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Original Article

Expression and significance of FXYD-3 protein in gastric adenocarcinoma

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Abstract. *Objective*: FXYD-3, also known as Mat-8, is a member of the FXYD protein family. It was reported that this protein can associate with and modify the transport properties of Na, K-ATPase, and may play an important role in a variety of physiological and pathological states. This protein is up-regulated in certain types of cancers (such as breast, prostate and pancreatic cancer), but down-regulated in other types of cancers (such as colon and kidney cancer). No study has been performed in gastric cancer; therefore, the aim of this project was to investigate FXYD-3 expression and its clinicopathological significance in gastric adenocarcinoma.
Patients and methods: FXYD-3 protein was examined by immunohistochemistry in normal gastric mucous ($n = 29$) and gastric adenocarcinoma ($n = 51$), obtained from surgical resection of gastric cancer patients.
Results: FXYD-3 protein was present in the cytoplasm of normal gastric epithelial cells or gastric cancer cells. The rate of FXYD-3 strong expression was significantly higher in cancer (51% of 51) than in normal mucosa (10% of 29, X^2=13.210, $p <$ 0.0001). FXYD-3 expressed strongly in ulcerative/infiltrating types of cancers compared to polypoid/fungating ones ($X^2 =$ 5.765, $p = 0.016$). However, FXYD-3 expression was not correlated with patient's gender, age, tumor size, lymph node status and histological grade ($p > 0.05$).
Conclusion: Up-regulated expression of FXYD-3 protein may be involved in tumourgenesis and invasion of gastric adenocarcinoma.

Keywords: FXYD-3, gastric cancer, immunohistochemistry

1. Introduction

The FXYD proteins constitute a family of conserved auxiliary subunits of the Na, K-ATPase, and have been the study focus in biomedicine field recently due to their ability to finely regulate the activity of the enzyme complex in various physiological and pathologixcal settings [1].

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The FXYD protein family contains seven members that are small, single-span membrane proteins characterized by a signature sequence containing an FXYD motif and three other conserved amino acid residues [1, 2]. Recent evidences suggest that all members including FXYD-1 (phospholemman) [3], FXYD-2 (gamma subunit of Na, K-ATPase) [4], FXYD-3 (mammary tumor marker 8, also called as Mat-8) [5], FXYD-4 (corticosteroid hormone-induced factor, CHIF) [6], FXYD-5 (protein related to ion channel, Ric) [7], FXYD-6 (phosphohippolin) [8]and FXYD-7 [9] associate with Na, K-ATPase in a tissue-specific way and modulate its transport properties.

FXYD-3 was identified as a transmembrane protein, which is expressed in a subset of murine breast tumors [5,10]. Although it is a member of the FXYD family, it differs from the most of the other members. It has a signal peptide that is uncleaved and completely different by being the only one with two transmembrane domains. The other members have only one transmembrane dormain [1]. It is reported that FXYD-3 is expressed not only in normal tissues but also in tumors. In normal tissues it is mainly expressed in the uterus, stomach, colon and skin. And in tumor tissue it has been found in breast, colon and prostate tumors. Interestingly, in some types of cancers such as prostate [11] and pancreatic cancer [12], FXYD-3 is up-regulated and in others such as colon and kidney cancer [12] it is down-regulated. Moreover, it was shown that FXYD-3 was expressed in breast tumor initiated by the oncogenes c-neu or v-Ha-ras, but not by c-myc [10]. And Grzmil et al. [11] showed that small interfering RNA (SiRNA)-mediated inhibition of FXYD-3 expression promotes a decreased proliferation in prostate cancer cell line. However, to our knowledge, no study has been performed in gastric cancer yet, therefore, in the present study, we examined FXYD-3 protein expression in normal gastric samples and gastric adenocarcinomas, and further explored its clinicopathological significance in the patients.

2. Materials and methods

2.1. Materials

For immunohistochemistry formalin fixed paraffin-embedded tissue samples were obtained from 51 gastric adenocarcinoma patients who underwent surgical resection at the First Hospital of Hebei Medical University (Shijiazhuang, Hebei Province, China), during 2001–2006. The study included 29 distant normal mucosa specimens (they all were matched with the primary tumors) taken from the margin of distant resection. Among the primary tumors, 34 cases had lymph node metastasis. The patients' gender, age, tumor size, macroscopical type, lymph node status and differentiation were obtained from surgical and/or pathological records at the hospital. The mean age of the patients was 62 years old. According to the WHO classification, tumor differentiation was graded as grade I (high), grade II (moderate) and grade III (low). All pathological slides including normal specimens and tumors were confirmed by two pathologists (Zhu ZL and Wang ZM).

