

UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
DOCTORAL SCHOOL

DISERTATION

A STUDY OF SOME MOLECULAR
MECHANISMS
IN PREMALIGNANT GASTRIC LESIONS

ABSTRACT

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Craiova 2013

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I. GENERAL PART

1. INTRODUCTION

Digestive cancers and especially gastric cancers, continues to be within the first 5 places regardless of the statistics that I could cite; in Europe in 2006 , the incidence of 159,900 new cases reported annually (5th place), producing a mortality by 118200 [1,2], indicating that there exists a significant variation over 3 times higher in eastern Europe compared to western Europe. Worldwide incidence of gastric cancers is almost 5 times higher in South America, and East Asia (China, India, and some of the outlying areas of non-industrialized Japan, where he reported a very high mortality 12.5 %, which situates gastric cancer 2nd after lung cancer [3]).

2. CARCINOGENIC RISK FACTORS regarding gastric cancer, were classified by the studies of Gomceli et al. into 3 groups: [8] *genetic factors* (male sex , family polyadenomatosis - Lynch II; diffuse gastric cancer in CDH1 gene with E- cadherin gene mutation, genetic polymorphism for cytokine pro and anti -inflammatory, Peutz-Jeghers syndrome), *exogenous environmental factors* (H. pylori, Epstein Barr virus, excess alcohol, hipersodium nutrition, smoking and dry food, nitrite, nitrate, N-nitroso compounds, smoke, dust, diets low in fiber and fresh vegetables, low intake of antioxidants - carotenoids, folate, tocopherols, ascorbic acid) local gastric risk factors - *pre-malignant lesions*.

3. PREMALIGNANT GASTRIC LESIONS. The general section summarizes, according to literature [25,35], few defining data regarding the most common disorders of the digestive tract that are included in most gastroenterology treaties as gastric pre-malignant lesions, ie pathological situations under certain conditions can progress to the development of gastric tumor disease (chronic atrophic gastritis, Barrett's esophagus, gastric adenomatosis; Menetrie gastritis, gastric polypoid adenomatous hyperplasia, gastric metaplasia, pernicious anemia, subtotal gastrectomy with an age of over 20 years, back gastric polyps, benign gastric ulcer, chronic gastritis with a high degree of dysplasia).

4. PATHOPHYSIOLOGY OF GASTRIC ONCOGENESIS. Polyamines are small molecules whose essential role is cell growth and proliferation in normal tissues, but unfortunately the same role in neoplastic tissues pathological type [50,51,52,53]. Since 1984 is known from studies of Tabor and Tabor [49] that polyamines and the activity of ornithine decarboxylase (ODC) , are closely correlated with proliferation and cell transformation. Antizymes (AZ) and their endogenous inhibitors (AZIN) are metabolism modulators and hence the production of ODC and polyamines are therefore negative regulators in the karyokinesis by decreasing or increasing the activity of proliferation, differentiation and cell transformation. The relationship between AZIN

level than that of AZ, at cellular level is particularly important, because the higher the AZ creates conditions to produce tumor regression, as AZIN mainly predisposes the activation of proliferation and tumor growth. As a natural consequence, finding methods for ODC degradation has become a long-term goal, if we look at this as a possible anti-tumor cure, so at this point there are several clinical trials using the ODC inhibitor regimen [86 , 87].

5. RESEARCH METHODS IN ONCOGENESIS.

CELL CULTURES. All medical research in recent years were mainly performed on cell cultures harvested from tumor tissue, for studying the mechanisms of initiation and maintenance in the karyokinetic process. Since 1984, The European Collection of Cell Cultures (ECACC) was founded, which was the first cell culture collection available to researchers from around the world, it later became the most authentic and recognized bank of cell line cultures from around the world.

Polymerase Chain Reaction (PCR) is a biochemical laboratory technology which is used for the synthesis of DNA, by base DNA pairs amplification. The technique is used not only in medical laboratories, but also in a in a large number of areas with different profiles: molecular biology, environmental science, forensic medical sciences, biotechnology, microbiology, food, epidemiology, etc.

II . SPECIAL PART

1 . MATERIALS AND METHODS.

