Altered expression of ETV1 and its contribution to tumorigenic phenotypes in gastrointestinal stromal tumors

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Received January 17, 2014; Accepted February 24, 2014

DOI: 10.3892/or.2014.3281

Abstract. Gastrointestinal stromal tumor (GIST) is the most common mesenchymal neoplasm of the gastrointestinal tract. ETV1 is a unique transcription factor specific to GIST that has been reported to date. The present study aimed to determine aberrant ETV1 expression and its contribution to tumorigenesis in GISTs. Altered expression levels of ETV1 and its relevant signaling pathways were assessed using western blotting and quantitative real-time PCR in 72 paired patient tissue samples. In addition, immunochemistry was performed on 156 GISTs using tissue microarray to analyze the correlation between ETV1 and clinical parameters. The results revealed that ETV1 was highly expressed in the GISTs at both the transcription and protein levels. Immunochemical analysis revealed that increased expression of ETV1 was correlated with KIT in the 156 patients. In addition, the frequency of ETV1 positivity was higher when compared with KIT [50.0% (9/18) vs 38.9% (7/18)] particularly in high-risk GISTs. Analysis of western blotting data showed that total protein isoforms of Raf, MEK and ERK were similar in the GIST tissues as well as in the uninvolved normal tissues. In contrast, the level of phospho-Raf, phospho-MEK and phospho-ERK were decreased in the tumor group. Moreover, enhanced signaling molecules such as Bcl-2 and Dvl2/GSK-3β/β-catenin/Smad2 were detected, showing a significant difference in comparison with the uninvolved normal cases. We conclude that ETV1, a member of the ETS family, is upregulated in GISTs, and its signaling is integrated into a cellular signaling network for resistance to apoptosis, tumor cell invasion and survival.

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Key words: ETV1, signaling pathway, tumorigenesis, gastrointestinal stromal tumor

Introduction

Gastrointestinal stromal tumor (GIST) which arises from interstitial cells of Cajal (ICC) or Cajal-like precursor cells (1) is the most common mesenchymal neoplasm of the gastrointestinal tract (2). Activating mutations in KIT and platelet-derived growth factor receptor α (PDGFRA) have been identified in ~85 and 10% of GISTs, respectively (3). Although inhibitors of KIT and PDGFRA such as imatinib mesylate (Gleevec; Novartis Pharmaceuticals) have increased progression-free survival in the majority of metastatic or recurrent GIST cases and have led to an unparalleled success in molecular-targeted therapy (4), <3% of patients achieve complete remission (5). Moreover, resistance to tyrosine kinase inhibitors is also a vital issue. Hence, KIT inhibition alone is unlikely to cure GISTs. Further studies must focus on other therapeutic targets and intracellular pathways.

ETV1, known as ETS-related protein 81 (ER81), encodes a member of the well known ETS family of transcription factors. It has been defined as an oncoprotein overexpressed in breast cancer (6,7), prostatic carcinoma (8,9), melanoma (10), Ewing sarcoma (11) as well as GISTs, and plays a key role in neoplastic formation, development and progression. ChiP (12) confirmed the observation that ETV1 is a lineage-specific survival factor which cooperates with KIT in GISTs. This observation showed that strong ETV1 expression and constitutively activated KIT are implicated in hyperplasia ICC set which may give rise to GISTs. Furthermore, ETV1 directly regulates ICC- and GIST-specific transcription. Notably, activated KIT can prolong ETV1 protein stability through KIT-independent signaling. Together these results demonstrate that ETV1 is essential for the growth of GIST cells and activated KIT may collaborate with ETV1 to promote tumorigenesis (5).

ETV1 is a unique transcription factor specific to GISTs that has been reported to date. In addition, it has been thought that downstream Ras/Raf/MAPK signaling cascade is reactive when KIT/PDGFRA kinases are activated by its ligand stem cell factor (SCF) in GISTs (13,14). These data raise the intriguing possibility that ETV1 cooperates with cellular signaling pathways to promote tumorigenesis. In the present study, we aimed to explore the relationship between ETV1 and cellular signaling pathways which may provide novel molecular-targeted therapies for GISTs.