2.2. Immunohistochemical staining

Immunohistochemical staining was performed on 5-um thick formalin-fixed paraffin-embedded sections. The sections were incubated at 60° for 12 hours, deparaffinized and then rehydrated. The sections were transferred to 0.01M Tris-EDTA buffer (pH9.0) and subjected to high pressure cooker for 8 minutes and incubated at room temperature for 30 minutes for antigen retrieval. The sections were then washed in phosphate buffered saline (PBS, pH7.4) and incubated with 3% H_2O_2 in methanol for 20 minutes, to block endogenous peroxidase activity. Nonspecific binding of antibody was prevented by preincubating the sections with 1.5% horse serum (Fuzhou Maixim Biology Technology Limited Company, Fuzhou, Fujian Province, China) in PBS for 10 minutes. After removing the blocking solution, the sections were incubated with a monoclonal anti-FXYD-3 primary antibody (received kindly from Drs. H. Hayed and J. Kleeff, University of Heidelberg, Heidelberg Germany) in 1:2 diluted in PBS (pH7.4) over night at 4° in a moist chamber. Subsequently, the sections were incubated with biotinlated anti-rabbit IgG antibody (Fuzhou Maxim Biology Technology Limited Company) for 30 minutes, followed by an incubation of an avidin-biotin-peroxidase complex (Fuzhou Maxim Biology Technology Limited Company) for 30 minutes. The sections were washed with PBS between each incubation step. The peroxidase reaction was developed using 3.3 diaminobenzidine (DAB) (Fuzhou Maixim Biology Technology limited Company) for 8 minutes. Then, the sections were rinsed with water and counterstained with Mayer's haematoxylin and then washed, dehydrated in ethanol and mounted with xylene-based mounting medium. The breast cancer sections known for positive FXYD-3 were included as negative or positive controls. For negative controls,

PBS or/and purified rabbit IgG (Fuzhou Maixim Biology Technology limited Company) were used instead of the primary antibody. In all runs, there was no staining in the negative controls, and the positive controls showed clear staining.

The stained sections were microscopically examined and evaluated independently by two pathologists (Zhu ZL and Wang ZM) in a blind fashion without any clinicopathological information. Cytoplasmic staining was considered as FXYD-3 positive. The intensity of the staining was graded as negative (no positive cells), weak ($<$ 20% positive cells), moderate (20–50% positive cells) and strong positive ($>$ 50% positive cells). In statistical analysis, we considered negative, weak and moderate staining as weak group, and strong staining as positive group. To avoid artificial effects, tissue in the areas with poor morphology, necrosis and in the margins of the sections were not considered.

2.3. Statistical analysis

The Chi-square method was used to examine the relationship of the frequencies of FXYD-3 expression in normal gastric mucosa and cancer, and the relationship between FXYD-3 expression in cancer and clinicopathological variables. All P-values cited were two-sided and P-values $<$ 5% were judged as statistically significant.

3. Results

3.1. FXYD-3 expression in normal mucosa and primary tumor

We examined FXYD-3 protein expression in normal gastric mucosa and gastric cancer, and found that the expression was in the cytoplasm of normal mucosa epithelial cells and cancer cells, and there was no nuclear staining. Among 29 normal specimens, 15 cases was negative (52%), and 14 cases was positive, including 6 (21%) cases with weak, 5 (17%) moderate and 3 (10%) strong staining (Fig. 1A). Among 51 cancers, there were 9 (18%) negative, 8 (16%) weak, 8 (16%) moderate and 26 (50%) strong expressed cases, the staining was mainly in the cytoplasm of the cancer cells (Fig. 1B).

Taking into account similar clinicopathological features and facilitating statistical analysis, the cases with negative, weak and moderate staining were grouped as weak group, and the cases with strong staining as strong group.

As shown in Fig. 2 of FXYD-3 expression in normal mucosa and cancer, the rate of strong FXYD-3 expression in cancer was 51% (26/51), which was significantly higher than that in the normal mucosa, 10% (3/29, $X^2 = 13.210, p < 0.0001$).

