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Correlation between Fas and FasL proteins expression in normal gastric mucosa and gastric cancer

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Abstract: The study's objective was to assess the expressions of Fas and FasL proteins in gastric cancer in correlation with chosen clinicohistological parameters. Fas and FasL expression was analyzed in 68 patients with gastric cancer, using the immunohistochemical method. The expression of Fas was found to be lower in gastric cancer cells than in healthy mucosa, both in the lining epithelium and in glandular tubes (28% vs. 48% and 44%; $p < 0.001$). The expression of FasL was also markedly lower in cancer cells than in glandular tubes, yet higher than in the lining epithelium (51% vs. 73% and 14%; $p < 0.01$). Positive expressions of FasL and Fas were lower in less advanced gastric cancer cells (T1, T2), than in more advanced tumors (T3, T4), but only in the case of FasL was this difference statistically significant ($p < 0.05$). Our findings seem to confirm the theory of the impact of apoptotic disorders at the level of Fas receptor and FasL protein in the process of gastric cancer formation and growth, which is manifested in the varied expressions of these proteins in gastric cancer and in the normal lining and glandular epithelium of the stomach. However, the lack of significant differences in the expressions of Fas and FasL in correlation to other clinicohistological parameters indicates the existence of mechanisms that have a greater impact on the process of differentiation of gastric cancers. This in our opinion eliminates these proteins as prognostic factors. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 1, pp. 142–147*)

Key words: gastric cancer, Fas, FasL, *Helicobacter pylori*

Introduction

Tumor grade at the time of diagnosis and histological type are key factors in the treatment of patients with gastric cancer, affecting their survival after surgery. Little is known of the factors that could determine the respective histological type or those that affect tumor growth rate or formation of distant metastases. These issues are closely connected to the abnormal processes of gastric mucosal cell proliferation and disturbances in apoptosis, which is a natural defense mechanism.

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A number of apoptosis-inducing factors have been known so far. Two main apoptotic pathways have been distinguished: external (membranous), due to membrane Fas receptor stimulation; and internal (mitochondrial), largely dependent on the Bcl-2 family of proteins. The ultimate effect is cell death as the result of enzyme activation.

The activation of TNF family membrane receptors, including Fas (Apo-1, CD95), occurs after binding to a receptor-specific ligand molecule, the Fas-ligand protein (FasL). Cytotoxic T lymphocytes that have a FasL molecule on their surface act through the Fas-FasL interaction. Following identification of cells that are to be eliminated (e.g. neoplastic or infected with viruses) T cells bind to their membrane Fas receptor and trigger cell destruction. The Fas receptor has in its structure the so-called 'death domain' (DD) at the cytoplasmic side. Fas-ligand binding to

the receptor causes activation of its death domain that binds to FADD (Fas-associated death domain) — the protein mediating caspase activation. The Fas — FADD complex via its domain — DED (the death effector domain) — activates precaspase 8 (the so-called 'FLICE' protein (FADD-like interleukin 1 beta converting enzyme). Caspase 8 (FLICE) through caspase 10 activates the apoptotic effector protein, caspase 3. There is another apoptotic model involving membrane receptors, through secondary intracellular transmitters engaged in apoptosis, with ceramide as the final product (originates from sphyngomyelin under the effect of sphyngomyelinase). Its activation depends on the stimulation of membrane receptors for TNF and Fas/APO-1, which results in the production of death protein — FADD/Mort 1 and RIP (after Fas receptor activation). These proteins affect sphyngomyelinase that affects sphyngomyelin, giving rise to ceramide which leads to caspase activation [1–4].

Despite growing understanding of apoptosis, the question remains unanswered: Which apoptotic pathway (and to what degree) becomes disturbed during neoplastic transformation?

The objective of the current study was to compare the expressions of Fas, FasL proteins in gastric carcinoma cells and healthy non-neoplastic gastric mucosa (lining and glandular epithelium). The expressions of Fas and FasL were analyzed in relation to tumor histological type, histological malignancy grade (feature G), degree of advancement (pT), location in the stomach and the presence of *Helicobacter pylori* infection in the stomach. The lifespan of patients was also investigated in relation to whether Fas and FasL could possibly be prognostic factors in gastric cancer patients.

Material and methods

Patients

The study involved 68 patients operated on in the II Department of General and Gastroenterological Surgery, Medical University of Bialystok, between 1999 and 2004. The study group included 21 women and 47 men aged 31–79 years. The study was conducted on the archive material consisting of paraffin cubes with embedded tissues of gastric cancer. Healthy gastric mucosa was obtained from the fragment of the stomach excised during surgery, and this served as the control group.