CELL CULTURES. Our study was conducted in two stages: the first period (October 2010 - December 2012) , began in the Gastroenterology and Hepatology Research Center, Department III of Internal Medicine and Gastroenterology, County Emergency Hospital, Craiova, under the careful guidance of Mr. Prof. Dr. TUDOREL CIUREA and was focused on deepening and accumulating knowledge and current data as related to gastric cancer, premalignant lesions, risk factors, morbidity. The second period (January-September 2013), has resulted in a daily work done in the UNIVERSITY OF HELSINKI, FACULTY OF MEDICINE, HAARTMAN INSTITUTE, under the guidance of Mr. Prof. Dr. LEIF ANDERSSON in the Department of Pathology, where we performed in vitro study that is the subject of this paper. We present below the latest methods of DNA identification and multiplication in cancer cells in three culture cell lines (SW 1353, K562 , HGT -1).

WESTERN BLOTTING, or immunoblotting is a method that is based on three important steps that result in the separation and detection of proteins in a mixture.

POLYMERASE CHAIN REACTOR is a precise technical, complicated and long lasting achievement, during several stages. **DNA matrix.** All PCR reactions begin with the selection of linear or circular DNA molecules which must be very pure, to ensure the integrity of freedom from the action of DNA polymerase, and further should be selected molecules containing the sequence which is subject to amplification. The **oligonucleotide primers**, which are produced in the PCR reaction should have a concentration from 0.1 to 06 mM, the complementary intra-chain must not be close to or even identical values of T_m to allow for the attachment temperature between 55 – 65 degrees C (optimum recommended 62-63 degrees C). **Thermostable DNA polymerases.** Given the importance of the stability of the optimal temperature for the success of the PCR, the DNA polymerase used today have been achieved by genetic manipulation of thermophilic microorganisms (*Thermus aquaticus*), which enzymatic equipment with optimum temperatures (above 70 degrees C) and are able of maintaining specific activities even after passing the high temperatures (above 100 degrees C). **Deoxynucleotide triphosphate (dNTP = dATP , dGTP , dCTP , dTTP)** . The four kinds of deoxynucleotide triphosphates molecules are introduced into the reactor in equimolar amounts (50 to 500 micrometre each dNTP) and DNA polymerase are used in the polymerization reaction. PCR is often used for **DNA amplification and quantification** IN forensics and archeology, the results with a high degree of accuracy, as well as **medical diagnostic method** used in recent times for diagnosis of diseases transmitted genetic identification cancers and hematological malignancies (RPS technique with a sensitivity of at least 10,000 times higher than

any other method used).

A. PERSONAL STUDY.

In the Pathology laboratory of Haartman Institute, University of Helsinki, I had to work on 3 cell lines: *Project 1* - cell line SW 1353, *Project 2* - cell line K565 and *Project 3* cell line HGT -1. The entire process of molecular study that I attended is a sequence of a survey work AZIN less investigated isomers (AZIN2, AZIN3).

PROJECT 1 - SW 1353. In this study, SW cell lines - human chondrosarcoma was investigated by analyzing the activity of AZIN2 - mRNA gene expression induced by treatment with IGF. This way, we created an in vitro model whose stability depends on the curve - time and dose required for qPCR. We tried to study and highlight its expression level in other tumor cell types and especially its dependence on dose, comparing results from acquired qPCR with housekeeping gene GADPH.

PROJECT 2 - K562. **Step 1** for Project 2 consisted of determining AZIN -2 induction, Rab27A and Rab27B in K562 cell cultures where megakariocitary differentiation attempted by treatment with 30nm *TPA (tissue plasminogen activator)*. In **step 2**, the second substance for induction of AZIN2, Rab27A and Rab27B in K562 *erythroid differentiation which has been made with 60µM Hemin*, a protoporphyrin IX -containing iron chloride and a ligand, used in the treatment of attacks of porphyria, especially in acute intermittent porphyria. The control sample was an untreated cell line with no reagent. They were subjected to western blot, PCR and IF techniques.

