Comparison of expression of selected proteins in the cells of intestinal and diffuse type gastric cancer – immunohistochemical analysis

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ABSTRACT

Introduction: Despite the progress in gaining knowledge about carcinogenesis, it is still unclear what processes are directly responsible for the differentiation of gastric cancer into its intestinal or diffuse form. Dividing of these two forms is based on one of the oldest, yet still commonly used classifications – the classification of Lauren. There are many factors that may influence the formation of gastric tumors of various aggressiveness.
Purpose: To evaluate the expression of proteins: fragile histidine triad (FHIT), E-cadherin, α-catenin, γ-catenin, cathepsin B, epidermal growth factor (EGF), HER-2, MMP-9, MCM-2, Bak, Bax, BID, Bcl-XL, p53, FasL, Bcl-2, caspase-8, procaspase-3 in gastric cancer cells, depending on the type of tumor by Lauren classification.
Materials and methods: Study group consisted of 91 patients treated surgically for gastric cancer in the Second Department of General and Gastroenterological Surgery, Medical University of Bialystok in years between 2000 and 2006.
Results: It is shown, that the expression of E-cadherin was significantly higher in the Lauren I gastric cancer cells than in Lauren II. In case of caspase-8 there has been significantly less frequent expression of this protein in Lauren I gastric cancer cells compared to Lauren II. The authors describe no statistically significant differences in the expression of other proteins taken into consideration.
Conclusions: These results suggest the role of adhesion and apoptosis-related proteins in the development of two different types of gastric cancer according to Lauren's classification.
Key words: Gastric cancer, apoptosis, Lauren classification, E-cadherin, apoptotic pathways.

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INTRODUCTION

Gastric cancer is a common cancer and despite advances in medicine, still burdened with a poor prognosis. There are many classifications in which gastric cancer is classified into different types depending on the parameters taken into consideration. The most popular are: Macroscopic Borrmann classification, Goseka classification, WHO classification and Ming's classification. One of the oldest yet still commonly used in the clinic is the classification of Lauren. It was developed in 1965 and is based on an evaluation of histopathological features of infiltration of cancer, cytological features of single cancer cells and differences in the secretion of mucus between them. It distinguishes two main types: intestinal and diffuse. Diffuse type is less common and some cancers have features of both, so an additional mixed type was discerned. In Lauren's classification, the diffuse type gastric cancer has the worst prognosis with the fastest progression in the same time [1,2].

Despite the development of knowledge about carcinogenesis, it is still unclear what factors influence the occurrence of various types of cancer. Despite that, there are many factors that may influence the formation of tumors of various aggressiveness. These include adhesion molecules, such as E-cadherin and some other proteins forming complexes with it, such as α, β and γ-catenins. Disorders of their functioning lead to the weakening of cell adhesion [3-7].

The other groups of factors are proteins involved in apoptosis. Some of them, like Bak and Bax, exert pro-apoptotic activity in the so-called internal-mitochondrial apoptotic pathway. Other molecules, such as Bcl-2 and Bcl-x1 inhibit apoptosis. Still further proteins stimulate apoptosis in the so-called outer – membrane pathway. These are Fas and FasL. Another protein, BID forms a bridge between the internal and external pathways integrating the pro-apoptotic signaling. The effect of the pro-apoptotic action of proteins is the activation of effector proteins - caspases, resulting in cell death [8-10]. Yet another factor influencing the induction of apoptosis and thereby affecting proliferation of cells is the fragile histidine triad (FHIT) protein [11-13].

Disorders of the functioning of all these proteins may result in the development of cancer, wherein unknown is their effect on the formation of particular forms of gastric cancer.

An important role in the formation and growth of the primary tumor is played by proteolytic enzymes, which include metallo-proteinases, including metalloproteinase 9 [14,15]. Secretion of these proteins by the cell is initiated, among others, by the epidermal growth factor (EGF). The epidermal growth factor receptor (EGFR) belongs to the family of tyrosine kinase receptors, which also include HER2. They affect the proliferation, invasion and tumor cell apoptosis [16,17].