Materials and methods

Patients and clinical tissue samples. Tumor tissue samples and corresponding tumor-adjacent normal samples were collected from patients with suspected GISTs who received surgical resection from May 2012 to September 2013 at The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China. The study protocol was approved by The First Affiliated Hospital Ethics Committee. Written informed consent statements were obtained from all the participants involved in the present study before surgery. Patients who had received any preoperative chemotherapy, molecular-targeted therapies such as imatinib mesylate or not diagnosed as having GISTs were excluded. The diagnosis of GIST was based on hematoxylin and eosin staining for histopathological appearance which was compatible with GIST and was confirmed by positive immunohistochemical staining for c-KIT. A total of 72 patients were included in the present study. Another 10 cases of other sarcoma types such as leiomyoma were utilized as the control group. Tissue specimens were placed into liquid nitrogen and store at -80˚C until RNA and protein extraction.

Western blot analysis. The total protein was lysed from 72 pairs of tissue samples using cell lysis buffer (Cell Signaling, Beverly, MA, USA) according to the manufacturer's protocol. Equal amounts of protein were applied to 8-12% gels and subjected to SDS-PAGE. The samples were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) for 1 h. Membranes were blocked for 2 h at room temperature with 5% dry milk in TBST and incubated overnight at 4˚C with primary antibodies against c-KIT (1:2,000); phospho-c-KIT (Tyr703) (1:2,000); c-Raf (1:1,000); phospho-c-Raf (Ser338) (1:1,000); MEK1/2 (1:2,000); phospho-MEK1/2 (Ser217/221 for MEK1 and Ser222/226 for MEK2) (1:2,000); ERK1/2 (1:2,000); phospho-ERK1/2 (Thr202/Tyr204 for ERK1 and Thr183/Tyr185 for ERK2) (1:2,000); Smad2/3 (1:2,000); caspase-3 (1:1,000); Bcl-2 (1:2,000); Bax (1:2,000); E-cadherin (1:2,000); vimentin (1:2,000); β-catenin (1:1,000); GSK-3β (1:1,000); Dvl2 (1:1,000); GAPDH (1:5,000) (Cell Signaling) and ETV1 (1:1,000) (Abcam, Cambridge, UK). Membranes were washed in Tris-buffered saline with Tween-20 (TBST) and incubated with 1:5,000 anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and washed again. Immune complexes were detected with an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and acquired in the linear range of the scanner and analyzed using Quantity One software (Bio-Rad).

Immunohistochemistry. Formalin-fixed, paraffin-embedded 4-μm sections of a GIST tissue microarray (TMA) (GIST 801-802) were purchased from Alinabio (Xi'an, China). TMA sections with 156 cases were deparaffinized in 100% xylene and re-hydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide for 20 min at room temperature. Heat-activated antigen retrieval was performed in sodium citrate buffer (pH 6.0). After blocking with 3% bovine serum albumin (BSA), TMA sections were incubated with a primary anti-ETV1 antibody diluted 1:100 or anti-KIT antibody diluted 1:200 in TBS containing 1% BSA overnight in a humidified chamber at 4˚C. After washing (5 min x3) in phosphate-buffered saline (PBS), the sections were incubated with anti-rabbit horseradish peroxidase-conjugated antibody at room temperature. Diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) was used as the chromogen, and color development was stopped by dipping the slides in distilled water. The nuclei were then counterstained with hematoxylin (Sigma). Blinded evaluations of the ETV1 immunostaining and independent observation were carried out simultaneously by two experienced pathologists. The immunohistological score calculated was the sum of the percentage of the positive area (negative, 0 point; <25%, 1 point; 26-50%, 2 points; 51-100%, 3 points) and the staining intensity (negative, 0; weak, 1; moderate, 2 and strong, 3). Five horizons were randomly selected and the average score in each case represented the final positive staining points: 0-2 points for each case was assigned as negative; 3-4 points was regarded as weak/moderate and 5-6 points was assigned as strong.

