UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA

DOCTORAL SCHOOL

DOCTORAL THESIS

INVOLVEMENT OF MAIN POLYMORPHISMS OF ANTIOXIDANT ENZYMES IN COLORECTAL CANCER

ABSTRACT

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INTRODUCTION

Colorectal cancer is a major problem of public health, having an increased incidence, at the moment represents the fourth place in frecquency over the world. (1,2)

The etiology of colorectal cancer is not completely known, but it is demostrated a multifactorial ettiology, including environmental factors, as well as genetic factors. Almost 80% of cases are spoaradic , in patients without any evidence of familal antecedents, and 20% of cases are described in patients with familial antecedents of colorectal cancer or colorectal adenomas and polyps.

The research in molecular biology, the elaborated studies of genetics are discernig the expectation of the management of this disease and will allow rational strategies of protection against pathologic mechanisms of neoplasic diseases.

In this actual context my study propose an evaluation of the polymorfisms of the main enzymes involved in cellular oxidative stress which could be associated with an increased risk of colorectal cancer.

Key words: colorectal cancer, cytokine, singlenucleotidic polymorfism, genotype, susceptibility

CURRENT STATUS OF KNOWLEDGE

CHAPTER 1

Epidemilogy and risk factors

This chapter presents recent dates in relation with the incidence and prevalence of colorectal cancer in Europe and Romania .

Colorectal cancer (CRC) is a major cause of morbidity and mortality, representing almost 25% of digestive cancers. In conformity with recent studies, the incidence rates of colorectal cancer at 100000 persons are variable over the world, for male from 4,1% in India to 59% in Czech Republic, and for women from 3,6% in India to 39,5% in New Zeeland. The differences in incidence among countries , and the rate of incidence at emigrants demonstrate that the incidence of colorectal cancer is dependent on envinromental factors.(3,4)

This chapter presents also the risk factors associated with this disease, like: diet, obesity, smoke, alcohol consumption, free radicals, genetic susceptibility of host.

CHAPTER 2

The role of oxidative stress in tumorigenesis

The free radicals are substances derived from incompletely oxidated compounds that have passed through partial combustion, having in their structure groups of oxygen capable to initiate at the surface of cellular membranes or even inside the cells aggressive oxidation reactions (5).

The most active free radicals are: superoxide , peroxide, hidroxid and nitric oxide. They have two major sources: endogene (free radicals that are constituted in the organism during the physiological processes or metabolic processes) and exogene (free radicals which penetrate the organism from outside.(6)

Free radicals are highly reactive molecules which determins important damages in the mollecular structure, as nucleic acids, lipidic structures and proteic structures. (7)

Reactions between reactive oxygen species and organic substrates are a complex event. ROS represent key components of signalling paths determining induction or suppression of cellular proliferation as well as the release or inhibition of apoptosis.(8)

Proteins are very susceptible to ROS, being a frequent target of increased production of free radicals. ROS oxidizes the protein structures and inhibits the proteolytic systems having as result irreversible alterations of the proteic structures and enzyme functions, and alteration of ADN polymerase capactity in ADN replication (9). The configuration of carbonyl group is considered a stabile and early marker of proteic oxidation, having an essential role in the pathogenesis of a high number of cancers(10,11)

DNA damages induced by oxidative stress promote carcinogenesis, a process suggested by a high susceptibility to develop cancer, found especially in pacients with different inflammatory affections such as viral hepatitis, ulcerative colitis, or Helicobacter pylori infection (12). In these affections cancer is induced by the increase of ROS level which determines DNA lesions with mutagenic potential by activating the oncogenes or by inactivating the tumor- suppressor genes(13, 14). The tumoral promotion and the tumoral progression can b the consequence of high levels of endogene ROS. At tumoral cells there are high levels of ROS, due to a high rate of cellular metabolism and to the deficiency in redox systems (15,16), characteristics that can contribute to the tumoral progression.

There are studies that suggests that the generation of ROS have involved in all phases of carcinogenesis- initiation, promotion and tumoral progression (17). Cellular and molecular events evidentiated during carcinogenesis are including DNA damage, increased proliferation and genetic instability (13, 18).