Another protein, cathepsin B is involved in all stages of neoplastic transformation, such as tumor growth, angiogenesis, invasion and metastasis. Overexpression of cathepsin B was observed in gastric, lung, breast and colon cancers, and overexpression of MCM-2 protein may be associated with an aggressive phenotype, and can be used as a prognostic marker of cancer [18-20].

P53 protein is a gene transcription and cell cycle regulator. Loss of function of *P53* gene is one of the most common genetic lesions in human cancers, and its presence has been found in lung cancer, pancreatic cancer, breast cancer and gastric cancer [21,22].

The aim of this study was to evaluate the expression of proteins: FHIT, E-cadherin, α-catenin, γ-catenin, cathepsin B, EGF, HER-2, MMP-9, MCM-2, Bak, Bax, BID, Bcl-XL, p53, FasL, Bcl-2, caspase-8, procaspase-3 in gastric cancer cells, depending on the type of tumor by Lauren classification.

MATERIALS AND METHODS

Patients

The study group consisted of 91 patients treated surgically for gastric cancer in the Second Department of General and Gastroenterological Surgery, Medical University of Bialystok in years between 2000 and 2006. In this group, there were 27 women and 64 men aged from 19 to 89 years (Average: 67).

Immunohistochemical studies were carried out on the archival materials which were paraffinic cubes containing gastric cancer tissue specimens. Studies have been done in the framework of the statutory work with the consent of the Bioethics Committee of the Medical University of Bialystok (No. RI-003/124/2004).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue specimens were cut on a sliding microtome into sections with a thickness of 4 microns. The slides were dewaxed in xylenes and hydrated in alcohols. The antigen was exposed to the antibody in citrate buffer (pH = 6.0) for 20 minutes at 97^0C and for 20 minutes at room temperature. The sections were then incubated with primary antibody (Table 1). As a detection system there was used a biotinylated anti-mouse and anti-rabbit antibody, streptavidin-conjugated horseradish peroxidase (Peroxidase Detection System, Novocastra Leica, Poland) and a polymer set directed against goat antibodies (En Vision FLEX Visualization System, Dako, Poland). The color reaction for peroxidase was performed

with DAB chromogen (DAB, Leica Novocastra, Poland). Protein expression was evaluated by two independent pathologists. The percentage positive cells were calculated in 500 cells in each preparation, at a magnification of 400x. The assessment of protein expression in tumor cells was performed in a semi-quantitative way, recognized as positive with the values given in Table 1.

Table 1. Antibodies used for detection, dilute solutions and examined the expression of proteins considered to be positive.

Protein	Antibody	Dilution	Positive reaction
Fhit	polyclonal, Abcam	1:200	≥30%
E-cadherin	monoclonal, clone 36B5, Novocastra Laboratories Ltd	1:50	≥30%
α-catenin	polyclonal, clone H-297, Santa Cruz Biotechnology	1:300	≥30%
β-catenin	monoclonal, clone 17C2, Novocastra Laboratories Ltd	1:100	≥30%
γ-catenin	Polyclonal, clone C-20, Santa Cruz Biotechnology	1:100	≥30%
Cathepsin-B	monoclonal, clone CB131, Novocastra Laboratories	1:40	≥30%
EGF	monoclonal, clone EGF-10, Sigma-Aldrich	1:50	≥50%
HER-2	monoclonal, clone 10A7, Novocastra Laboratories	1:50	≥50%
MMP-9	monoclonal, clone15W2, Novocastra Laboratories	1:40	≥30%
MCM-2	polyclonal, clone N-19, Santa Cruz Biotechnology	1:400	≥50%
Bak	polyclonal, clone G-23, Santa Cruz Biotechnology	1:100	≥20%
Bax	polyclonal, clone P-19, Santa Cruz Biotechnology	1:50	≥10%
BID	polyclonal, clone N-19, Santa Cruz Biotechnology	1:100	≥20%
Bcl-xl	polyclonal, clone A-20, Santa Cruz Biotechnology	1:300	≥15%
Bcl-2	polyclonal, clone N-19, Santa Cruz Biotechnology	1:100	≥10%
p53	monoclonal, clone DO-7, DAKO	1:100	≥30%
FasL	polyclonal, clone N-20, Santa Cruz Biotechnology	1:250	≥20%
Caspase-8	monoclonal, clone 11B6, Novocastra Laboratories Ltd	1:250	≥25%
procaspase-3	monoclonal, clone E61, Abcam	1:100	≥25%