PROJECT 3 - HGT -1. Studies on the molecular mechanisms involved in digestive cancers, especially gastric cancer are few and inconclusive. We used a human gastric adenocarcinoma cell line, HGT -1 taken from the primary tumor, from a patient aged 60 years, diagnosed with gastric adenocarcinoma [108 119]. HGT -1 cell line was characterized in terms of morphology and karyotype. Line HGT -1 is epithelial and proved to be tumorigenic when transplanted into mice, was immunosuppressed with a hyperdiploid karyotype with a number of 57 chromosomes. Thus HGT-1 cell lines , possess histamine H2 receptor (H2R) functional, Sintex mediating adenosine 3' - 5'- cyclic monophosphate and consequently the production and activation of adenylate cyclase. H2R are specific receptors for normal parietal cells, secreting hydrochloric acid. HGT -1 cells do not secrete mucus or carcinoembryonic antigen. In this study, HGT -1 cell line was transfected with Fugene 6, Promega, USA. Transfection is the intentional introduction of nucleic acids into cells. The term is often used for non -viral methods used for eukaryotic cells [120]. We used transfection to try to follow the way in which the gene expression in cell culture line type HGT -1, derived from a human gastric adenocarcinoma. At last reading at 48h, it changes again in the cell transfection, add culture medium containing D- MEM + FBS + glutamine + PS (penicillin - streptomycin) and an

new antibiotic G418, which has the particularity of blocking the synthesis of polypeptides by inhibiting elongation in the two types of specific cells both prokaryotic and eukaryotic cells [123]. G418 is commonly used in laboratory research to select particularly genetically modified cells [124].

1. RESULTS AND DISCUSSION .

A. DISCUSSION ABOUT GASTRIC CANCER MORBIDITY.

1. Carcinogenic risk factors. There are still some elements scientifically documented and clearly stated by the medical world to be taken into account ie. *endogenous risk factors can not be altered* (genetic factors, gender, family premalignant lesions), while monitoring and testing of reduction of exogenous risk factors, do nothing to improve the prognosis of people at risk. Intense and sustained combat hypersaline food seems to be one of the easiest methods and handy to decrease the risk of gastric cancer among population groups at risk in certain geographical areas, totaling more risk factors, with or without chronic infestation with H. Pylori. Therefore, monitoring and control of risk factors and the digestive premalignant lesions can be very helpful for people at high risk.

2. Gastric premalignant lesions. Many of the premalignant lesions that we presented, with their objective and subjective clinical characteristics, are often easily treated, by the patient and the physician. The patient often gives up easily to carry out regular checks to track how the disease progresses, after being diagnosed with a new disease.

3. Pathophysiology of gastric oncogenesis. Mostly, the balance between the two enzymes, is the primary influence on the level of ODC and thus the production and acquisition of polyamines . The polyamine uptake mechanism, into cells, is not yet fully understood, so any new identification of a protein that may be involved in this process can give new hope to find treatments for human cancer [109] .

B. RESULTS AND DISCUSSION ON AZIN -2 EXPRESSION IN GASTRIC CANCER CELL – A Personal Study

We chose to study AZIN2 expression in osteosarcoma tumor cells (SW1353) , from megakariocytes (K565) and tumor cells of gastric cancers (HTG -1 in human gastric adenocarcinoma), so other than those cited in the literature namely specialized brain cells and testicular. The role and place AZIN2 in the oncogenesis is well established, but unfortunately poor enzyme stability made it to be studied less.

PROJECT 1 - SW 1352 . Our results at the end of the first experiments on cell line SW 1353 are presented in tables and graphs from the thesis, where four readings were made each 24 hours where quantification of AZIN2 gene expression in the control sample and shaded in the IGF. Although all readings AZIN2 difference between mean values measured for control samples and those treated with IGF increases, we can not say that it is a statistically significant difference as p value obtained by Student 's t test $p = 0.085 > 0, 05$, so over the maximum threshold of significance. The lack of statistical significance due to high variability found between individual test results.