In the last years there were studied many associations between oxidative sress and colorectal cancer.

Most studies are concentrated on the investigation of genetic factors with a role in the susceptibility to CRC. It is demonstrated that several singlenucleotid polymorphisms (SNPs) of genes that encode enzymes involved in the defense antioxidant mechanism is associated with an increased susceptibility to cancer development. Singlenucleotid polymorphisms have an important role in colorectal cancer progression, being potential biomarkers for the risk of colorectal cancer.

An extensive case –control study on the incidence of carcinogenesis in South-west of Germany by Silvia Funke et al in 2009 estimated association between carcinogenesis risk, smoking and

some genetic polymorphisms of the enzymes with a crucial role in oxidative stress: catalase(CAT) (rs 100179), superoxiddismutase (SOD), myeloperoxidase (MPO) and endothelial nitric oxide synthetase – e(NOS):CAT(rs100179), MnSODVal^{9}Ala(rs4880), MPOG^{463}A(rs2333227) and eNOS Glu^{298}Asp(rs1799983), but none of the genetic polymorphisms described was associated with colorectal carcinogenesis , none important interaction between the durate of smoking and these polymorphisms has been identificated (19).

The association between SOD Val16 Ala (rs4880) polymorphism and GPX1 (rs1050450) and the risk of malign tumors has been evaluated by Blein S and Brendt S in 2014, in an case- control study on a lot of 10726 cases of breast cancer and 7532 cases of prostate cancer. There were no identificated associations between these polymorphisms and the risk of cancer(20).

Our study is based on the association between oxidative stress and genetic factor. We evaluated the association between the presence of some enzyme polymorphisms, such as CAT, SOD2 and SOD 3 and the susceptibility or the resistance of the appearance of colorectal cancer.

ORIGINAL CONTRIBUTION

CHAPTER 3 MATHERIALS AND METHODS

Inclusion criteria

For the accomplish of this project there were studied 2 lots of patients from Emergency Hospital of Craiova: a lot of subjects diagnosed with colorectal cancer and a control lot. All subjects were informed about the aim of the study and signed an informal consent before including in the study.

Prelevation of samples and biological matherial

The biological material was represented by blood samples (3 ml of venous blood preleved on EDTA and maintained at 4 degrees until the moment of DAN extraction) and samples of tumoral tissue for the studied lot and only blood samples for the control lot.

DNA extraction

For genomic DNA isolation from blood samples we used Wizard® Genomic DNA Purification Kit (Promega, Madison, WI).

This method has four main phases through the final result.The protocol begins with cellular and nuclear lysis (in the beggining with red blood cells, then leucocytes and their nucleus lysis). The next phase is optional and consists inRNA digestion by some enzymes named RN-ase. Then, the proteins released from cells are remoted by precipitation with a saline solution, which precipitates the proteins, while in the solution remains genomic DNA. Ulterior, by precipitation in isopropanol, DNA is concentrated. The last phase is deshidratation in etanol, and rehidratation of DNA.

The detection of genetic polymorfisms by Real-Time PCR

Genetic analyses for identification of the polymorfisms of genes studied were elaborated in Molecular Genetics Laboratory of University of Medicine and Pharmacy of Craiova, which possess the necessar equipement for these tests.

Real- Time PCR with Taqman probes is a moderne tehnique where the magnified fragment is visualisated once with the progress of the amplification process.This real time following of the amplification process is possible by fluorescent marking of primers or probes.

Statistic process of dates

With Microsoft®Office Excel® 2007 and Genex Pro 4.4.2.308© we realised correlations and associations between the studied parameters and the results from Real- Time PCR reactions.

For descriptive analyse of dates we used Microsoft Acces 2007 Program. We used this program to realise the database for the statistic process.

For the characterisation of value series we used statistical indicators.

CHAPTER 4

RESULTS

In this study are involved 314 subjects: 117 were diagnosed with colorectal cancer (CRC), and 197 subjects- the control lot.

