group of patients with gastric cancer,
- Biological sampling
    - blood sample from both groups,
    - surgical or endoscopic biopsies from the gastric cancer group,
    - gastrectomy resection parts from gastric cancer group,
- Genetic tests
    - specific TaqMan probes using as method RealTime PCR for each of the studied polymorphisms
    - electrophoresis in agarose gel,
    - PCR technique.
- Statistical analysis in the studied groups through:
    - X^2 test- Hardy-Weinberg

- The odds ratio (OR) with 95% confidence interval, p test - to determine any correlations between the studied polymorphisms and risk of gastric cancer, as well as:
  - ✓ tumor location,
  - ✓ histopathologic type according to Lauren classification - intestinal or diffuse adenocarcinoma.

The research was conducted on samples available in the Laboratory of Human Genomics as well as new biological samples harvested from patients enrolled during the thesis to increase the scientific impact of the published articles.

In the Laboratory of Human Genomics s there is a section containing samples from patients with gastric adenocarcinoma. Available biological samples from patients with this pathology are blood samples, genomic DNA, cDNA, biopsies of cancer tissue, and normal tissue biopsies.

Also within this section there is a set of biological samples from subjects without chronic conditions that constituted the control group for the study of the selected polymorphisms.

Patients diagnosed with gastric adenocarcinoma during the period of 01.01.2014 until 06.30.2015 in the Department of Gastroenterology and Surgery I and II of the Emergency County Hospital Craiova represented the prospective part of PhD research. Their introduction in the study met inclusion and exclusion criteria initially established.

Patients who presented with dyspeptic syndromes in the same period to whom the upper gastrointestinal endoscopy revealed no injuries were also included in the control group.

RESULTS

My research assessed the involvement of NOD2 Arg702 Trp polymorphism in gastric cancer development and for this matter I have genotyped 322 biological samples, of which 72 patients with gastric cancer with histopathological confirmation of gastric adenocarcinoma and 250 healthy controls.

The rigor of the exclusion caused removal from the study of patients who had histological confirmation of the precancerous lesions or gastric lymphoma.

The distribution of the controls was carried out so as age, gender, or ethnicity are similar.

NOD2 polymorphism rs2066844 C / T (Arg702Trp) was not in the control group significantly deviated from the values expected from the Hardy-Weinberg equilibrium balance ($p > 0.05$, $X2 = 0.24$) in the control group.TT genotype was not found in any patient.

There were no statistically significant differences between the two groups by comparing each genotype (genotype CC served as a reference) (OR 0.45, 95% CI: 0.10 - 2.05) or when we compared the frequency of allele (C allele served as a reference) (OR 0.46, 95% CI: 0.11 - 2.04).

NOD2 rs2066844 polymorphim did not associate with tumor location and histopathologic type as these parameters were examined separately and my study reports significant differences between tumor location and histopathologic gastric cancer type (intestinal/diffuse) or control group along the stratified analysis.

CC genotype was most common among the group with gastric cancer, but also in similar proportions in the population control.

C allele was found in 98.61% of gastric cancer cases compared with 97% in the control group.

T allele was found in 1.39% of gastric cancer cases, compared with 3% in the control group.

In the study concerning the involvement of ATG5 polymorphism rs2245214 C / G in gastric cancer development I have genotyped 138 biological samples of which 33 were from patients with gastric cancer with gastric adenocarcinoma histological confirmation and the rest of 105 from healthy controls.

The gastric cancer batch contained 33 gastric cancer patients with a mean age of 67.6 years, the gender distribution of 21 men and 12 women.

The control group of 105 healthy volunteers aged between 17 and 75 years with a gender distribution of 49 men and 56 women.

Gastric adenocarcinomas were classified according to the Classification Lauren in adenocarcinomas of intestinal type and diffuse of which 23 cases were intestinal type and 10 cases were diffuse.

Regarding the degree of tumor differentiation, the total of 34 cases of gastric were separated in:

- 9 G1 (well differentiated)
- 13 G2 (moderately differentiated)
- 11 G3 (poorly differentiated and undifferentiated).

The staging of gastric adenocarcinoma used TNM system which allowed me to report that most of the patients suffering from gastric cancer were diagnosed in a late stage (stage IV):

- ✓ 4 cases stage I,
- ✓ 6 cases stage II,
- ✓ 6 cases stage III
- ✓ 17 cases stage IV (50%).

Regarding the analysis study of healthy volunteers the genotypic frequencies of ATG5 polymorphisms did not deviate significantly from the values expected under Hardy-Weinberg equilibrium.

Genotyping of studied polymorphism in the controls indicated:

- CC genotype in 53 subjects,
- CG genotype in 40 subjects,
- GG genotype in 12 subjects.

DISCUSSIONS

The results of my study report the lack of statistically significant correlations between the studied polymorphisms and susceptibility to gastric cancer since polymorphism genotypes distribution in the studied groups is similar.

