

THE UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
THE FACULTY OF MEDICINE

PhD Dissertation

**THE MMR GENES (MSH2, MSH6, EXO1)
IMPLICATION IN ESO-GASTRIC CANCER
(ABSTRACT)**

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Introduction

The Gastric Cancer (GC) represents a major health issue nowadays, the pathogenesis of this illness being the result of the interaction between the environmental, epidemiologic and genetic factors.

In gastric cancer, genetic and epigenetic alterations occur which define the biological characteristics of the cancerous cell and which could serve as therapeutical targets.

It is the genomic instability that can be clasified into microsatellites (MSI) and chromosomal instability that contributes to the accumulation of these alterations.

The emergence of nucleotidic substitutions or the deletion or insertion of repetitive units, within microsatellites structure, a phenomenon which characterises their instability, is the consequence of the MMR genes deteriorations which are involved in DNA replication errors.

The microsatellites instability (MSI) created by the MMR deficiency represents a mutator phenotype related to the accumulation of secondary mutations, being a biomarker of the loss of MMR activity in tumoral cells. (Shah et al. 2010; Yamamoto et al., 2012; Zhang et al., 2013)

The discovery of the direct connection between the MMR genes and the hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) has demonstrated that these genes require extra investigations in other neoplasias.

Key words: gastric cancer, gene expression, MMR, MSH2, MSH6, EXO1

I. THE STAGE OF KNOWLEDGE

Chapter 1., entitled „**General considerations regarding the ethiopathogeny of gastric cancer**” describes the epidemiology of gastric cancer, the risk factors involved, as well as the morphopathology of these illnesses.

Chapter 2., entitled „**Molecular mechanisms involved in gastric carcinogenesis**” sums up the main genetic and epigenetic alterations that emerge in gastric neoplasma, as well as the implication of growth factors, cytokines, angiogenic factors in carcinogenesis.

Chapter 3., named , „**The MMR Genes**” describes the main components of the DNA mismatch repair system (MMR) and their mode of action.

II. PERSONAL CONTRIBUTIONS

This study’s purpose was to compare the MMR: MSH2, MSH6 and EXO1 genes expression in tumoral and peritumoral tissue of the patients diagnosed with eso-gastric cancer, as well as the analysis of correlations between the expression of these genes, the localization and the evolution of pathology.

Chapter 4. Material and method.

Biopsies have been taken from tumoral gastro-esophagus formations from 45 patients submitted to superior digestive endoscopy and eco-endoscopy at the Gastroenterology and Hepatology Research Centre from Craiova between 2008-2010, among these 15 were females and 30 males.

The average age was 62 years old.

The main localization was in most cases non-proximal (n=23), followed by the cardia (n=14) and esophageal (n=8).

The histologic type was represented by adenocarcinoma for gastric cancers and squamous cell carcinoma for the esophageal cancers.

From each patient pair samples have been taken both from the tumoral tissues and the peritumoral tissue.

All patients included in the study were infected with *Helicobacter pylori*.

The peritumoral tissue has been analyzed from the histo-pathologic point of view and no transformed malign cells have been identified.

From each patient informed consent for molecular studies has been asked for. Some of the samples have been collected in formalin.

Others have been collected in RNA stabilization solution, Ambion and kept at -80°C until the RNA isolation, after which the histopathologic analysis followed, the total RNA isolation the RNA quality evaluation (concentration, purity), the total RNA reverse-transcription from the complementary DNA, the qRT-PCR and the results analysis.

The total RNA isolation

The utilized kit has been represented by SV Total RNA Isolation System (Promega) for the total RNA isolation and purification from tumoral and peritumoral tissues submitted to biopsy.

The concentration and the purity of total RNA from the studied samples have been measured spectrophotometrically through Eppendorf Biophotometer, the quality of the RNA being established through Agilent 2100 Bioanalyzer (Agilent Technologies Inc., US) and electrophoresis of agarose gel.

qRT-PCR

For the right dosage of gene expression, I have used quantitative Real-Time PCR technology in two steps: 1µg of RNA has been reverse-transcribed in

single-stranded complementary DNA (DNAc) using Capacity cDNA Reverse Transcription Kit (Applied Biosystems); then I used Real- Time PCR by means of TaqMan® Gene Expression Master Mix (Applied Biosystems) with TaqMan® primers and probes for target genes and the reference gene.

The amplifying reactions have been realized in 20 µl volumes, in triplicate utilizing a Mastercycler®ep realplex (Eppendorf) system.

