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Immunohistochemical screening for ALK fusion gene in signet-ring cell gastric carcinoma.

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Abstract

The aim of this study was to prescreen the expression of ALK-positive in signet-ring cell gastric carcinoma by IHC assay. We selected 84 FFPE samples with signet-ring cell gastric carcinoma (55 cases of GC, 29 cases of EGJ) and performed the detection of ALK-positive using IHC. The correlation of ALK-positive and clinicopathologic characteristics was statistically analyzed. The results showed, of 84 cases prescreened, 11 (13.09%) were ALK-positive. For the 6 cases with IHC 2+ (7.14%), and the 5 cases with IHC 1+ (5.95%). We noted that 8 (72.73%) cases were never smoker, 8 (72.73%) cases were >5 cm tumor size and 9 (81.82%) cases were T4 in invasive depth. All of the 11 cases were III of pathologic TNM stage. The ALK-positive patients showed significantly statistical difference in lymph node metastasis (p=0.0285) and TNM stage (p=0.0497), compared with the ALK-negative patients. In conclusion, the expression of ALK fusion is found in signet-ring cell gastric carcinoma by IHC assay.

Keywords: Immunohistochemistry, Screening, Signet-ring cell gastric carcinoma, ALK fusion gene.

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Introduction

The anaplastic lymphoma kinase (ALK) gene rearrangements were initially identified in anaplastic large cell lymphomas (ALCL) for over 20 years [1]. Subsequently, ALK gene was also recognized in diffuse large B-cell lymphoma (DLBCL) and in inflammatory myofibroblastic tumors (IMT) [2-4]. In 2007, the fusion of ALK gene with echinoderm microtubule associated protein [4] like (EML4) gene locus was detected in a subset of non-small cell lung cancer (NSCLC) in Japan [5]. The incidences of EML4-ALK fusions in NSCLC, mostly involving East Asian patients, were around 3%-13% with no significant differences between Asian and western countries [6-9]. By extrapolation, recent estimates indicate that approximately 5% of NSCLC contain an EML4-ALK fusion, which would mean that over 70,000 patients will get clinical benefit from crizotinib. It was a small molecule inhibitor of ALK and c-MET receptor tyrosine kinases and approved by the U.S. FDA for the treatment of EML4–ALK positive NSCLC patients [10-12]. Clinical characteristics associated with EML4-ALK fusion are younger age, never/light smoking status, or adenocarcinoma histology. Meanwhile, the majority of EML4-ALK fusion tumors were demonstrated a solid growth pattern with >10% signet-ring cells [13-15].

This distinct cytologic characteristic is reminiscent of the signet-ring cells more commonly seen in gastric carcinoma than in lung cancer. Signet-ring cell gastric carcinoma, characterized by cells with abundant mucin in the cytoplasm and nuclei located at the cell periphery, is a histologic diagnosis by the World Health Organization [16,17]. It has long been thought to have a worse prognosis than other forms of gastric cancer. However, several studies have begun to question this idea and found that is not the case [18-20]. The molecular basis of their growth and metastasis still remains unclear, and the genetic background has rarely been researched. Therefore, efficient screening for the ALK fusion gene is a crucial issue in molecular basis and clinical practice of signet-ring cell gastric carcinoma.

Currently, there are three methods for the EML4-ALK fusion gene detecting in NSCLC: 21 fluorescent *in situ* hybridization (FISH), reverse transcriptase-polymerase chain reaction (RT-PCR) and Immunohistochemistry (IHC). FISH is sensitive and specific to detect EML4-ALK as an eligibility criterion in NSCLC, but it is not readily available due to technical and financial problems in routine pathology practice in China. Theoretically, although RT-PCR is a standard method for testing the EML4-ALK, it requires fresh frozen tissues for extracting RNA, so limited its clinical application. Compared with FISH and RT-PCR, the conventional IHC assay is more cost-effective and convenient screening test in most pathology practices, especially in pathology labs without a VENTANA IHC platform. Many research groups also reported good correlations between IHC stain and FISH for detection of EML4-ALK fusion gene [22-24]. For these reasons, conventional IHC seems suitable for a large-scale screening of patients with ALK positive carcinoma.