Immunohistochemistry

The expressions of Fas and FasL were determined using the immunohistochemical method, in neoplastic and healthy tissues (glandular and lining epithelium). Formalin-fixed and paraffin-embedded tissue specimens were cut on a microtome into 5 μm thick sections. The sections were deparaffinized in xylenes and hydrated in alcohols of decreasing concentrations. In the case of Fas antibody, antigens became exposed through citrate buffer heating (pH = 6.0) for 15 minutes. Following rinsing in PBS buffer (pH = 7.4), the sections were incubated with primary antibodies: Fas (mouse monoclonal antibody, Sc-8009, Santa Cruz Biotechnology) and FasL (goat polyclonal antibody, Sc-834-G, Santa Cruz Biotechnology). After performing the reaction in LSAB technique (LSAB +System HRP, Dako, Poland) the antigen-antibody complex was visualized using chromogen DAB (S3000, Dako, Poland).

The expressions of Fas and FasL were semi-quantitatively assessed in the neoplastic cells. The expression was considered positive when found in more than 20% of neoplastic cells, and negative when observed in fewer than 20% of these cells.

Helicobacter pylori

Giemsa's method was used in order to confirm the presence of the bacteria *Helicobacter pylori.*

Statistical analysis

The results underwent statistical analysis using the Pearson's χ^2 test. Distant survivals were assessed using the Kaplan–Meier curve, whereas Gehan–Wilcoxon test was applied to compare the survivals. The differences found were considered statistically significant at $p < 0.05$, with greater differences at $p < 0.01$ and $p < 0.001$. Pairs of missing values were removed.

Results

The expression of Fas was found to be significantly lower in gastric cancer cells than in healthy mucosa, both in the lining epithelium and glandular tubes (28% vs. 48% and 44%; $p < 0.001$). Similarly, the expression of FasL was markedly lower in cancer cells than in glandular tube cells, but higher than in the lining epithelium (51% vs. 73% and 14%, $p < 0.01$) (Table 1, Figure 1).

Positive expressions of FasL and Fas were lower in the cells of less advanced gastric cancer (T1 and T2), compared to the more advanced ones (T3 and T4), although only in FasL was the difference statistically significant (33% vs. 63%; $p < 0.05$). Protein expressions were compared in relation to tumor location in the stomach, feature G, Lauren's and Bormann's clas-

Table 1. Comparison of Fas and FasL expressions in gastric cancer cells and in healthy gastric mucosa (lining and glandular epithelium, control group)

Variables	Fas		p	Fas L		p
	+	–		+	–	
Study group Gastric cancer	21 (28%)	55 (72%)		36 (51%)	35 (49%)	
Control group						
Lining epithelium	13 (48%)	14 (52%)	$p < 0.001$*	3 (14%)	19 (86%)	$p < 0.01$*
Glandular tubes	12 (44%)	15 (56%)	$p < 0.001$*	16 (73%)	6 (27%)	$p < 0.01$*

*Comparison with study group (gastric cancer)