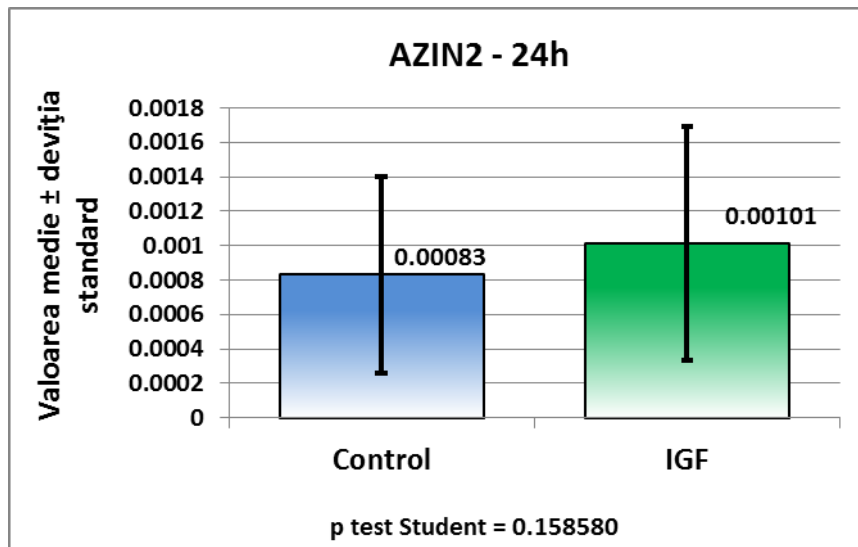


FIGURE no. 1. AZIN2 - GADPH,IGF la 24h

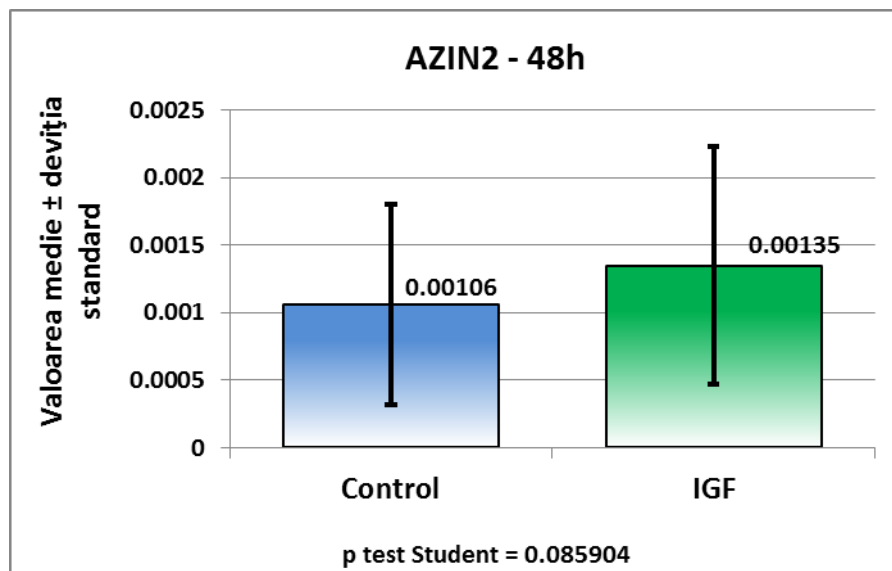


FIGURE no. 2 AZIN/2 AND IGF 48h

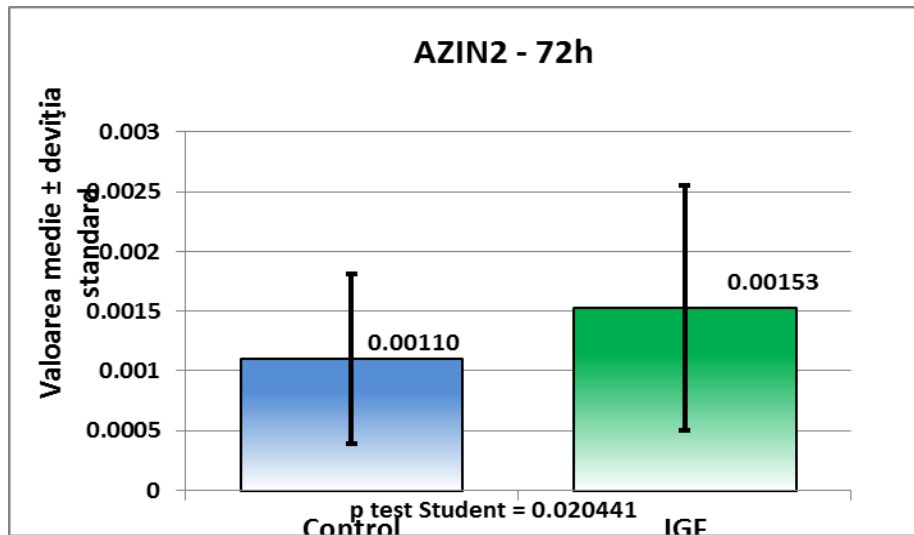


FIGURE no. 3 AZIN-2 - GADPH, IGF - 72h.

