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ORIGINAL ARTICLE

KLF4 expression and apoptosis-related markers in gastric cancer

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Summary

Purpose: *To correlate the expression of Kruppel-like factor 4 (KLF4) with clinicopathological properties of gastric cancer (GC) and to evaluate any possible correlation between KLF4 expression and the expression of apoptosis-related markers p53, Fas, Bcl-2, survivin and FLICE inhibitory protein (Flip-l).*

Methods: *Formalin-fixed, paraffin-embedded tissue specimens obtained from 96 patients with GC who had undergone gastric surgery were analyzed for pathological parameters, while KLF4, p53, Fas, Bcl-2, survivin and Flip-l expression was assessed by immunohistochemistry.*

Results: *TKLF4 immunohistochemical staining was noted in 78.1% of the cases. Strong positivity was found in 15.6% and weak in 62.5% of the samples. Positive expression of p53, Fas, Bcl-2, survivin, Flip-l was found in 56.2%, 44.8%, 15.6%, 41.7% and 38.5% of the samples, respectively. KLF4 expression was significantly associated with p53 nuclear staining and Fas immunoreactivity. p53-positive tumors demonstrated more often high KLF4 staining compared to p53-negative tumors. Fas-positive tumors were associated with decreased KLF4 expression. Logistic regression analysis of apoptosis-related markers to KLF4 expression revealed that Fas positivity significantly decreased the probability of strong KLF4 expression, and inversely, Bcl-2 expression improved the prediction of KLF4 staining. When all 5 predictive variables were considered together (p53, Fas, survivin, Bcl-2, Flip-l) they significantly predicted the type of KLF4 expression in GC cells (p=0.019).*

Conclusion: *Our results suggest that the decrease or loss of KLF4 expression correlates with diffuse-type GC and immunoreactivity to Fas, and are inversely linked with p53 nuclear accumulation. The significance of KLF4 in GC requires further studies and should be more thoroughly investigated for potential use in the evaluation and better stratification of GC patients.*

Key words: *apoptosis, Fas, gastric cancer, KLF4, p53, survivin*

Introduction

GC remains the fourth most common cancer worldwide, and the second most common cause of cancer-related deaths [1], although incidence and mortality rates have been slowly decreasing in many countries over the last 5 decades. Despite the major improvements in diagnosis and treatment, overall 5-year survival rate is less than 25% because most cases are diagnosed when the tumor has invaded the muscularis propria [2]. The aggressive nature of gastric carcinoma has been associated with numerous molecular abnormalities, including disarrangement in apoptotic mechanisms and resistance to programmed cell death [3-5].

GKLF, a transcription factor that can either activate or suppress gene expression, has been found to play important roles in the regulation of proliferation and differentiation of gastrointestinal tract epithelial cells [6].

Recent studies suggested that KLF4 contributes to the development and progression of GC [7,8]. KLF4 expression was found to be decreased or lost in GC and inactivated KLF4 has been proposed as a marker of poor prognosis [8].

KLF4 was shown to physiologically interact with p53 tumor suppressor protein (one of the key factors involved in apoptosis) by mediating the p53-dependent cell-cycle arrest process in response to DNA damage [9]. It was found that KLF4

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Table 1. Correlation between KLF4 expression and clinicopathological parameters