Similar results were reported in a Portuguese study which assessed NOD2 mutations and gastric cancer susceptibility. The study was performed on a group of 150 patients with gastric cancer and 202 healthy controls. The genotype frequencies for R702W proved no association between various histological types of gastric cancer and

the studied polymorphism. The same study implied that the 3020insC variant represents a risk factor for developing gastric cancer in Portugal, precisely intestinal histological type.

The Portuguese results are sustained by results in a German study that included 171 patients suffering of gastric cancer and 153 healthy controls and whose results implied that the studied NOD2 gene polymorphisms are not risk factors for gastric cancer.

On the other hand an Italian study published earlier in 2009 which included 170 gastric cancer patients and 156 healthy controls admited that R702W and 1007fs polymorphisms were highly correlated with gastric cancer. The authors incriminated environmental carcinogens and NOD-induced proinflammatory cytokines as possible reasons by which the studied polymorphisms may increase the risk for gastric cancer.

A meta-analysis published in PloS one in February 2014 by Liu and co. evaluated the evidence that NOD2 polymorphisms might be related with increased cancer risk. The results of the already published studies were not conclusive. The meta-analysis evaluated the NOD2 polymorphisms such as rs2066842 C/T, rs2066844 C/T, NOD2 rs2066845 C/G, NOD2 rs2066847. The NOD2 rs2066844 C/T polymorphism was associated with increased risk of cancer for individuals with TT or CT genotype compared to individuals with CC genotype. The subgroup of analysis revealed that TT+CT genotype was associated with high risk of colorectal cancer but there was no important association with the development of gastric cancer. The study included the analysis of 16 studies that consisted of 4507 cancer cases and 4780 healthy patients that assessed the risk of cancer associated with NOD2 rs2066844 C/T polymorphism. The meta-analysis concluded that several NOD2 polymorphisms might be associated with increased risk of developing gastrointestinal cancers.

The differences in results were explained in 2011 by Kutikhin when he explained the reasons why studies show different results and he attributed these variants in results to environmental factors that may increase risk of cancer in various populations, different bacterial and viral impacts in the aetiology of cancer, as well as diagnostics, genotyping methods, differences in sample size, clinic and histological characteristics and least but not last chance.

A greek study from 2005 performed by Papaconstantinou and co. suggested that NOD2 mutations may be a risk factor in developing colorectal cancer after assessing R702W,G908R and 3020insC variants in 104 Greek patients suffering of gastric cancer and 100 healthy controls with results showing much higher frequency of all the mutations in the gastric cancer group compared to the healthy group.

Furthermore, NOD2 polymorphisms have been evaluated in relation with other pathological entities such as inflammatory bowel diseases (IBD), especially since 2001 when NOD2 was identified as the first gene related to Crohn Disease.

On other hand the research concerning the frequency of ATG5 rs2245214 C / G polymorphism on two groups of subjects with Romanian ethnicity is the first reporting of its kind in south-western Romania.

Our allele frequency results are slightly different when we compared with other Caucasian cohorts, yet the deviation was higher for genotypes. In a Dutch case-control study, including 189 healthy controls, the genotypes were 35% CC, 52% CG and 13% GG. This research showed that patients carrying the ATG5 rs2245214 polymorphism have a higher probability to develop thyroid carcinoma. In a replication study of previously reported systemic lupus erythematosus risk loci, including ATG5 rs2245214 polymorphism, the risk G allele frequency was 0.37 in 12188 controls. A separate comparation including only American and Swedish controls show a 0.353 respectively 0.407 of G allele.

In another case-control study including 952 Javanese controls (Indonesia) no association was observed between this polymorphism and susceptibility to pulmonary tuberculosis. The frequency of ATG5 genotypes in control group were 35.8% CC, 48,5% GC and 15,7% GG, and minor G allele frequency was 39.9. The minor C allele was found in 47.4% controls in a Han Chinese population case-control study, including 807 systemic lupus erythematosus patients and 938 healthy controls, and no correlation with systemic lupus erythematosus was found . Minor allele frequency in our study was lower than those found in HapMap public database: 0.30 (G) in our study versus 0.43 (G) in Caucasians, 0.45 (G) in African Americans, 0.47 (G) in Han Chineses and 0.49 in Japanases (National Center for Biotechnology Information dbSNP). In addition to the

small number of samples, an explanation for our results could be the ethnicity of subjects.

Other ATG5 variants were investigated in several case-control studies. No association was found between Atg5 M129V (rs34793250) and age at onset of Huntington disease. Polymorphism rs6568431 in ATG5 was initial associated with rheumatoid arthritis in a UK cohort, but the association did not remain statistically significant after Bonferroni correction. Harley et al., identified ATG5 rs573775 (G/A) as a susceptibility locus for systemic lupus erythematosus, but their results were not replicated in a Finnish study.