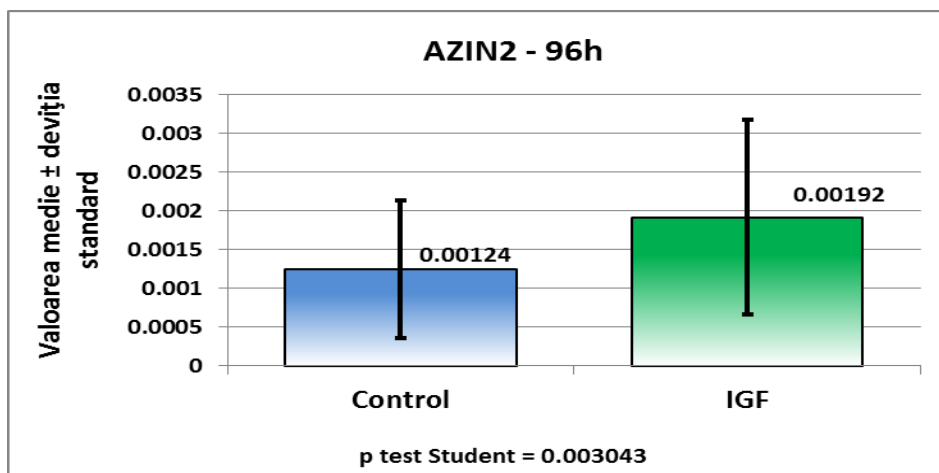


FIGURE no 4 AZIN2 - GADPH, - IGF la 96h.

At the end of the experiment conducted under the name of Project 1 to reinforce the data presented in tables and graphs, the objective of gene expression in cell culture by illustrating immunofluorescence images showing granular level type stains membrane and/or nuclear for antibody used as cell markers.

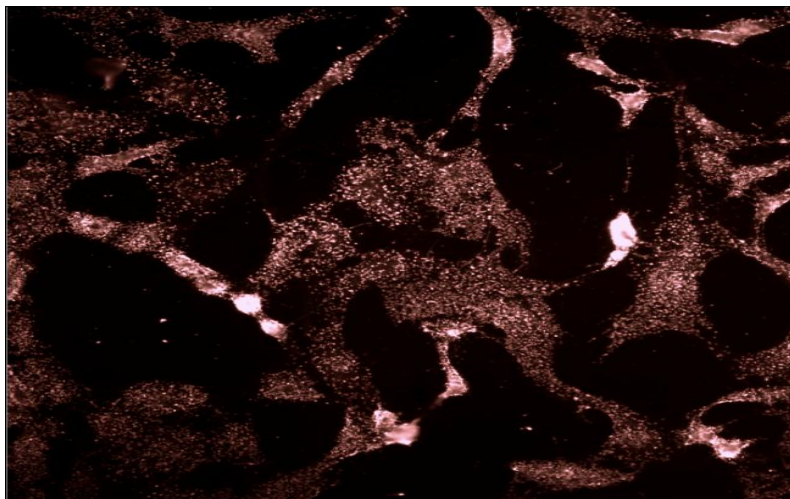


FIGURE nr 5 Imaginea microscopică a liniei SW 1353 tratate cu IGF, anticorp primar Rab27A, anticorp scundar anti-mouse TRITC