For an evaluation of target gene expression, GAPDH endogenous control has been used both for the tumor and the adjacent mucosa. Due to the fact that the primers efficiency and the used samples in all reactions was 100%, the proportion between the initial value and the final value in pair samples has been calculated using $2^{-\Delta\Delta Ct}$ method. (Livak et al., 2001)

The statistics Data

For pair samples, the difference of gene expression has been taken into account, if the fraction of values obtained was higher than 1,8 or below 0,55 and insignificant the values in between.

The Chi-square test has been used to observe if the difference between the number of patients from each group is significant.

When the variables have not been normally distributed , I made use of non parametric statistic test (the Wilcoxon test of pair ranks, the Mann-Whitney test, the Kruskal-Wallis test, the Dunn's test).

The data obtained have been analyzed by means of GraphPad Prism 5 and GraphPad InStat programmes.

To see if there are correlations between genes studied, were applied Spearman coefficients.

Chapter 5. Results

The comparative evaluation of relative expression of each analyzed gene both for the tumor and for the peritumoral tissue is presented in **table 1**.

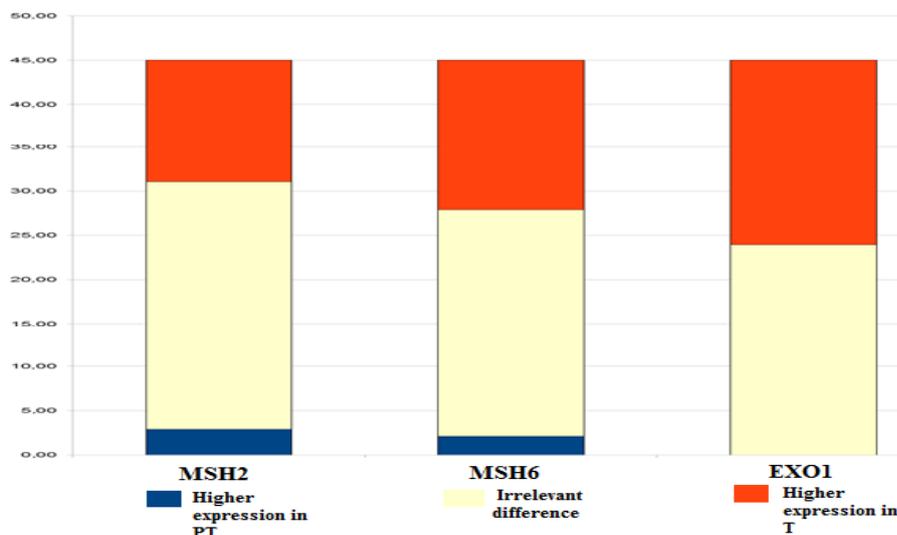
Tab.1 The expression profile of MSH2, MSH6, EXO1 genes for the analyzed lot.

MSH2	MSH6	EXO1	
14 (31,11%)	17 (37,78%)	21 (46,67%)	>1,8 overexpression in T
31	28	24	< 1,8
0	0	0	0/0
3 (6,67%)	2 (4,44%)	0 (0,00%)	<=0,55 overexpression in PT
28 (62,22%)	26 (57,78%)	24 (53,33%)	0,55-1,8 irrelevant difference

I have applied the Chi-square test to see if the difference between the number of patients from each group is significant, noticing the fact that for each gene analyzed, p was < 0,0001. (Fig.1)

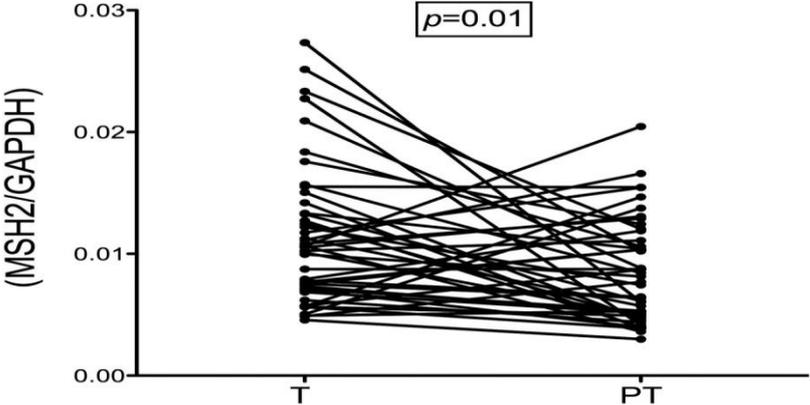
Fig. 1 The level of analyzed gene expression.