In this study, we examined the ALK fusion gene using conventional IHC in signet-ring cell gastric carcinoma and analyzed the correlation between ALK fusion gene and clinicopathologic characteristics of the disease. The present study was aimed at preliminary screening for ALK fusion gene in signet-ring cell gastric carcinoma.

Materials and Methods

Patients and tissues

This study included 84 archival formalin-fixed paraffin-embedded (FFPE) samples with signet-ring cell gastric carcinoma (55 cases of gastric cancer, 29 cases of esophagogastric junction carcinoma) at the Fourth Hospital of Hebei Medical University from 2011 to 2013. It was approved by the Ethics Review Board at the Fourth Hospital of Hebei Medical University. All samples were surgically resected tissues and were proved to the signet-ring cell carcinoma by experienced pathologists. They were collected and used after obtaining informed consent from the patients. According to the TNM staging for carcinoma of the stomach by AJCC (7th ed., 2010), the TNM stage was postoperative pathologic stage. Medical records were reviewed to extract data on clinicopathologic characteristics, including age, gender, smoking history, tumor size, invasive depth, lymph node metastasis and cancerembolus.

Immunohistochemistry

Immunohistochemical staining was performed on unstained 4 μm thick FFPE tissue sections. Briefly, after deparaffinization and rehydration, the slides were heated for antigen retrieval in steam cooker for 2 minutes in 1mM EDTA, pH 9.0(ZSGB-BIO, China). Then the slides were incubated with monoclonal for ALK (Clone SP8, Abcam, USA) and overnight at room temperature with a dilution of 1:100. Immunoreactivity was visualized with DAB detection kit (Dako, CA) according to the manufacturer's protocol. The IHC stains were evaluated for the expression of ALK protein by two pathologists. The criteria for scoring ALK fusion gene were as follows with reference to ALK-positive lung cancer: 23 No staining (0); Faint or weak staining intensity with >5% tumor cells or any staining intensity with ≤ 5% tumor cells (1+); Moderate staining intensity with >5% tumor cells (2+); Strong and granular staining intensity with >5% tumor cells (3+).

Statistical analysis

The statistical analyses were carried out by using Statistical Analysis System V8 (SAS Institute Inc. USA). To analyze correlations between ALK status and clinicopathologic features, we used the conventional chi-square association test or Fisher's exact test. All statistical tests were two-sided, and a value of $P<0.05$ was considered to be statistically significant.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the medical ethics committee of fourth hospital of Hebei medical university.