Statistics

Association between expression of particular proteins and Lauren classification was assessed using Fisher's exact test. These differences were considered as statistically significant at a confidence level of $p < 0.05$, and the larger differences at $p < 0.01$ were underlined. Statistical calculations were performed in IBM SPSS statistical package Statistica 20.0.

RESULTS

The expression of E-cadherin was significantly higher in the Lauren I gastric cancer cells than in Lauren II (67.2% vs 45.2%, $p < 0.05$) (Table 2).

It was also demonstrated that BID protein expression was significantly higher in Lauren I cancer cells than in Lauren II (57.1% vs 30.4%, $p < 0.05$) (Table 2.). In the case of caspase-8 there has been the significantly less frequent expression of this protein in Lauren I gastric cancer cells compared to Lauren II (39.6% vs 75.0%, $p < 0.01$) (Table 2).

There were no statistically significant differences between the expression of gastric cancer cell proteins: FHIT, β-catenin, γ-catenin, cathepsin B, EGF, HER-2, MMP-9, MCM-2, Bak, Bax, Bcl-XL, p53, FasL and Procaspase 3, depending on the classification according to Lauren (Table 2).

Table 2. Selected protein expression in gastric cancer cells depending on Lauren's classification.

Protein	Lauren I		Lauren II		P value
	Positive	Negative	Positive	Negative	
	N (%)	N (%)	N (%)	N (%)	
FHIT	27 (50.9%)	26 (49.1%)	17 (65.4%)	9 (34.6%)	0.241
E-cadherin	41 (67.2%)	20 (32.8%)	14 (45.2%)	17 (54.8%)	**0.047***
α-catenin	25 (52.1%)	23 (47.9%)	10 (40.0%)	15 (60.0%)	0.459
β-catenin	27 (58.7%)	19 (41.3%)	10 (47.6%)	11 (52.4%)	0.437
γ-catenin	9 (47.4%)	10 (52.6%)	3 (20%)	12 (80%)	0.152
Cadhepsin-B	22 (68.8%)	10 (31.2%)	6 (66.7%)	3 (33.3%)	1.00
EGF	11 (64.7%)	6 (35.3%)	5 (45.5%)	6 (54.5%)	0.441
HER-2	11 (68.7%)	5 (31.3%)	7 (70%)	3 (30.0%)	1.00
MMP-9	19 (70.4%)	8 (29.6%)	5 (71.4%)	2 (28.6%)	1.00
MCM-2	20 (41.7%)	28 (58.3%)	8 (50%)	8 (50%)	0.772
Bak	33 (64.7%)	18 (35.3%)	10 (40.0%)	15 (60.0%)	0.152
Bax	23 (57.5%)	17 (42.5%)	5 (31.2%)	11 (68.8%)	0.138
BID	28 (57.1%)	21 (42.9%)	7 (30.4%)	16 (69.6%)	**0.045***
Bcl-xl	26 (72.2%)	10 (27.8%)	8 (53.3%)	7 (46.7%)	0.328
p53	15 (83.3%)	3 (16.7%)	9 (90%)	1 (10%)	1.000
FasL	13 (50.0%)	13 (50.0%)	6 (40.0%)	9 (60.0%)	0.746
Bcl-2	17 (50%)	17 (50%)	4 (26.7%)	11 (73.3%)	0.275
Caspase-8	21 (39.6%)	32 (60.4%)	15 (75.0%)	5 (25.0%)	**0.009****
procaspase-3	61 (100%)	0 (0%)	30 (100%)	0 (0%)	1.000