(The patients appear on the abscissa and the gene expression on the ordonate)



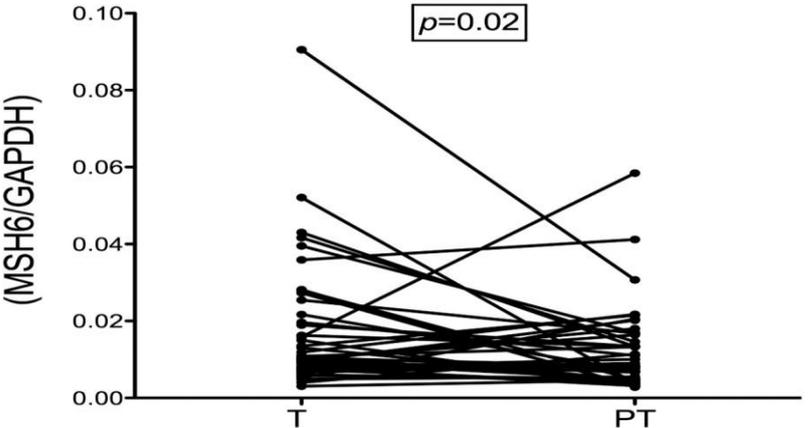
The evaluation of expression for each gene of interest has been realized by parallelism in pair samples, the data being presented as relative expression of the RNAm for each gene, as compared to GAPDH (Wilcoxon matched-pairs signed rank test). (Fig.2,3,4)

Fig. 2 The comparative expression of MSH2ARNm at tumoral/peritumoral level



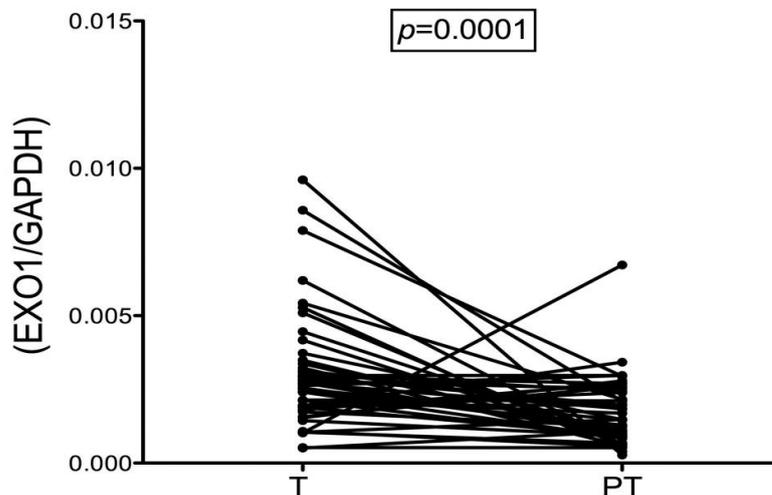
The relative expression of MSH2 is noticed to have been significantly increased at tumoral level as compared to peritumoral level.

Fig. 3 The comparative expression of MSH6ARNm at tumoral/peritumoral level.



Also the relative expression of MSH6 has been significantly increased at tumoral level as compared to peritumoral level.

Fig. 4 The comparative expression of EXO1ARNm at tumoral/ peritumoral level.



Just like in the case of MSH2, MSH6, the EXO1 expression was significantly higher at tumoral level, as compared to peritumoral level.

The correlation degree has been analyzed between the studied genes using Spearman coefficients and I noticed the following:

- there is a strong positive association between MSH2 and MSH6 at tumoral level ($p < 0,0001$);
- there is a strong positive association between MSH2 and MSH6 at peritumoral level ($p < 0,0001$);
- there is a positive association between MSH and EXO1 at peritumoral level ($p < 0,0001$).

As for the correlation between relative gene expression with tumor localization (applying Kruskal Wallis Test and then Dunn's Multiple Comparison Test), I noticed that the relative expressions of MSH2 and MSH6 was significantly associated with the distal tumoral localization (**Fig.5, 6**)

Fig. 5 The relative expression of MSH2 as reported to tumoral localization.