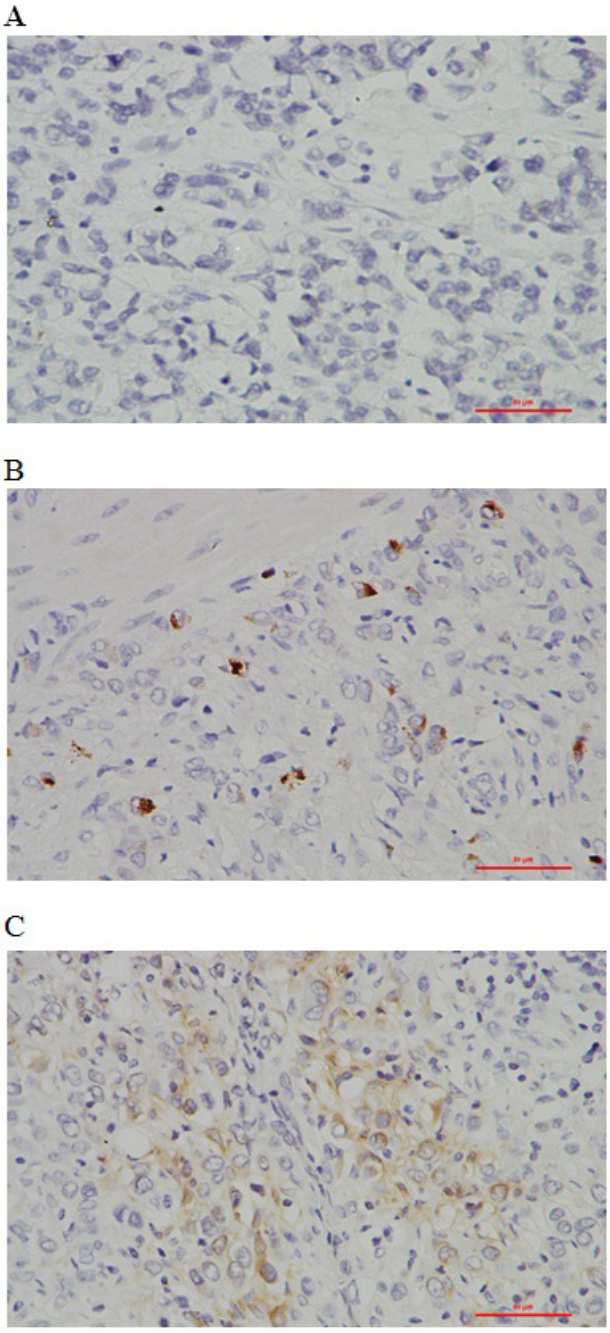

Figure 1. *The ALK protein expression in signet-ring cell gastric carcinoma. (A) Negative immunostaining. (B) Weak cytoplasmic staining (1+). (C) Moderate cytoplasmic immunoreactivity (2+), x400.*

Results

ALK protein expression by IHC in signet-ring cell gastric carcinoma

In the preliminary study, we analyzed a panel of 84 signet-ring cell gastric carcinoma samples, comprising 55 cases of gastric cancer, and 29 cases of esophagogastric junction carcinoma (EGJ). ALK rearrangement was detected in 11 of 84 cases (13.09%). All the ALK-positive cases exhibited cytoplasmic staining pattern in tumor cells (Figures 1A-1C). For the 6 cases with IHC 2+ (7.14%), and the 5 cases with IHC 1+ (5.95%). Of the 11 ALK-positive cases, 8 were gastric cancer, and 3 were EGJ. Individual patient characteristics of ALK-positive cases were listed in Table 1.

Table 1. *Individual patient characteristics of ALK Fusion-positive Patients in Signet-ring Cell Gastric Carcinoma.*

N	ALK (IHC)	Gender	Age	Smoking	Location	Tumor size (cm)	Invasive depth	Lymph node metastasis	TNM stage	Cancerembolus
1	1+	male	53	smoker	EGJ	6	T3	N3a	IIIB	negative
2	2+	male	36	never	EGJ	7	T4a	N3a	IIIC	negative
3	1+	female	72	never	EGJ	3.5	T4a	N1	IIIA	negative
4	2+	female	58	never	GC	3.5	T4a	N3a	IIIC	negative
5	1+	female	55	never	GC	8	T3	N3a	IIIB	negative
6	2+	female	48	never	GC	13	T4a	N3a	IIIC	negative
7	2+	male	67	smoker	GC	6	T4a	N1	IIIA	negative
8	2+	male	59	smoker	GC	3	T4a	N2	IIIB	negative
9	2+	female	66	never	GC	11.5	T4a	N2	IIIB	negative
10	1+	male	74	never	GC	6	T4a	N3a	IIIC	positive
11	1+	male	45	never	GC	6	T4a	N1	IIIA	negative

EGJ: Esophagogastric Junction Carcinoma; GC: Gastric Cancer

As summarized in Table 2, the cases composed of 6 (54.55%) men and 5 (45.45%) women with a median age 57.5 years (from 36 to 74). Smoking, 3 (27.27%) were smoker, and 8 (72.73%) were never. The tumor size was ≤ 5 cm in 3 (27.27%), and >5 cm in 8 (72.73%). In invasive depth, 2 (18.18%) were T3, and 9 (81.82%) were T4. The lymph node metastasis was N1 in 3 (27.27%), N2 in 2 (18.18), and N3 in 6 (54.55%). All of the 11 cases were III of pathologic TNM stage, and only 1 case was cancerembolus positive.