Two other ATG5 polymorphisms (rs12201458 and rs510432) were associated with asthma. The minor allele (A) of ATG5 rs12201458 was associated with a decreased risk of asthma, while the minor allele (G) of ATG5 rs510432 was associated with increased asthma risk. Alonso-Perez et al. showed that Atg5 rs573775 was more associated with systemic lupus erythematosus susceptibility in Central than in Southern Europeans, and T allele was a risk factor for systemic lupus erythematosus in carriers of the high IL-10 producer genotype.

A Chinese study published in 2011 on 45 patients with gastric cancer and 58 patients with colorectal cancer assessed mutations of autophagy-related genes such as ATG2B, ATG5, ATG12 and concluded that ATG mutations affect the autophagy process and can contribute to the development of gastrointestinal cancers. Similar conclusions were reported by another Asian study whose data suggested that altered ATG5 gene can play a role in carcinogenesis by affecting autophagy and therefore the cell death.

In a later study, published in 2014, Jang-Ming lee and al. concluded that ATG5 and COL4A3 gene polymorphisms can serve as unfavorable prognostic predictors for patients with esophageal squamous cell carcinoma .

Studies that assess autophagy-involved gene variants and outcome of gastric cancer are limited but there is literature data has proven that several genetic variants involved in authophagy can result in poor outcome for patients with prostate cancer. A Chinese study performed on 962 patients concluded that lower ATG16L1 expression levels were correlated with worse outcome in patients with prostate cancer.

Significantly more evidence emerges to support the involvement of autophagy and inflammasome activation in the process of immunogenic tumor cell death. Inflammasomes are large intracellular protein-complexes that occupy an important role in innate immunity and whose activation is essential for the defense of the host against various intracellular pathogens suchs as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). In 2011 Kersse et al. stated that the activity of the inflammasomes can be regulated via autophagy which acts by degrading them in macrophages in order to impose a limit to their over-activation.
Some studies have claimed that ATG5 variants can prove to be beneficial. In this matter a recent italianItalian study suggested that ATG5 rs573755 was protective for patients in matter of developing erosive inflammatory arthropathies. Similar results were found for ATG5 rs2245214.

My research indicated that there is no statistically significant association between ATG5 rs2245214 polymorphism and gastric cancer. Similarly, the stratified analysis is not able to suggest the association of the studied polymorphism with the histological grade of tumor differentiation, tumor location or disease stage.

CONCLUSIONS

Gastric cancer is a disease of complex etiology in which the host genetic susceptibility interacts to other risk factors such as environmental factors, diet and Helicobacter pylori infection.

Gastric cancer does not have specific symptoms and is often asymptomatic. Frequently, gastric cancer is diagnosed in advanced stages due to the fact that there is no national screening program, and patient approach medical help extremely late. My research demonstrated that over 60% of patients were diagnosed in stage III or IV according to the TNM classification.

The need for early gastric cancer diagnosis is imminent from increasing percentages of survival at 5 years.

The emergence of new generation of endoscopic devices that allows describing in high resulotion the mucosal pattern increased the rate of early gastric cancer diagnostics.

My research is the first report of the involvement of ATG5 and NOD2 gene polymorphisms in the occurrence of gastric adenocarcinomas in Romania.

NOD2 gene polymorphism rs2066844 C / T (Arg702Trp) is not related with gastric cancer risk in the population studied Romanian and future investigations are needed to fully elucidate additional contribution of NOD2 gene in gastric carcinogenesis.

ATG5 polymorphism does not affect susceptibility to gastric cancer.

G allele risk frequency of the polymorphism rs2245214 was different in the control group from other reports in the literature due to ethnic variations worldwide.

The aggressiveness of gastric cancer is supported by the loco-regional invasion and silent metastasis processes that were observed in most diagnosed cases. Metastasis as well as invasion in serous and adjacent structures has been found in 50% of gastric cancer patients enrolled in ATG5 rs2245214 genotype studying group.

There is an increasing percentage of poorly differentiated and undifferentiated adenocarcinoma (G3) which supports the recent literature data which certifies the increased incidence of diffuse adenocarcinomas and decreased incidence of intestinal type adenocarcinomas.

The stratified analysis of the cases denied an association between ATG5 rs2245214 C / G polymorphism with histological grade of tumor differentiation (G1 - well differentiated G2 - moderately differentiated G3 - poorly differentiated and undifferentiated), tumor location (cardia and non-cardia) or tumour staging.

The absence of correlation between the studied polymorphisms and gastric cancer risk can be explained by a possible involvement of genetic heterogeneity in gastric carcinogenesis.

In order to fully clarify the influence of gene polymorphisms involved in the autophagy process and susceptibility to gastric cancer it is required to be able to assess different large group studies within the same ethnic populations.

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