We also compared the staining intensity of the inner tumor cells with the tumor cells at the invasive margin; there was no difference in the expression intensity.

3.2. FXYD-3 protein expression in relation to clinicopathological variables

Table 1 shows the relationship between FXYD-3 expressions in tumors with the clinicopathological variables. None of the tumors with polypoid/fungating growth pattern had strong FXYD-3 expression, while 57% (26 of 46) ulcerative/infiltrating tumors did ($X^2 = 5.765$, $p = 0.016$). Besides, FXYD-3 expression was not significantly correlated with patients' gender ($p = 0.811$), age ($p = 0.903$), tumor size ($p = 0.691$), lymph node status ($p = 0.490$) and histological grade ($p = 0.492$).

4. Discussion

FXYD-3 is able to associate with and modify the transport proteins of Na, K-ATPase [13], and to act as chloride channel or a chloride channel regulator [1]. Although FXYD-3 is a member of the FXYD protein family, it displays some uncommon characteristics that differ from the other members of the FXYD family. In addition, Bibert S et al. [14] identified two splice variants of human FXYD-3 in CaCo-2 cells, namely, short human FXYD-3 and long human FXYD-3. Their results showed that short human FXYD-3 had 72% sequence identity with mouse FXYD-3, whereas long human FXYD-3 was identical to short human FXYD-3 but had a 26-amino acid insertion after the transmembrane domain. Short and long human FXYD-3 RNAs and proteins were differentially expressed during differentiation of CaCo-2 cells. Long human FXYD-3 was mainly expressed in undifferentiated cells and short human FXYD-3 in differentiated cells.

Recently, it has been shown that FXYD-3 not only expresses in some normal tissues (such as the liver, pancreas, intestine, prostate, lung, brain and skin etc) [2,10], but also in a growing number of tumors and tumor cell lines. For example, Morrison et al. used RT-

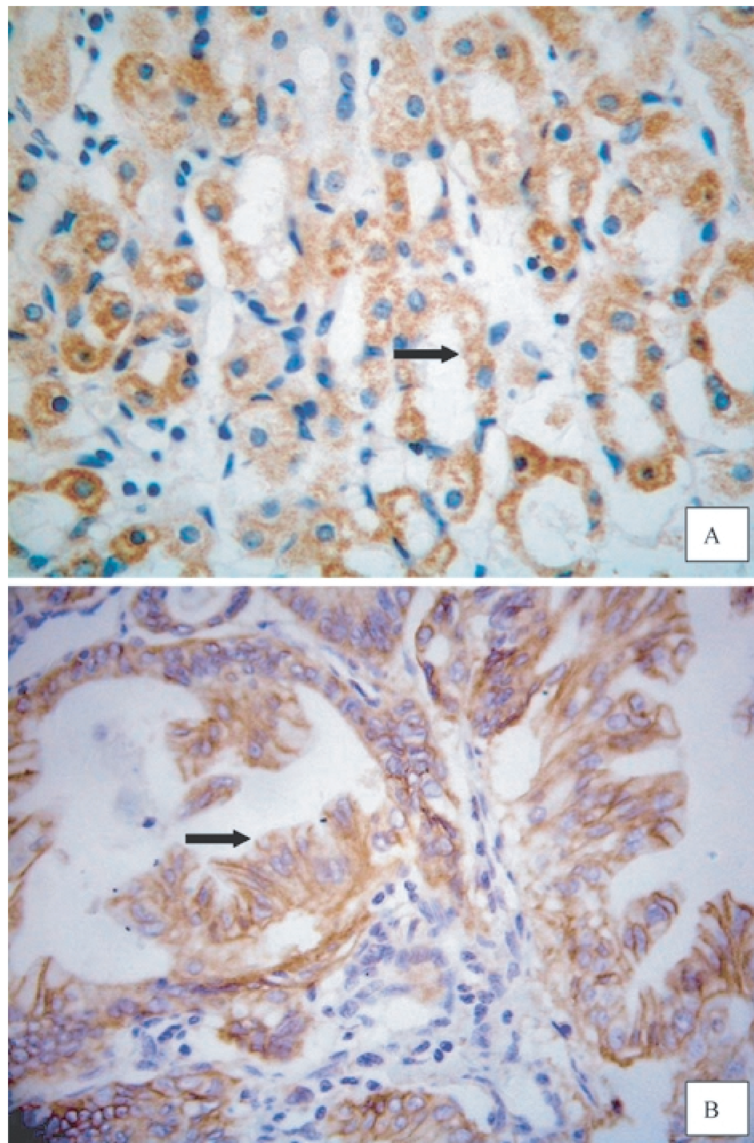

Fig. 1. The positive FXYD-3 immunohistochemical staining was in the cytoplasm and/or membrane of glands cells (➡) in normal gastric mucosa (A) and of cancer cells (➡) in gastric adenocarcinoma tissue (B). there was no nuclear and stromal staining.