Table 2. *Clinicopathologic Features of ALK Fusion-positive Patients in Signet-ring Cell.*

Clinicopathologic features (N=11)	ALK-positive	
	(n)	(%)
Gender		
Male	6	54.55
Female	5	45.45
Age (median age 57.5)		
≥ 60	4	36.36
<60	7	63.64
Smoking		
Smoker	3	27.27
never	8	72.73
Tumor size		
≤ 5 cm	3	27.27
>5 cm	8	72.73
Invasive depth		
T3	2	18.18
T4	9	81.82
Lymph node metastasis		
N1	3	27.27
N2	2	18.18
N3	6	54.55
TNM stage		
III	11	100
cancerembolus		
Negative	10	90.91
Positive	1	9.09

Correlation with clinicopathologic features

In the present study, a total of 84 cases had successful IHC stain for detection of ALK fusion gene expression. Eight of 55 patients with gastric cancer were ALK-positive (14.55%), for 2 patients with IHC 1+ (3.64%), and 6 patients with IHC 2+ (10.91%). Three of 29 patients with EGJ were ALK-positive (10.35%), but all of 3 patients were IHC 1+. As the median ages of the ALK-positive and ALK-negative groups were 57.5 and 60.7 years, respectively, there were no significantly difference (p>0.05). The clinicopathologic features of the ALK-positive and ALK-negative patients were compared and the results were shown in Table 3. The ALK-positive patients showed significantly statistical difference in lymph node metastasis (p=0.0285) and TNM stage (p=0.0497), compared with the ALK-negative patients. No appreciable correlation was demonstrated between ALK-positive and ALK-negative groups in gender, smoking habit, tumor size, invasive depth, or cancerembolus.

Table 3. *Frequency of the ALK Fusion Gene Expression in Signet-ring Cell Gastric Carcinoma and its Association with Clinicopathologic features.*

Clinicopathologic features (N=84)	**ALK fusion gene**		P-value
	Positive n=11 (%)	Negative n=73 (%)	
Gender	6 (9.68)	56 (90.32)	0.2337
Male	5 (22.73)	17 (77.27)	
Female			
Age	4 (10.0)	36 (90.0)	0.4227
≥ 60	7 (15.91)	37 (84.09)	
<60			
Smoking	3 (9.37)	29 (90.63)	0.6456
Smoker	8 (15.38)	44 (84.62)	
never			
Tumor size	3 (7.69)	36 (92.31)	0.1718
≤ 5 cm	8 (17.78)	37 (82.22)	
>5 cm			
Invasive depth	0	13	0.3722
T1	0	3	
T2	2 (20.0)	8 (80.0)	
T3	9 (15.52)	49 (84.48)	
T4			
Lymph node metastasis	0	19	0.0285
N0	3 (33.33)	6 (66.67)	
N1	2 (6.90)	27 (93.1)	
N2	6 (22.22)	21 (77.78)	
N3			
TNM stage	0	25	0.0497
I+II	11 (18.64)	48 (81.36)	
III+IV			
Cancerembolus	10 (15.15)	56 (84.85)	0.4993
Negative	1 (5.56)	17 (94.44)	
Positive			

Discussion

Signet-ring cell gastric carcinoma is a distinct entity. Its diagnosis is based on an adenocarcinoma containing a majority of signet-ring cells by the identification of pathologist. A study shows that signet-ring cells in EGJ have a more aggressive biological behaviour [25]. Meanwhile, signet-ring cells present a low sensitivity to chemotherapy [19,20]. Therefore, we would try to research the molecular basis and find the potent molecular targets of Signet-ring cell gastric carcinoma. Considering the majority of EML4-ALK fusion lung cancer was demonstrated a solid growth pattern with >10% signet-ring cells, it is reminiscent of the signet-ring cells more commonly seen in gastric carcinoma than in lung cancer [13,14]. In this preliminary study, we show that ALK fusion gene was detected in 11 of 84 (13.09%) patients with Signet-ring cell gastric carcinoma. By using IHC stain, of the 11 ALK-positive cases, 6 cases were IHC 2+ (7.14%) and 5 cases IHC 1+ (5.95%). It seems to bring the hope and surprise to the disease.