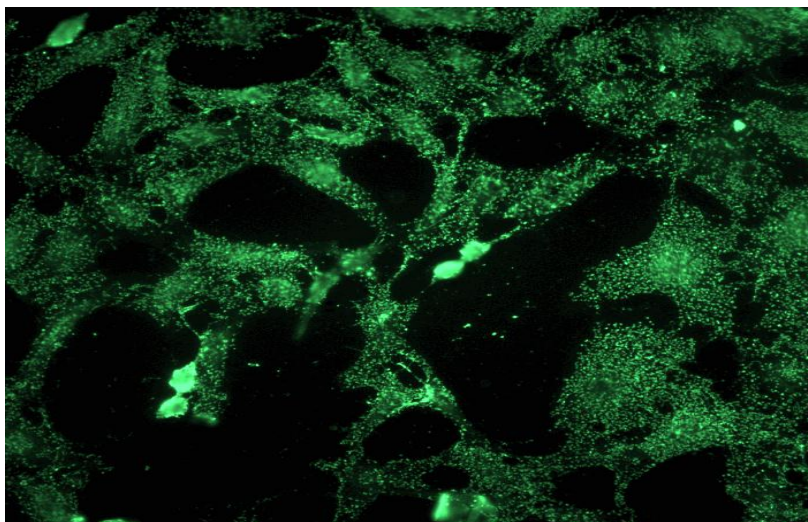


FIGURA nr. 6 Imaginea microscopică a liniei celulare SW 1353 tratate cu IGF, anticorp primar Rab27A, anticorp scundar anti-rabbit FITC

PROJECT 2 - K562, was conducted largely on the same frame as the protocol type cell line SW1353 culture. Thus the project began with induction of AZIN2, Rab27A and Rab27B in K562 cells, leading to megacariocitary differentiation by treatment with 30nM TPA. Another sample was treated with Hemin allowing erythroid differentiation of K562 line. Following the same steps performed in project 1, two K562 cell lines have been differentiated readings every 24 hours for 4 days, in order to appreciate the AZIN2 gene expression by comparing them with the control sample. In this way, the AZIN2 gene expression was found significantly increased as compared with the sample of TPA and control, and also correlated with the time variable in the sense that the highest values were found in the reading of the 72 hours. Finally, we made a comparison between the two lines studied and found that gene expression is higher in samples from K562 cell line treated with Hemin, so AZIN2 has greater specificity to tumor cell type blood erythrocytes.

PROJECT 3 - HGT -1. Results obtained by Xin- Pu Miao et al [107] suggest that ODC, can be used as a biomarker for screening and diagnosis of premalignant lesions, can sometimes predict when malignant transformation and proliferation of apparently benign lesions at time of diagnosis. The HTG -1 cell line will be a good experimental model for studying molecular mechanisms involved in gastric secretion and by default gastric premalignant lesions.

CONCLUSIONS .

1. Premalignant lesions and gastric cancer, remain in second place in the hierarchy of global morbidity from cancer, despite medical advances, along with excess salt, gastric premalignant lesions and infection with *Helicobacter pylori*, have direct implications in gastric oncogenesis.
2. Microbiological and biochemical studies have demonstrated that the cellular balance of the two enzymes, AZ/AZIN, which is the primary influence the level of ODC, and consequently the production and acquisition of polyamines, responsible for proliferation and cell growth in both normal cells and in the tumor cells.
3. This study attempts to prove the existence of AZIN2 gene expression, in other cells lines than those studied and accepted so far (brain cell and testicular), ie three distinct types of cell lines: SW1353 (ostosarcoma) , K562 (megakaryocytes) and HGT -1 (gastric adenosarcoma).
4. AZIN2 gene expression in both cell culture lines were more clearly highlighted in K562 line to cell line SW1353, if we compare the results obtained by IF staining or treated with Hemin line. So we can say that AZIN2 gene expression in both bone tumor cells and blood cells, along with those described so far in cerebral and testicular cancer.
5. Highlighting the presence of AZIN2 in HGT -1 seems to be confirmed, but at much lower levels. The results are not definitive nor complete, as this study has just started and besides of immunofluorescence and transfection with Fugen 6 and G418, have not yet tried other types of antibiotics that could better highlight the presence of gene expression AZIN2 in HGT -1 cells. The study is still in progress.
6. The reason to continue studying cell line HGT -1 gene expression is also that poor expression in this cell type may lead to the creation of an experimental model for studying the molecular mechanisms of gastric secretion and the intimate mechanisms of pathological cell proliferation in gastric cancers, or the study of oncogenic mechanisms that lead to the evolution of malignant gastric premalignant lesions.

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