PCR and RNA blot methods to determine the expression of FXYD-3, and found that FXYD-3 expressed in 16 breast cancers and also 11 cell lines of breast cancer [5]. At the same time, there were studies showing that FXYD-3 also expresses in Chinese hamster ovary-K1 (CHO-k1) cells [15] and human colorectal cancer cells [16], the positive staining was distributed in intracellular membranes, being not only detected around the nuclear envelop but also partly overlapping with an endoplasmic reticulum marker. Subcellular fractionation by density gradient centrifugation supported this partial overlapping. The spherical structures observed were not co-localized with markers for lysosomes, endosomes, and Golgibodies, suggesting that FXYD-3 is distributed in a distinct endoplasmic reticulum region and the nuclear envelope after synthesis on membranebound ribosomes [15,16]. In present study, our results also show that the positive staining of FXYD-3 is located in the cytoplasm of gastric gland cells and gastric adenocarcinoma cells, and there was no nuclear staining.

FXYD-3 protein expression was also analyzed in normal pancreatic tissue and pancreatic ductal adenocarcinoma (PDCA) by Iacobuzio-Donahue et al. [17]

Table 1
The relationship between FXYD-3 protein expression and clinicopathological variables in the patients with gastric adenocarcinoma

Variables	N	FXYD-3 expression		X^2	P value
		Weak (%)	Strong (%)		
Gender					
Male	38	19 (50)	19 (50)	0.057	0.811
Female	13	6 (46)	7 (54)		
Age (years)					
⩽ 62	29	14 (48)	15 (52)	0.015	0.903
> 62	22	11 (50)	11 (50)		
Tumor size (cm)					
⩽ 3	15	8 (53)	7 (47)	0.158	0.691
> 3	36	17 (47)	19 (53)		
Macroscopical					
Polypoid/fungating	5	5 (100)	0	5.765	0.016
Ulcerative/infiltrating	46	20 (43)	26 (57)		
Lymph node status					
Non-metastasis	18	10 (56)	8 (44)	0.476	0.490
Metastasis	33	15 (45)	18 (55)		
Grade					
I	15	6 (40)	9 (60)	2.410	0.492
II	15	10 (67)	5 (33)		
III	21	9 (43)	12 (57)		

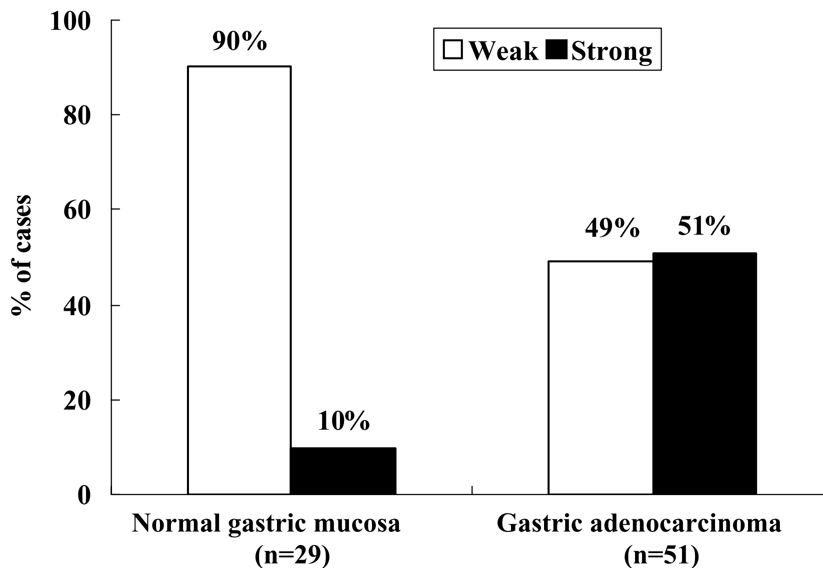

Fig. 2. Frequency of FXYD-3 protein immumohistochemical staining in normal gastric mucosa and gastric adenocarcinoma.