1. **CAT-262 C□T (rs1001179) ploymorfism**

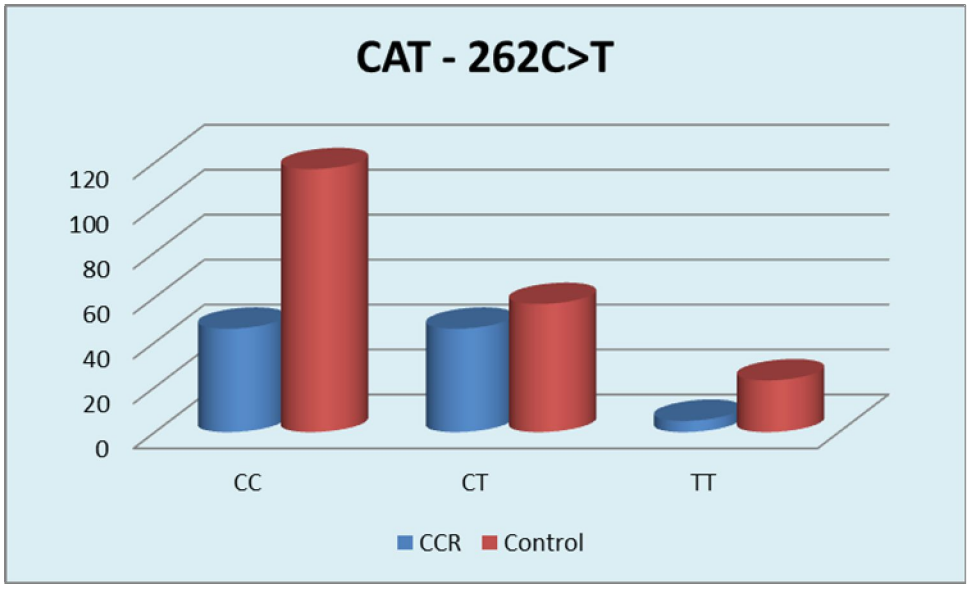

Fig. 1: Genotype frecquncy CAT -262C>T polymorfism

Comparing the genotypes and statistic dates obtained, it result that for **CAT-262 C□T there is a high association with colorectal cancer for CT genotypes (heterozygotes).**

Comparing the frequency of CT genotype with CC genotype (refernce genotype for for CAT-262 C□T polymorfism) we observed that the presence of CT polymorfism is associated with a two times higher risk of colorectal cancer . CT and TT genotypes have a 1.1 times higher risk of colorectal cancer.

By stratificated analyse of this polymorfism weobtained interesting results. For CAT-262 C□T polymorfism, there is a high association for CT genotype (heterozygotes) with the risk of colorectal tumors with high cellular differentiation (G1), and a low association with medium and low differentiated tumors.