* $p<0.05$, ** $p<0.01$

DISCUSSION

Despite the progress in gaining knowledge about carcinogenesis and some of the factors affecting the progression of the disease, it is still unclear what processes are directly responsible for the differentiation of gastric cancer into its intestinal or diffuse form. Analyzing the expression of proteins associated with apoptosis, depending on the type of gastric cancer in histopathological studies the authors found no significant differences in the expression of factors associated with the inner mitochondrial apoptosis pathway. This applies both to proapoptotic proteins, such as Bax and Bak as well as the apoptosis-inhibiting proteins Bcl-2 and Bcl-xl. There were also no significant differences in the expression of the marker of outer-membrane apoptotic pathway - FasL. This may indicate a lack of effect of disorders in the expression of these proteins when different Lauren types of gastric cancer are considered. Also the expression of the effector protein precursor – procaspase-3 did not differ in the cells of the intestinal or diffuse type caner.

Among the proteins associated with apoptosis in our study there were significant differences in the expression of caspase-8 and BID protein depending on the histological form of gastric cancer.

Caspase-8 is an essential element of both membrane and mitochondrial apoptotic pathways. On the external or membrane apoptotic pathway the activation of caspase-8 occurs due to stimulation of the Fas receptor by its ligand FasL with the participation of FADD protein. Together with caspase-10 it belongs to the so-called initiator caspase group, and is important effector activation competent. In this signaling pathway the activation of caspase-3 leads directly to cell death. On the mitochondrial pathway caspase-8 activates protein BID, which is a factor linking the two pathways of apoptosis. BID belongs to the family of Bcl-2 and causes the release of mitochondrial cytochrome c. The effect on mitochondria is therefore partially modulated with the function of other proteins of the Bcl-2 family, such as anti-apoptotic Bcl-2 and Bcl-XL and pro-apoptotic Bax [8-9].

It has been shown in clear that a significantly more frequent expression of caspase-8 occurs in cancer cells of Lauren type II cells compared to Lauren I. The occurrence of differences in the expression of this protein in

various types of cancer may be indicative of different disorders of apoptosis occurring in the various subtypes of gastric cancer. The role of caspase-8 in diffuse-type gastric cancer is hard to explain using conventional schemes of apoptosis.

Higher expression of caspase-8 should lead the cell into the apoptotic pathway and, consequently, cause it to self-destruct .This is typical for the less aggressive forms of cancer. This may indicate the existence of non-membrane disorder of apoptotic pathway, possibly based on intracellular mechanisms involved in the formation of diffuse form of gastric cancer. The results obtained in the works of other authors are not explicit. Some authors observed no differences in the expression of caspase-8 depending on the histological form of gastric cancer [23-25], and others found its higher expression in the intestinal type of cancer [26].

The BID protein demonstrated significantly more frequent expression in Lauren I cancer cells. These results can be explained by the fact, that BID is a protein that activates apoptosis. As BID has a crucial role in apoptosis, loss of its function could be important in the development of Lauren II gastric cancer [10].

Although a number of authors reported that the occurrence of poorly differentiated gastric cancers burdened with a poor prognosis is associated with the inactivation of the *FHIT* gene and decreased expression of the protein encoded by it, in our study, there was no difference in the expression of FHIT protein depending on the form of gastric cancer [11-13].

Cadherins are transmembrane proteins responsible for cell adhesion. They play an important role in the cancer formation, invasion and metastasis. The extracellular domain of E-cadherin is responsible for the regulation of cell adhesion, whereas its intracellular domain is combined with the actin cytoskeleton of the cell. It is possible due to the formation of a complex with another protein: β- and γ-catenin, which is also connected to α-catenin. The E-cadherin-catenin complex maintains cell adhesion, their shape and polarity and is a regulator of cell migration. Dysfunction of any component of the E-cadherin-catenin complexes may result in impairment or loss of cell adhesion [3-6].