Searching for targetable oncogenes in lung cancer have not been stopped and made steady progress in recently years. The EML4-ALK fusion gene represents one of the newest molecular targets in NSCLC. Although it is a minor genetic abnormality in approximately 5% of NSCLC, the incidence of NSCLC is increasing in Asian and western countries, so the absolute number of ALK-positive lung cancer is not trivial. The potent oncogenic activity of EML4-ALK fusion can be effectively blocked by crizotinib, a small molecular ALK tyrosine kinase inhibitor, which demonstrated dramatic response and longer PFS in ALK-positive lung cancer [26,27]. The patients with ALK-positive in NSCLC are more likely to be young, never/light smoker, and adenocarcinoma histology [7-9]. In our study, we note that 8 (72.73%) cases were never smoker, 8 (72.73%) cases were >5 cm tumor size and 9 (81.82%) cases were T4 in invasive depth. Meanwhile, all of the 11 cases were III of pathologic TNM stage. Although ALK-positive cases share several clinicopathologic features, compared with the ALK-negative groups, there is no appreciable correlation was demonstrated. We also find that lymph node metastasis (p=0.0285) and TNM stage (p=0.0497) show significantly statistical difference between ALK-positive and ALK-negative groups.

Current diagnostic methods to detect ALK fusion gene include IHC, FISH and RT-PCR. Duo to the higher requirements for fresh frozen tissue, RT-PCR unlikely becomes the standard test in clinical practice. Although a criterion method for ALK-positive in NSCLC had not been established, FISH has been used as the current gold standard for detecting ALK fusion gene in clinical trials with crizotinib [28-30]. However, FISH for the routine large-scale detection in China remains to be

challenging because of the technological complexity, time consumption and high cost. Meanwhile, interpretation of FISH in ALK-positive lung cancer tends to be more difficult than in ALCL or DLBCL, because ALK gene variants in NSCLC is an intrachromosomal rearrangement, so the break-apart probes can be subtle and relatively difficult to recognize [22-24,30]. Rodig and colleagues have also shown that FISH as a pre-screening method did not detect all cases with ALK-positive in NSCLC [8]. Since IHC is rapid, easy, sensitive and relatively inexpensive for detecting ALK fusion protein in NSCLC by pathologists, it remains a mainstay of routine surgical pathology diagnosis. A few validated antibodies in IHC for ALK protein have been widely used to diagnose ALK-rearranged ALCL today. However, the IHC used to ALCL is inadequate for detecting the majority of ALK-positive lung cancer. In an initial research, Rodig et al. found that only 4 ALK-positive in lung cancer by the ALCL standard test, and through FISH proved to be 10 cases [8]. Subsequently, some studies showed that ALK expression was detected in all ALK-positive lung cancer to be substantially lower than in ALK-rearranged ALCL [31]. Therefore, the College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC), and Association for Molecular Pathology (AMP) established the molecular testing guideline for selection of lung cancer patients for ALK tyrosine kinase inhibitors [8]. They found the interpretation criteria for ALK-positive lung cancer in IHC. In this preliminary study, according to the scoring criteria of ALK-positive lung cancer, we examined the ALK fusion gene using conventional IHC in signet-ring cell gastric carcinoma. Of the 11 ALK-positive cases, 6 cases were IHC 2+ (7.14%) and 5 cases IHC 1+ (5.95%). We thought that this was inappropriate in signet-ring cell gastric carcinoma. With the deep research and increased cases, we should find the diagnostic standard for ALK-positive in signet-ring cell gastric carcinoma.

Currently, the consistency of FISH and IHC is still controversial in detecting ALK-positive NSCLC. Some studies have found the good concordance between FISH and IHC [32,33]. Paik et al. also showed that none of IHC 1+ and 30% of IHC 2+ was FISH-positive and the specificity of IHC 2+ or more was 95.2% in detecting ALK-positive NSCLC [34]. However, Park et al. reported that 83.3% (5/6) of IHC 1+ and 84.6% (11/13) of IHC 2+ were FISH-positive, respectively. They thought that the high FISH-positive rate of IHC 1+ and 2+ in this study may result from different cutoff value of fluorescence, but there is no found standard for separation distance to define spilt signal [35]. In our present study, we performed the prescreening of ALK-positive in signet-ring cell gastric carcinoma by IHC assay, but we have not finished the further confirmation using FISH or RT-PCR. This is just a preliminary result, and don't rule out the possibility of false positive. One important goal of this preliminary study was to prescreen the expression of ALK-positive in signet-ring cell gastric carcinoma. In conclusion, our data show that the expression of ALK fusion gene is found in signet-ring cell gastric carcinoma by IHC assay. The further study will be required to performed FISH and RT-PCR assay as ALK-positive confirmation.

Informed Consent

Informed consent was obtained from all individual participants included in this study.

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