2. SOD 2-201 A□G (rs4880) polymorfism

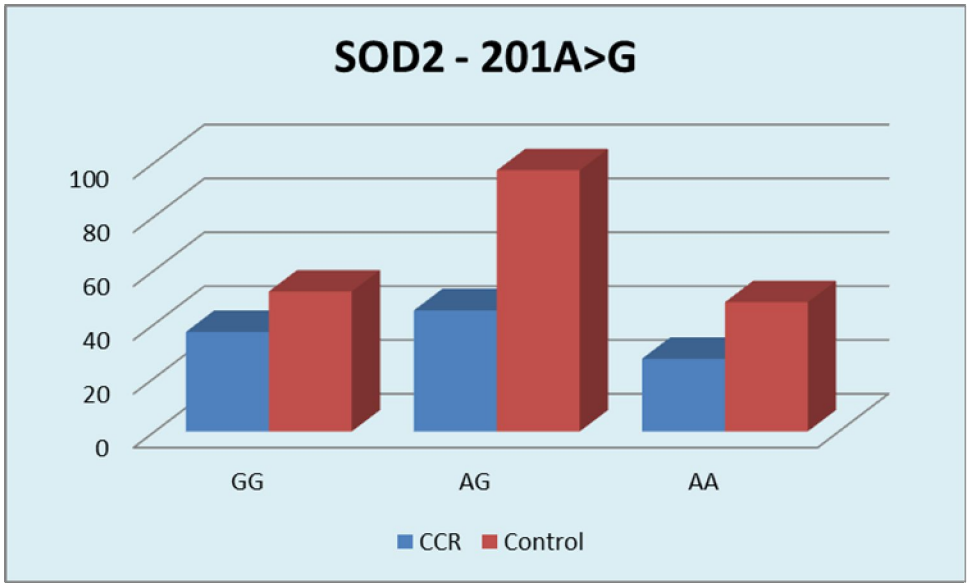

Fig. 2: Genotype frequency for SOD2–201A>G polymorfism

Comparing the genotypes (with reference GG genotype) and obtained statistic dates , we observed that SOD2-201 A□G polymorphism is not associated with colorectal cancer. Also, GG and AG genotypes represent 76% from variants in control lot, and 76% in study lot.

By stratificated analyse of cellular tumor differentiation, we observed for SOD 2-201 A□G there is no association with high susceptibility or resistance to colorectal cancer.

3. SOD 3-896 C□G polymorphism

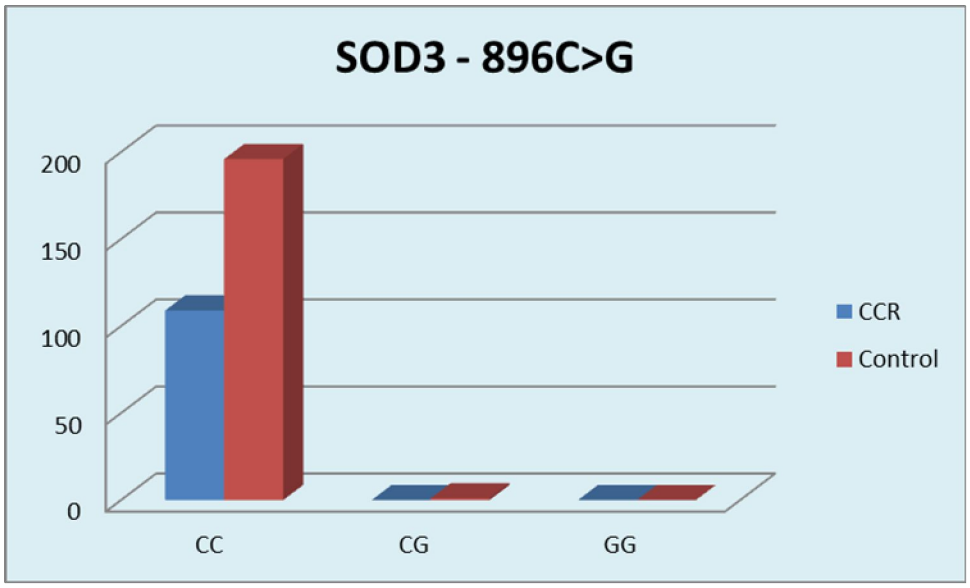

Fig. 3: Genotype frequency for* SOD3-896C>G *polymorfism

In our study SOD 3 -896C□G polymorfism is not associated with colorectal cancer. Also it is observed that CC genotype represents 100% from variants for study lot, and an appropriated value for control lot.

CHAPTER 5

CONCLUSIONS

1. We observed that for CAT-262C□T polymorfism, CT genotype is associated with a two higher risk of colorectal cancer. CT+TT genotypes have 1.1 higher risk of malign colorectal tumors.
2. By stratificated analyse of the study lot based on the cellular differentiation, we observed that CAT-262C□T polymorfism is high associated with colorectal cancer, having a two and a half higher risk of developing high differentiated colorectal tumors.
3. We observed that SOD 2-201A□G polymorfism is associated with a higher risk of developing high differentiated colorectal cancers. This result needs to be confirmed by future studies.

4. The results for SOD 3-896C□G have not been statistic corelated with an increased risk of colorectal cancer.
5. The study lot respected the Hardy-Weinberg statistic equilibrum . The results are concording with studies published by now.

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