In this study, the authors found significantly more frequent expression of E-cadherin in Lauren I gastric cells than in those of Lauren II. However, there were no statistically significant differences in the expression of other adhesion molecules studied (α-, β- and γ-catenin). A lower expression of E-cadherin, probably as a result of its gene dysfunction, could be one of the elements leading to a faster and more aggressive progression of diffuse type gastric cancer and therefore, affect the formation of a typical embodiment of the tumor. The emergence of this form of gastric cancer, resulting from the loss of function of the gene encoding E-cadherin, is described by many authors [6-7, 27-31]. There are also reports where there was no difference observed in the expression of E-cadherin between the cells of intestinal and diffuse types [32].

B-cathepsin is a member of lysosomal protease family and is involved in numerous biological processes, including the extracellular matrix degradation, antigen presentation, apoptosis, and angiogenesis. Its deficiency may lead to carcinogenesis [19]. It is involved in all stages of neoplastic transformation that is tumor growth, angiogenesis, invasion and metastasis. Over-expression of cathepsin B was also observed in gastric cancer [18]. This study showed no changes in the expression of this protein in different gastric cancer cells, depending on the classification of Lauren.

The MCM-2 protein belongs to the minichromosome maintenance protein complex family and plays an important role in the two stages of the cell cycle: replication of DNA and cell division. Overexpression of this protein may be associated with an aggressive phenotype of the stomach cancer and may serve as a prognostic factor [20]. Despite this, the authors of this study found no differences in the expression of MCM-2 protein according to the histopathological form of gastric cancer.

The p53 protein is involved in the DNA repair and synthesis as well as in the process of cell differentiation, angiogenesis and induction of apoptosis. It also acts as a regulator of gene transcription and cell cycle. Loss of function of the *p53* gene is one of the most common genetic defects in tumors, and its presence has been found, inter alia, in lung cancer, pancreatic, breast, and gastric cancer. In gastric cancer, it is usually associated with lower differentiation and a worse prognosis [21-22]. Our study did not confirm this relationship.

Metalloproteinase 9 plays an important role in tumor invasion and metastasis. It has the ability to degrade type IV collagen, thereby modulating endothelial permeability, with consequent migration of tumor cells allowing tumor growth and metastasis formation [14-15]. Its expression is initiated among others by EGF – epidermal growth factor [16]. This factor also plays an important role in the proliferation, differentiation and maturation of germinal cells; therefore, it may be involved in neoplastic transformation. Increased expression of EGF and its receptor EGFR was observed in the course of various types of cancer also in gastric cancer [16-17]. The main function of HER2 is the modulation of proliferation, carcinogenesis, apoptosis, and invasion of tumor cells. *HER2* gene amplification or overexpression

of HER2 protein in the course of cancer is usually associated with an increased risk of relapse and a poor prognosis [33].

In our study, there were no significant differences in the expression of MMP-9, EGF and HER-2, depending on the histological type of gastric cancer.

CONCLUSIONS

Different expression of caspase-8 in the two different gastric cancer types according to Lauren demonstrate disorders in apoptosis during differentiation of gastric cancer types. More frequent expression of caspase-8 in the diffuse type gastric cancer suggests that the formation of aggressive forms of this cancer may involve mechanisms other than membrane pathway of apoptosis. One of them may be the reduction of BID protein expression, the function of which is the integration of the membrane and mitochondrial apoptotic pathways. Disorders of pro-apoptotic signal transduction through internal pathway of apoptosis could be a factor in the formation of more aggressive forms of gastric cancer.

Another very important mechanism in the development of the two types of gastric cancer can be a reduction of the function of E-cadherin and its complex, which disturbs the adhesion of cells and consequently the formation of aggressive forms of cancer.

Conflicts of Interest

There is no conflicts of interest.

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