using cDNA microarrays and Kayed et al. [12] using QRT-PCR and microarray analysis, in situ hybridization and immunohistochemistry, their results showed that the levels of FXYD-3 protein were increased in PDAC compared to the normal specimens. In addition, a similar result has been reported by other research groups [18,19], in which the expression of FXYD-3 was higher in pancreatic ductal adenocarcinoma than in the chronic pancreatitis tissue. All above results indicated that overexpression of FXYD-3 in pancreatic cancer might contribute to the proliferative activity of the cancer. Grzmil et al. [11] examined the expression of FXYD-3 protein using the cDNA array and RT-PCR technique in a set of prostate tumors and the matched normal prostate tissue, and found that the expression of FXYD-3 protein was highly increased in prostate carcinoma in comparison to the matched normal prostate tissue, moreover, the expression of FXYD-3 was al-

so higher in prostate carcinoma cell lines than in the matched normal epithelial cell samples, in addition, their results also showed that FXYD-3 silencing by siRNA promotes a decreased proliferation in prostate cancer. Together with, those results suggested that FXYD-3 played an important role in cellular growth of prostate carcinoma.

However, there were some contrary results on the expression of FXYD-3 in prostate cancer. Vaarala et al. [20] studied the expression of FXYD-3 protein in the human prostate cancer cell line and in benign prostatic hyperplasia tissue, and found that FXYD-3 protein was highly expressed in benign prostatic hyperplasia tissue, but it was down – regulated expression in prostate cancer cell line, even non-expression.

In the present study, we also found that FXYD-3 strongly expressed in tumors than in normal gastric musoca samples. The results were similar to the majority of all above results such as in breast cancers [5], Chinese hamster ovary-K1 (CHO-k1) cells [15], human colorectal cancer cells [16], pancreatic cancer [12, 17–19] and prostate cancer [11], indicating that the expression of FXYD-3 was higher in cancer tissue than it in the corresponding normal tissue, and suggesting that FXYD-3 protein may be related to tumorgenesis.

To study the fine role of FXYD-3 in tumorogenesis, Bibert S et al. [21] used human colon adenocarcinoma cells (Caco-2) to investigate the effect of FXYD-3 silencing on cell proliferation, differentiation and apoptosis, and Na, K-ATPase activity and expression. Their results showed that FXYD-3 silencing had no effect on cell proliferation. This result is compatible with the result reported by Grzmil et al. [11]. In addition, Bibert S et al. [21] also showed that FXYD-3 silencing could promote cell apoptosis and prevent cell differentiation of Caco-2 cells. From those results, they supposed that the most possibility was that FXYD-3 silencing prevented a proper regulation of Na, K-ATPase, which led to perturbation of cellular Na+ and K+ homeostasis and changes in the expression of Na, K-ATPase isozymes, whose functional properties were incompatible with Caco-2 cell differentiation. Overall, further studies are needed to definitively establish the mechanism of FXYD-3 in tumorogenesis.

In addition, the gross appearance of tumors is affected by many factors such as benign, malignant, location, growth rate, invasive ability, necrosis and hemorrhage etc. Therefore, to a certain extent, the gross type of tumor can reflect the invasive ability of tumor. In general, the invasive ability of gastric cancer in ulcerative/infiltrating pattern is stronger than it in polypoid/fungating pattern. In the present study, we also found that none of the five tumors with polypoid/fungating growth pattern had strong expression for FXYD-3, while 26 of 46 tumors with ulcerative/infiltrating pattern showed strong expression of FXYD-3. The result suggested that overexpression of FXYD-3 in gastric adenocarcinoma might be related to the invasive ability of cancer. Gorden et al. [22] studied the expression of FXYD-3 protein in the adenocarcinoma of the lung and found that FXYD-3 expression was positively related to poorer survival of the patients. Unfortunately, we did not have survival data for the patients in the present study, we could not see if the FXYD-3 was further related to patient survival. However, our result showed that FXYD-3 expression may be involved in invasion of gastric cancer.

5. Conclusion

Up-regulated expression of FXYD-3 protein may be involved in tumourgenesis and invasion of gastric adenocarcinoma.

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