Parameters		*KLF4 expression*		*H*	*df*	*p-value*
		Absent/low N (%)	*Strong N (%)*			
Gender	Male	58 (82.9)	12 (17.1)	0.13	1	0.722
	Female	23 (85.5)	3 (11.5)			
Age at diagnosis (years)	≤60	26 (100)	0 (0)	5.08	1	0.024
	>60	55 (78.6)	15 (21.4)			
Location	Upper half of the stomach	36 (85.7)	6 (14.3)	0.001	1	0.972
	Lower half of the stomach	45 (83.3)	9 (16.7)			
Gross appearance (Borman type)	Exophytic	20 (83.3)	4 (16.7)	0.34	2	0.844
	Infiltrative	39 (83)	8 (17)			
	Ulcerated	22 (88)	3 (12)			
Lauren's classification	Intestinal	50 (78.1)	14 (21.9)	4.36	1	0.037
	Diffuse	31 (96.9)	1 (3.1)			
WHO pathological type	Tubular	29 (78.4)	8 (21.6)	4.14	4	0.387
	Papillary	8 (80)	2 (20)			
	Poorly cohesive	22 (88)	3 (12)			
	Mucinous	8 (80)	2 (20)			
	Mixed	14 (100)	0/ (0)			
Differentiation	Well/Moderate	45 (80.4)	11 (4)	1.00	1	0.318
	Poor	36 (90)	4 (10)			
Primary tumor (pT)	T1	4 (100)	0 (0)	3.86	3	0.277
	T2	39 (81.3)	9 (18.8)			
	T3	31 (91.2)	3 (8.8)			
	T4	7 (70)	3 (30)			
Regional lymph nodes status (pN)	No (N0)	34 (85)	6 (15)	0.00	1	1.000
	Yes (N1/N2/N3)	47 (83.9)	9 (16.1)			
Distant metastases (M)	Absent (M0)	75 (85.2)	13 (14.8)	0.06	1	0.799
	Present (M1)	6 (75)	2 (25)			
TNM stage	I	6 (75)	2 (25)	1.35	3	0.717
	II	20 (90.9)	2 (9.1)			
	III	27 (84.4)	5 (15.6)			
	IV	28 (82.4)	6 (17.6)			
Lymph/angio invasion	No	52 (83.9)	10 (16.1)	0.00	1	1.000
	Yes	29 (85.3)	5 (14.7)			

acts as a transcriptional repressor of p53, through direct binding to its promoter, thus causing resistance to apoptosis induced by DNA damage [10]. Considering the established significance of apoptosis in cancer development and progression [11,12], we aimed at investigating whether KLF4 expression is associated with the expression of p53 and, in addition, to investigate the possible link of KLF4 with the expression of other important apoptotic proteins in GC carcinogenesis.

Therefore, in this study, we correlated the expression of KLF4 to clinicopathological properties of GC and evaluated any possible correlation between KLF4 expression and the expression of apoptosis-related markers p53, Fas, Bcl-2, survivin and Flip-l.

Methods

Patients and clinicopathological characteristics

Formalin-fixed, paraffin-embedded specimens from 96 patients who had undergone surgery for GC were studied. All analyzed cases of GC were diagnosed at the Institute of Pathology, Faculty of Medicine, Nis, Serbia. The mean patient age was 63.46 ± 7.9 years; the

youngest patient was 46, and the oldest 79 years old. Seventy patients (72.9%) were male and 26 (27.1%) female. In 42 (43.8%) cases GC was localized in the upper and in 54 (56.2%) in the lower half of the stomach. Staging was performed according to the TNM Staging Classification for Carcinoma of the Stomach (7th Edn, 2009) [13]. Patient and tumor characteristics are summarized in Table 1.

The histological sections were processed by standard techniques, and stained with hematoxylin and eosin (HE). HE-stained slides were used to assess the pathological type according to Lauren's and WHO classification, tumor differentiation and TNM stage.

Immunohistochemical analysis

Representative sections of GC and the surrounding non-neoplastic tissue were analyzed for KLF4 and apoptosis-related proteins by standard immunohistochemical procedures. The following primary antibodies were used: a rabit polyclonal antibody against human KLF4 (clone H180, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal mouse antibodies to p53 protein (DO-7,1:100 dilution), survivin (clone 12C4,1:100) and Bcl-2 (clone 124, 1:100), all purchased from Dako, Glostrup, Denmark; rabbit polyclonal antibodies to Fas (C-20, 1:100) and Flip-l (H-150,1:250) obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Three μm thick tumor tissue sections were deparaffinized in xylene and rehydrated in series of ethanol and deionized water. Following heat-induced epitope retrieval, sections were incubated with 0.3% hydrogen peroxide in methanol for 10 min. After the primary antibody application and 60 min incubation at room temperature, the slides were treated with standard immunoperoxidase methods using a streptavidin-biotin-peroxidase complex (LSAB+Kit/HRP, Dako, Glostrup, Denmark). Staining was developed using a liquid 3,3'-diaminobenzidine (DAB) substrate kit, where the positive reaction was indicated by brown precipitate in the nuclei, cytoplasm and cell membrane. Sections were counterstained with Mayer's hematoxylin. Negative controls were carried out by omitting the primary antibodies and were processed in parallel with positive controls and the investigated sections.