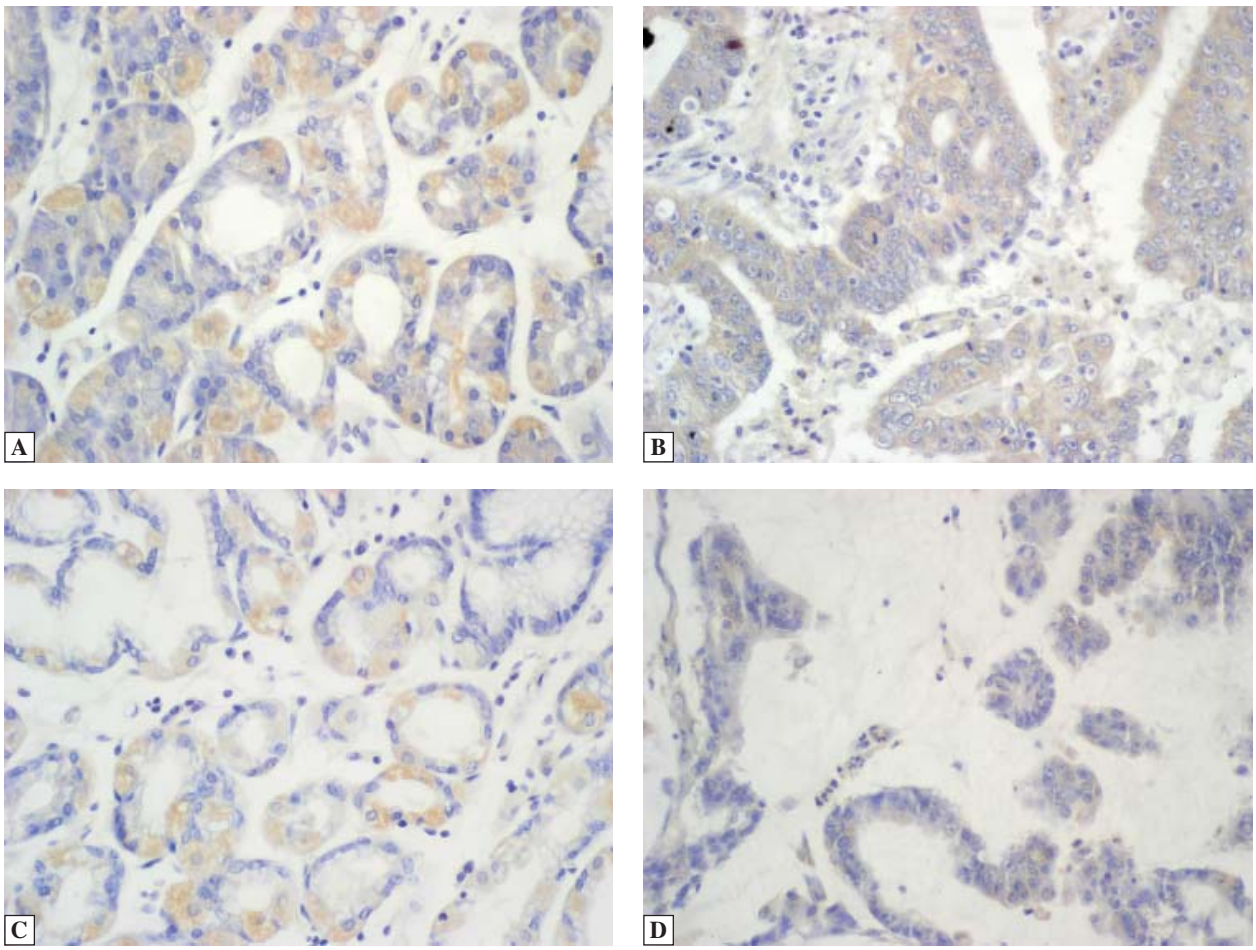

Figure 1. Immunohistochemical staining. **A.** Fas expression in normal mucosa. **B.** Lower cytoplasmic Fas reaction in gastric cancer cells. **C.** High FasL expression in main glandular tube cells. **D.** Weak FasL expression in gastric cancer cells. Original magnification × 400

sifications, patients' age and gender and *Helicobacter pylori* infection. No significant differences were found between the respective groups of patients (Table 2).

The average distant survivals were assessed after surgery, using the Kaplan–Meier curve, in Fas- and FasL-positive patients and in those lacking Fas and FasL expressions, and then compared using the Gehan–Wilcoxon test. Positive protein expression had no effect on the survival time of the patients (Figures 2, 3).

Correlations were compared between patients with positive and negative expressions of Fas and FasL. No

Table 2. Expressions of Fas and FasL in gastric cancer cells in correlation with clinicopathomorphological factors and *Helicobacter pylori* infection

Variables	Fas		p	FasL		p
	+	–		+	–	
Age						
< 60	5 (24%)	16 (76%)	SN	12 (46%)	14 (54%)	SN
≥ 60	16 (34%)	31 (66%)		23 (55%)	19 (45%)	
Gender						
Female	5 (24%)	16 (76%)	SN	10 (48%)	11 (52%)	SN
Male	16 (34%)	31 (66%)		25 (53%)	22 (47%)	
Infiltration depth (pT):						
pT1,2	4 (15%)	23 (85%)	SN	9 (33%)	18 (67%)	$p < 0.05$
pT3,4	13 (32%)	28 (68%)		26 (63%)	15 (37%)	
Lauren's feature						
Intestinal type	8 (19%)	34 (81%)	SN	22 (52%)	20 (48%)	SN
Diffuse type	9 (33%)	18 (67%)		13 (52%)	14 (48%)	
G feature						
G1, G2	4 (16%)	21 (84%)	SN	14 (56%)	11 (44%)	SN
G3	17 (33%)	34 (67%)		22 (43%)	29 (57%)	
Tumor location in the stomach						
Upper 2/3	6 (19%)	26 (81%)	SN	16 (50%)	16 (50%)	SN
Lower 1/3	11 (31%)	24 (69%)		19 (54%)	16 (46%)	
Bormann's classification						
0, 1, 2	12 (29%)	30 (71%)	SN	23 (51%)	19 (49%)	SN
3, 4	4 (21%)	15 (79%)		11 (52%)	8 (48%)	
Helicobacter pylori infection						
Present	9 (22%)	32 (78%)	SN	18 (51%)	17 (48%)	SN
Absent	12 (34%)	23 (66%)		18 (56%)	23 (44%)	