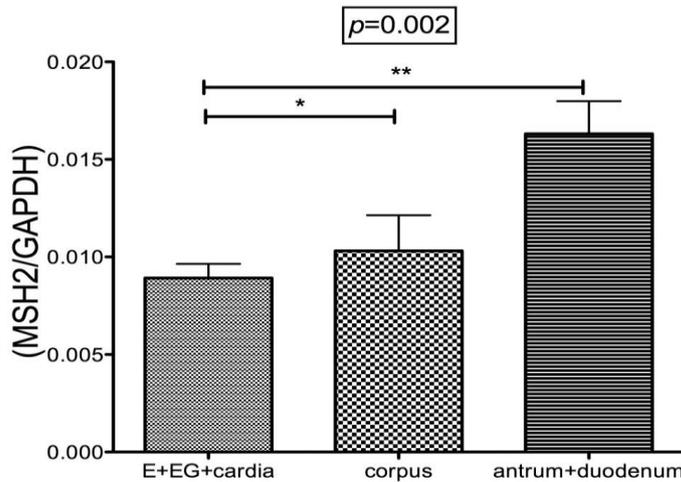
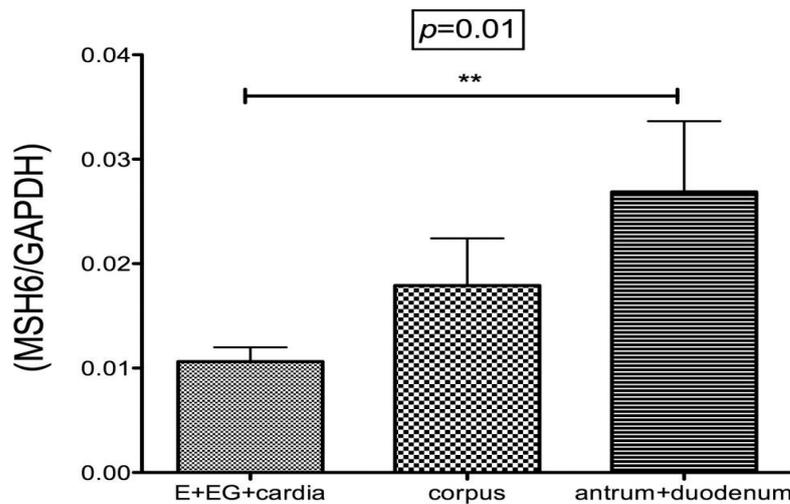


Fig. 6 The relative expression of MSH6 as reported to tumoral localization.



The histologic type and the tumoral stage haven't influenced the levels of relative expression of studied genes.

Alternatively, the third degree of tumoral differentiation has significantly correlated to MSH2 expression and that of MSH6. (**Fig.7, 8**)

Fig. 7 The relative expression of MSH2 as reported to the tumoral degree

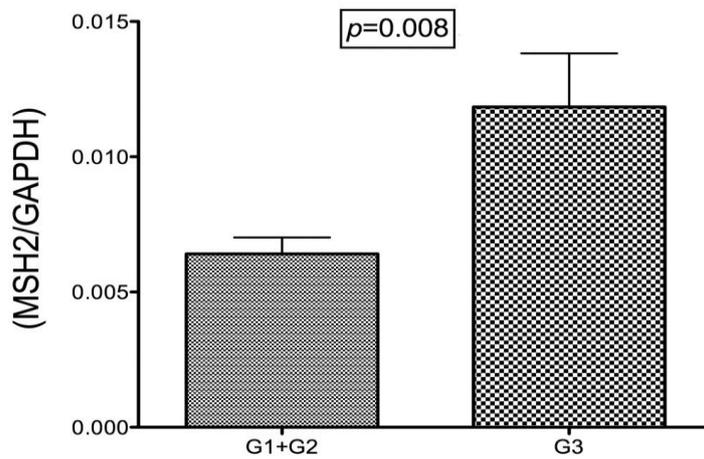
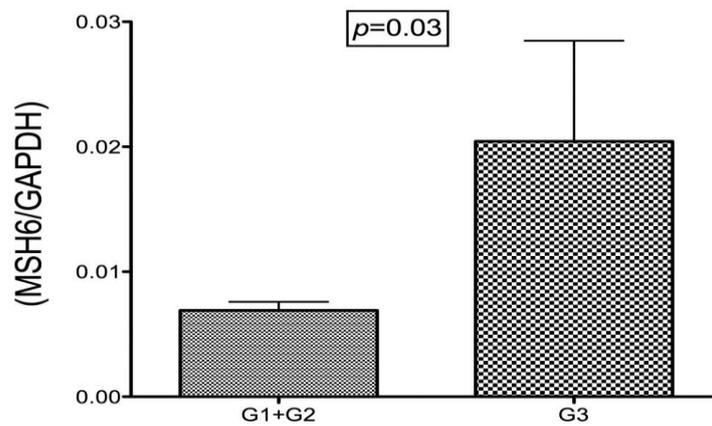


Fig. 8 The relative expression of MSH6 as reported to the tumoral degree



Chapter 6. Discussions

If in the literature with regard to Lynch syndrome MMR deficiency occurs in tumors, the gene expression pattern that I obtained indicates the tendency of superior activation of MMR genes in neoplasia (overexpression), the positive association of MSH2 and MSH6 at tumoral level and their correlation to distal localization of tumor.