Immunohistochemical staining scoring

Stained tissue sections were reviewed and scored independently by two investigators (MK and SS). Interobserver discrepancies were resolved using a double-headed microscope. KLF4 and Flip-l staining was noted in nuclei and the cytoplasm, while Bcl-2 showed cytoplasmic, and Fas cytoplasmic and membranous staining pattern. For p53 and surviving, nuclear staining was observed. The percentage of immunoreactive cells and staining intensities in each section were evaluated. The percentage of positive cells was divided into 5 grades (percentage scores): ≤10% (0), 10–25% (1), 25–50% (2), 50–75% (3), and >75% (4) [7]. The staining intensity was graded using a scale from 0 to 3 (0-absent staining, 1-weak, light brown, 2-intermediate, yellowish brown, 3-strong, dark brown). For the purposes of statistical analysis, the expression of the marker was considered positive if the staining was noted in ≥10% of cancer cells with staining intensity of ≥2. Specifically, for the analysis of KLF4 staining an additional classification was established, where the tumors were separated into two groups: tumors with strong expression (≥50% of tumor cells stained with intensity of at least 2) and tumors negative or with low expression in all other cases [7].

Statistics

All data analyses were processed using the Statistical Package for Social Sciences, version 15.0 statistical software (SPSS, Chicago, IL). A p-value of 0.05 or less was considered as statistically significant. Continuous variables like age were represented as mean±SD. Categorical variables were analyzed by chi-square and Fisher's exact test with Yates correction. Binary logistic analysis was performed with SPSS usage.

Results

FKLF4 immunohistochemical staining of paraffin-embedded GC tissue specimens sections was noted in 75 (78.1%) cases, while it was absent in 21 (21.9%). Strong positivity was found in 15 (15.6%) samples, and weak in 60 (62.5%). KLF4 staining was confined in the cytoplasm and nuclei with the more prominent loss of nuclear reactivity (Figures 1 and 2). In the adjacent non-neoplastic tissue, strong cytoplasmic and nuclear staining in the glandular epithelium was observed.

The correlation of KLF4 expression and clinicopathological parameters is displayed in Table 1. Significant difference in the distribution of KLF4 positivity according to patient age and Lauren's histology classification was observed (p=0.024 and p=0.037, respectively). Strong KLF4 staining was more frequently found in older (>60 years) GC patients. In addition, intestinal-type GC was significantly correlated with preserved KLF4 positivity. Diffuse-type GC more often displayed decreased or loss of expression of nuclear and cytoplasmic KLF4 stain (Figure 2).

Positive expression of p53, Fas, Bcl-2, survivin, Flip-l was found in 54 (56.2%), 43 (44.8%), 15 (15.6%), 40 (41.7%) and 37 (38.5%) of the cases, respectively (Figures 1 and 2). The correlation between KLF4 expression and apoptotic markers staining is shown in Table 2. KLF4 expression was significantly associated with p53 nuclear staining and Fas immunoreactivity. p53-positive tumors demonstrated more often high KLF4 stain-

Table 2. Correlation between KLF4 expression and apoptotic markers staining

Parameters		*KLF4 expression*		*H*	*df*	*p-value*
		Absent/low N (%)	*Strong N (%)*			
p53	Negative	39 (92.9)	3 (7.1)	3.01	1	0.044
	Positive	42 (77.8)	12 (22.2)			
Fas	Negative	41 (77.4)	12 (22.6)	3.31	1	0.036
	Positive	40 (93)	3 (7)			
Bcl-2	Negative	70 (86.4)	11 (13.6)	0.80	1	0.371
	Positive	11 (73.3)	4 (26.7)			
Survivin	Negative	48 (85.7)	8 (14.3)	0.02	1	0.887
	Positive	33 (82.5)	7 (17.5)			
Flip-l	Negative	52 (88.1)	7 (11.9)	0.99	1	0.321
	Positive	29 (78.4)	8 (21.6)			