SN — statistically nonsignificant

statistically significant correlations were noted between the two proteins examined ($p > 0.05$).

Discussion

We presented the expressions of Fas and FasL proteins — mediators of the receptor pathway of apoptosis in gastric cancer cells in correlation with chosen morphological parameters and *Helicobacter pylori* infection. Our own findings confirm the occurrence of disorders in the membrane apoptotic pathway in gastric cancer cells. The expressions of Fas and FasL in cancer cells differed significantly from those noted in healthy gastric mucosa. In the case of Fas, the expression was lower in cancer cells than in normal mucosa, both in the lining and glandular epithelium (28% vs. 48% and 44%; $p < 0.001$).

On the other hand, the expression of FasL in cancer cells was markedly higher than in the lining epithelial cells (51% vs. 14%; $p < 0.01$), and lower than in the glandular epithelial cells (51% vs. 73%). A similar correlation concerning differences in the expression of Fas ligand in the stomach wall was observed by Ishihara et al. [5] As shown by their findings, positive FasL expression in the gastric mucosa associated with the presence of *Helicobacter pylori* infection occurs only in glandular epithelial cells, not in the lining epithelium.

Such results would be in agreement with the theory of disturbances in the membrane apoptotic pathway in cancer cells as the cause of neoplastic transformation and would also confirm the cancer immune escape. According to this theory, a reduction in the Fas receptor expression causes disor-

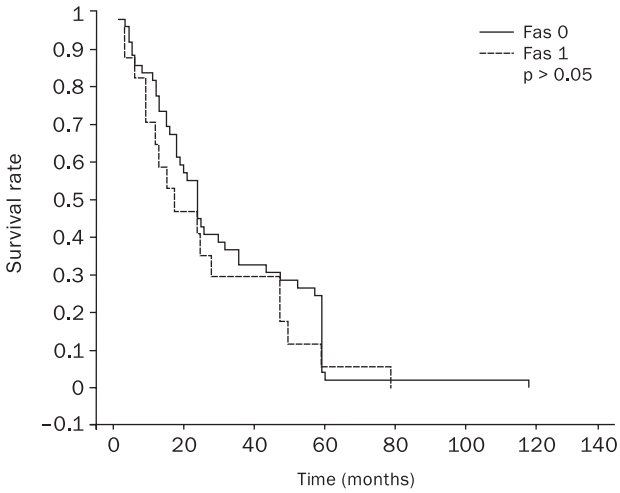

Figure 2. Comparison of survival rates in patients with positive Fas expression and its lack

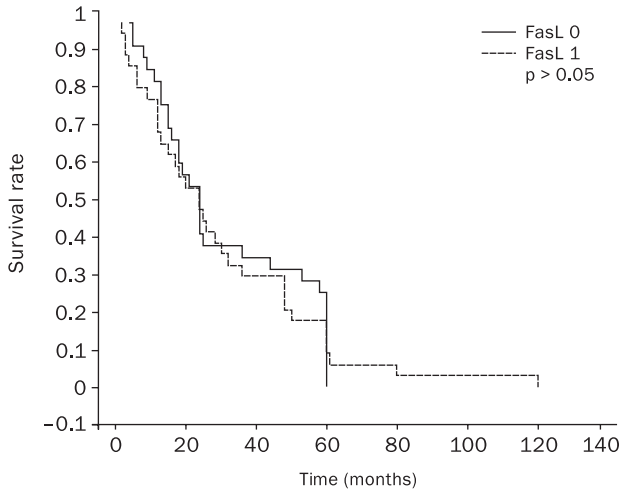

Figure 3. Comparison of survival rates in patients with positive FasL expression and its lack

ders in the apoptotic pathway in cancer cells which become resistant to stimulation by FasL. At the same time, high expression of FasL on the surface of the neoplastic cells results, through Fas receptor binding, in apoptosis in T lymphocytes, which decreases the immune response of the patient [1–3, 6–10]