A possible explanation could be that, taking into account the role of EXO1 in DNA double stranded excision and of MutS α (MSH2-MSH6) of singular error repairing of base-base type and 1-2 bases IDL, the overexpression of these genes in gastric cancer could represent a response of the

body to the rapid increase of the number of carcinogenesis replication errors, taking into consideration the capacity of increased proliferation of tumoral cells. The results of this study are consistent with those obtained by Li and his colleagues (2008) on MSH2 overexpression in tumors.

Besides this, Li and his collaborators (2008) analyzed the gene expression values as related to the Ki67 cell proliferation marker without obtaining significant statistic results.

The data mentioned by Li and his collaborators didn't suggest the existence of correlations to certain parameters such as: age, sex, gender, H.pylori infection, differentiation degree or metastasis in lymphatic nodes.

On the other hand, it has been noticed that the MMR overexpression in cancer isn't translated as a more efficient replication error repairing.

The increased values of MMR protein in neoplastic cells could be determined by its accumulation at tumoral level, but without exhibiting the original functions.

It is known that gene mutation can determine overexpression of proteic product. (Li et al., 2008)

Shin and his collaborators (2002) reported in one HNPCC case of gastric cancer that mutation of MSH2 promoter (G-C transversion at -225 position) made the transcriptional efficiency rise with 466%, suggesting that MSH2 mutation can lead to proteic product expression increase.

By analyzing these data related to association of MMR overexpression at tumoral level with high metastatic potential and low survival rate, noticed by Sarasin and Kauffman (2008), taking into account more types of neoplasias (malign melanoma, breast cancer, bladder cancer), an ipotesis can be issued: this increase has the role of conferring the neoplastic cell a certain degree of genetic stability necessary for invading and producing distance metastasis.

The destruction of the cell capacity of providing restoration of the genome can lead to decrease of the capacity of invasion and agresivity.

As far as the results of our study are concerned referring to correlation between tumoral overexpression of MSH2 and the third degree of differentiation of gastric cancer, numerous studies lead by other authors have shown the association of MSI status with low differentiation tumors.

Bacani and his collaborators (2005) exposed the existent correlation between MSI, the intestinal type of gastric cancer and the low differentiation degree, similar results to those obtained by Seruca and colleagues (1995).

Although the data of various studies are not similar, we can still regard the MMR implication in gastric cancer as a certainty.

All patients included in the analyzed lot have exhibited infection with *H. pylori*.

The presence of *H. pylori* leads to gastric mucosa inflammation, which subsequently lead to cell cycle modifications that stimulate the epithelial cell replication, thus increasing the apoptotic rate and oxidative substance release. (De la Riva et al., 2004)

As a side effect of these events and of the antioxidant defense exhaustion, the DNA mutations is favored, thus resulting in intensifying of MMR system activity.

Because the infection with *H. Pylori* occurs more often in individuals who exhibit MSI status, there could be the possibility of a direct interaction with MMR. (Leung et al., 2000; Kim et al., 2002)

Besides, the gastric epithelial cells inoculated with *H. pylori* exhibited the decrease of protein expression MutS and MutL. (Kim et al., 2002)

Park and his collaborators studied the level of MLH1 and MSH2 expression in patients infected with *H.pylori* before and after the eradication and reported the increase of MMR protein expression after the infection had been eradicated , thus the results sustaining this theory.

Halling and his collaborators (1999) noticed that the MSI positive gastric neoplasias frequently exhibit the loss of MLH1 activity and more seldom that of MSH2.

Similar results concerning the level of these proteins were obtained by Mirzae and colleagues (2008).

Taking into account that in our study we noticed the increased expression of the three genes of MMR group, in patients infected with H.pylori, we can consider that in this respect, our results are not in accordance to the studies presented before.

Chapter 7. Conclusions

- The increase in the expression of the 3 MMR (MSH2, MSH6, EXO1) genes under discussion at peritumoral level can be considered a tumoral marker of an early neoplasia;
- The overexpression of these genes at tumoral level can be interpreted as being:
 - the answer of the body to the increase of errors of replication involved in carcinogenesis;
 - the result of genetic mutations that increase the synthesis of proteins lacking the initial functions;
 - the way through which the neoplastic cell could ensure the genomic stability necessary to invade.
- Coexistence with H.pylori did not influence negatively the expression of the 3 genes;
- The association of MSH2 and MSH6 overexpression with G3 can be considered a negative prognostic factor.

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