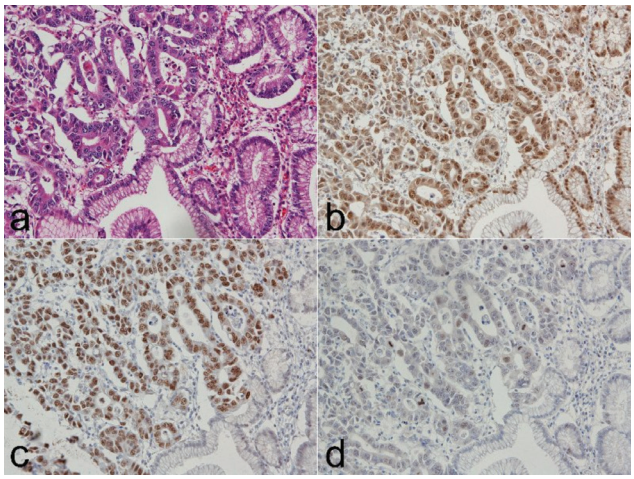

Figure 1. Representative photomicrographs of tubular gastric adenocarcinoma with adjacent mucosa containing regular glands and glands with dysplasia: (**a**) hematoxylin and eosin stain; (**b**) immunohistochemical staining of KLF4 (GKLF): non-cancerous cells show nuclear expression of KLF4, while cancer cells display strong nuclear and intermediate cytoplasmic staining; (**c**) positive p53 nuclear expression; (**d**) weak nuclear survivin expression exclusively in cancer cells (original magnification x200).

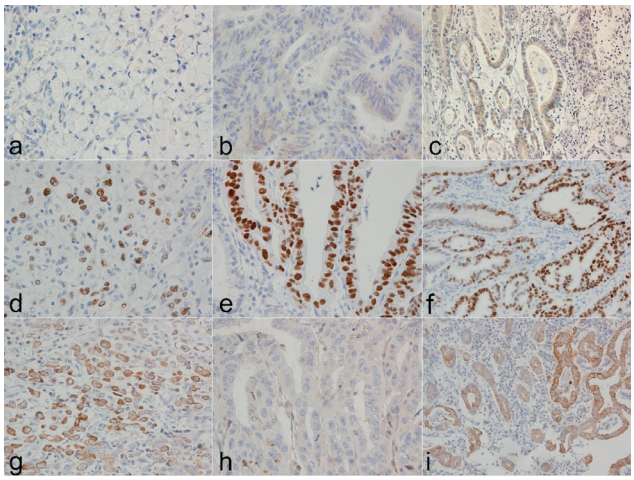

Figure 2. Immunohistochemical staining of KLF4 and apoptosis-related markers in diffuse-type (signet ring cell) GC (**a, d, g, j**), intestinal-type papillary (**b, e, h, k**) and tubular gastric carcinoma (**c, f, i, l**). (**a**) Absent, (**b**) poor cytoplasmic and (**c**) intermediate immunoreactivity to KLF4; (**d, e, f**) p53 positivity; (**g, i**) strong cytoplasmic and membranous staining pattern of Fas and (**h**) negative Fas stain; (**j**) high expression of Flip-l; (**k**) weak Bcl-2 and (**l**) survivin expression.

ing compared to p53-negative tumors (p=0.044). Nevertheless, Fas-positive tumors showed more often simultaneous decrease of KLF4 expression (p=0.036).