We found a slightly higher expression of Fas protein in more advanced cancers (T3 and T4) than in the less advanced ones (T1, T2) (32% vs. 15%). This difference, however, was not statistically significant. Also, Osaki et al. [11] did not observe any significant differences in Fas expression between adenomas and early and advanced forms of cancer (38% vs. 43% vs. 37%). Similarly, Liu et al. [7] found no differences in the expressions of Fas and FasL in relation to cancer advancement. On the contrary, Ohno et al. [8] showed higher expression of Fas protein in early forms of cancer compared to advanced ones (70% vs. 64%), explaining this as a possible protective effect of this protein inhibiting tumor growth through apoptosis induction.

We found a significantly higher expression of FasL in more advanced tumors (T3 and T4) than in the less advanced lesions (T1 and T2) (63% vs. 33%; $p < 0.05$), which would be consistent with the theory of the 'immune escape' that accelerates tumor growth. A similar correlation has been reported by other authors [11, 12].

Zheng et al. [10] found significantly higher FasL expression in gastric cancer cells than in normal mucosa (53% vs. 34%; $p < 0.001$) and its lower expression in metastatic tumors than in primary lesions (51% vs. 81%; $p < 0.05$).

Similarly to our findings, most authors showed no significant correlations between FasL expression and depth of gastric wall infiltration [7, 8, 11, 13].

Our own findings, as well as the reports of some authors showing varying expressions of apoptosis-associated proteins in gastric cancer cells depending on tumor grade, could indicate that the factors affecting tumor growth or metastasizing may result from various apoptotic disorders. This, however, requires further study.

Like most authors, we found no significant correlation between protein expressions and patients' age or gender [7], although higher Fas and FasL expressions were noted in patients over 60 years of age (Fas: 19% vs. 38%; FasL: 46% vs. 55%). This difference, however, was not statistically significant ($p > 0.05$). Ohno et al. [8] noted a higher expression of FasL in patients over 60.

In our study, Fas expression according to Lauren's classification was observed in 33% of diffuse cancers, compared to 19% of intestinal-type tumors. Ohno et al. [8] showed higher Fas expression in intestinal cancer cells than in the diffuse type, explaining this as more substantial disorders in the membrane apoptotic pathway in the case of more malignant cancers. We found no difference was in the expression of FasL in various histopathological forms of cancer (52% vs. 52%). These results are consistent with literature data [7, 9]. On the other hand, other authors have demonstrated higher FasL expression in intestinal-type cancer cells as compared to the diffuse type [1, 10, 14].

The assessment of the protein expression depending on the feature G, tumor location in the stomach and Bormann's classification did not show any significant differences between the respective groups of patients.

Similar results were obtained by Ohno et al. [8] and Benett et al. [13] who revealed, like Zheng [10], higher FasL expression in larger tumors.

The results could suggest that the type of apoptotic disorders is to a lesser degree dependent on tumor location in the stomach and that the macroscopic form of cancer (Bormann) depends on other factors than the disturbed membrane pathway.

The effect of *Helicobacter pylori* infection, one of the commonest carcinogenic factors, on Fas and FasL expressions was also assessed. No significant difference was noted in the expressions of proteins examined depending on the presence of the infection, although the expressions of both proteins were slightly lower in the infected patients. These results prove a slight effect of *Helicobacter pylori* infection on the disturbances of this apoptotic pathway and are consistent with literature data [15, 16].

We found no significant differences in the survival rate of patients depending on the protein expression. Some authors have reported longer survival in Fas-positive patients suggesting that high expression of this protein may have an inhibitory effect on tumor growth, associating the degree of Fas-dependent apoptotic pathway disorders with the occurrence of more aggressive forms of tumor and shorter survival of patients [9].

Our own results, presented here, confirm the theory of the effect of apoptotic disorders at the level of the Fas receptor in the process of formation and progression of gastric cancer. However, lack of significant differences in correlation with tumor malignancy, differentiation, macroscopic form, location in the stomach, or *Helicobacter pylori* infection seems to suggest the existence of mechanisms that have a greater effect on gastric cancer differentiation. Additionally, lack of significant difference in distant five-year-survival depending on Fas and FasL expressions excludes these proteins from being the prognostic factors some authors have suggested them to be [8]. However, it should be emphasized that our knowledge of the impact of apoptotic disorders on tumor formation and growth, or on the occurrence of distant metastases, is still slender. The problem requires thorough study in order to improve the future diagnosis and treatment of gastric cancer [3].

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