Tumor characteristics and the level of immunohistochemical expression of the investigated markers were tested in logistic regression analysis models. In the first logistic regression model which assessed the predictive influence of Lauren's classification, WHO pathology type, TNM stage, lymph/angio invasion and differentiation of the GC with regard to KLF4 expression, none of the investigated parameters showed significant correlation with KLF4 expression. When all 5 variables were considered together, they still did not significantly predict type of KLF4 expression (x^2=8.1, df=5, N=96, p=0.151; Table 3). In the second model that evaluated the association of KLF4 expression and apoptotic markers in GC, it was found that Fas positivity of the tumor significantly decreased the probability of strong KLF4 expression. Inversely, Bcl-2 expression improved the prediction of KLF4 staining. When all 5 predictors were considered together they significantly predicted the type of KLF4 expression in GC cells (x^2=13.5, df=5, N=96, p=0.019; Table 4).

Discussion

GC is a multifactorial disease with significant

Table 3. Logistic regression analysis of KLF4 expression in gastric cancer: pathological features of gastric cancer as model predictors

Parameters	*B*	*S.E.*	*Odds ratio*	*p-value*
Lauren's classification	-2.26	1.29	0.11	0.08
WHO pathological type	-0.11	0.24	0.90	0.65
TNM stage	0.27	0.34	1.30	0.43
Lymph/angio invasion	-0.15	0.66	0.86	0.83
Differentiation	0.24	0.75	1.27	0.75
Constant	-1.91	1.21	0.15	0.12

Table 4. Logistic regression analysis predicting the type of KLF4 expression by immunoreactivity of apoptosis-related markers

Parameters	*B*	*S.E.*	*Odds ratio*	*p-value*
p53	1.17	0.78	3.22	0.132
Fas	-1.57	0.78	0.21	0.045
Bcl-2	1.99	0.86	7.31	0.020
Survivin	-0.05	0.64	0.95	0.936
Flip-1	0.91	0.67	2.48	0.173
Constant	-2.74	0.78	0.07	0.000

geographical variations, intricate and perplexed pathogenesis determined by numerous environmental and host genetic factors [14]. Due to high worldwide incidence, poor prognosis and high mortality rates, the molecular basis of GC has been investigated in numerous studies [15,16]. GC shows great histological diversity which is reflected in the availability of numerous histopathological classification systems [17]. The most commonly used are the WHO and Lauren's classifications. The prevailing and more useful histological classification is based on the studies published by Lauren and Jarvi, which described two histologically distinct variants of gastric adenocarcinoma each with different pathophysiological characteristics, depending on the presence of features resembling the intestinal mucosa, intestinal-type and diffuse-type GC [18]. These two types follow different precancerous processes and show clinical and epidemiologic differences.

The epithelial zinc-finger transcription factor KLF4, also called gut enriched Krüppel-like factor or GKLF, is an important regulator of genes involved in cell-cycle arrest and differentiation, and acts as a potent inhibitor of proliferation in untransformed cells [10,19,20]. KLF4 is highly expressed in the post-mitotic and terminally differentiated epithelial cells, including the gastrointestinal tract epithelium [5]. In neoplastic disease, KLF4 expression seems to be downregulated in various solid tumors. It was found that GC, colonic adenocarcinoma, esophageal carcinoma, bladder and prostatic cancers were associated with significant decrease of KLF4 expression [7,21-24]. In addition, the restoration of KLF4 expression inhibited GC growth *in vitro* and tumorigenicity in animal models [24]. However, it was found that KLF4 expression increased in breast ductal carcinoma and oral squamous carcinoma [25]. This inconsistency considering KLF4 may reflect the pleiotropic nature of this molecule that may play roles of both tumor suppressors and oncogenes in a context-dependent manner [10,20]. The underlying molecular mechanisms by which the loss of KLF4 contributes to the development and progression of GC has not been elucidated so far and require further investigation.

In the present study, we found that KLF4 immunohistochemical expression was significantly decreased or lost compared with the surrounding non-neoplastic gastric tissue, which is in accordance to the results of previous studies [7-9]. However, we did not observe significant correlation of KLF4 negativity with tumor stage or with the propensity to spread via lymph/angio invasion to the regional lymph nodes or distant metastatic sites. Intestinal GC type, which is associated with less aggressive behavior and better prognosis, correlated significantly with strong KLF4 expression when compared to diffuse GC. Diffuse-type GC has poorer prognosis compared to the intestinal GC and its incidence has been increasing in some countries [2,26]. Only one case of diffuse GC showed KLF4 expression similar to that of the normal gastric tissue, with diffuse staining of nuclei and cytoplasm of cancer cells. The higher frequency of KLF4 down-regulation in diffuse GC may be related to KLF4 modulation of β-catenin/TCF4 signaling [8,27]. In addition, we found significant difference in the distribution of KLF4 immunoreactivity according to patient age, with the more frequent decrease or loss of KLF4 in patients younger than 60 years of age diagnosed with GC. Generally, diffuse-type GC more commonly affects younger people, which may contribute to the explanation of this finding.

Recent studies suggested that KLF4 constitutive expression inhibits DNA synthesis and reduces cell proliferation [28]. It was found that KLF4 is sufficient mediator of p53 for cell cycle arrest at G1/S checkpoint [29]. p53 plays an important

role in apoptosis and is considered "guardian of the genome" [30]. Failure of apoptotic mechanisms is a well known hallmark associated with many types of cancer, including GC. The programmed cell death is a complex and active cellular process that requires involvement of large number of molecules and is precisely regulated and well coordinated [5]. Two major apoptotic pathways, intrinsic or stress-induced, and extrinsic, death receptor-mediated path, include numerous pro-apoptotic and anti-apoptotic molecules. The accumulation of mutant, ineffective p53 and down-regulation of pro-apoptotic transmembrane Fas protein seem to play a critical role in gastric carcinogenesis and contribute to the dissemination of neoplastic cells. In addition, the overexpression of important anti-apoptotic proteins Bcl-2, survivin and Flip-l also have major influence in cancer progression and have been correlated with more invasive phenotype, metastasis and poor prognosis [31-34].

The findings of this study showed that positive expression rates of p53, Fas, Bcl-2, surviving and Flip-l were 56.2, 44.8, 15.6, 41.7 and 38.5% respectively, which are similar to the results of previously published studies [3,35-38]. p53 immunopositivity was found to be associated with the histological type of GC, with higher p53 expression noted in poorly differentiated GCs, however the investigation of p53 prognostic value yielded controversial results [35,39]. The results of this research implied that p53-positive staining is significantly associated with KLF4 immunoreactivity. Despite the KLF4's quality to downregulate p53, frequent simultaneous expression may not reflect this relationship, however, it may suggest more aggressive tumor phenotype.

The extrinsic apoptotic pathway following Fas binding activates caspase-dependent apoptosis

in susceptible cells when triggered by its ligand [33,38]. Moreover, a recent study found that Fas signaling contributed to epithelial-mesenchymal transition and promoted motility in gastrointestinal cancer cells [40]. In human cancers, Fas receptor is frequently downregulated during the progression of cancer [41]. However, GC cell lines express large quantities of Fas and are susceptible to Fas-mediated apoptosis [42]. In addition, soluble Fas serum level has been recently recognized as a non-invasive tool for early diagnosis of GC [42]. In GC, Fas immunoexpression was found to correlate with favorable clinicopathological features [38]. Our findings suggested that Fas positivity was inversely correlated with KLF4 expression. The logistic regression model emphasized Fas staining as a good predictor of decreased or absent KLF4 expression.

Flip-1, that can effectively block Fas-induced apoptosis and survivin, inhibitor of apoptosis protein linked to poor prognosis in GC and other human cancers, did not correlate significantly with KLF4 expression. However, in logistic regression analysis, the expression of Bcl-2, the well known anti-apoptotic molecule, improved the prediction of KLF4. Up until today, the clinical significance, the precise role and the prognostic value of Bcl-2 in GC have not been unraveled satisfactorily. This study's limitation of relatively small number of investigated GC samples and the small percentage of tumors with positive Bcl-2 expression suggests that this predictive value may have limited power.

In conclusion, this study demonstrated that decrease or loss of KLF4 expression correlates with diffuse-type GC and immunoreactivity to Fas, and is inversely linked with p53 nuclear accumulation. The significance of KLF4 expression in GC requires further studies and should be more thoroughly investigated for potential use in the evaluation and improved stratification of GC patients.

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