Quantitative real-time PCR. The total RNA of 72 frozen tissue specimens was extracted using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real-Time) (Takara, Dalian, China). DNA products were amplified with SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara). The primer sequences were as follows: KIT, forward 5'-AATGGCACGGTTGAATGTAAG-3' and reverse 5'-GGATGGATTTGCTCTTTGTTGT-3'; ETV1, forward 5'-GCAGTCAAGAACAGCCCTTTA-3' and reverse 5'-TCAGGTTTCGGTGTATGAGTTG-3'; GAPDH, forward 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3'. Thermocycling conditions were 10 min at 95˚C as the initial denaturation step, followed by 40 cycles at 95˚C for 30 sec and 60˚C for 34 sec. Reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System. Gene expression levels were measured using the threshold cycle (Ct) value, and relative fold-expression changes were normalized to the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Calculation of the gene copy number was carried out using the ΔΔCt method.

Statistics. Statistical analyses were conducted using the SPSS Software Package version 16.0 for Windows (SPSS, Chicago, IL, USA). Two-tailed paired sample t-test and independent sample t-test were utilized to determine differences between the tumor group and the uninvolved normal group as well as the control group. The relationship between expression levels of ETV1 and clinicopathological factors was examined using χ^2 test or Fisher's exact test in cross tables. A P-value <0.05 was considered to indicate a statistically significant result.

Results

In the first step, semi-quantitative RT-PCR analysis of 72 paired GIST patient tissue samples confirmed that ETV1 mRNA expression was significantly upregulated in the clinical tissue specimens when compared with that in the corresponding tumor-adjacent normal samples (P<0.05) (Fig. 1A); 55 patients (76.4%) were considered to have positive ETV1 expression.

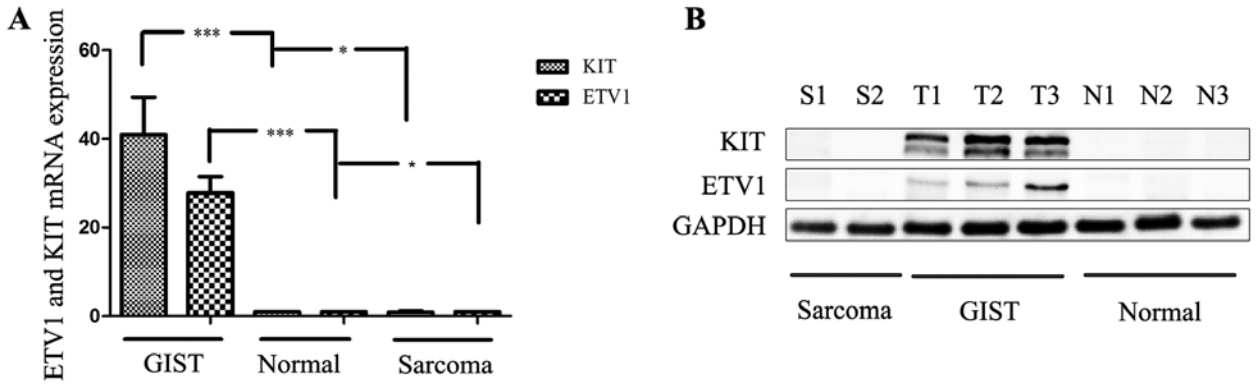

Figure 1. ETV1 expression in gastrointestinal stromal tumor tissues. (A) qRT-PCR analysis of ETV1 and KIT in 72 GIST tissues, their corresponding adjacent normal tissues and other sarcoma types. The results of the relative mRNA expression in three groups are presented as a histogram. The vertical axis represents the normalized ETV1 and KIT expression in the same tissue sample ($^{***}P<0.01$, $^{*}P<0.05$ compared with the control group). (B) Western blot analysis of ETV1 and KIT in the identical 72 patient samples. T1-3 denote the three representative GIST tumors and N1-3 denote the corresponding normal tissues. S1-2 denote the other types of sarcoma except GIST. GIST, gastrointestinal stromal tumor.

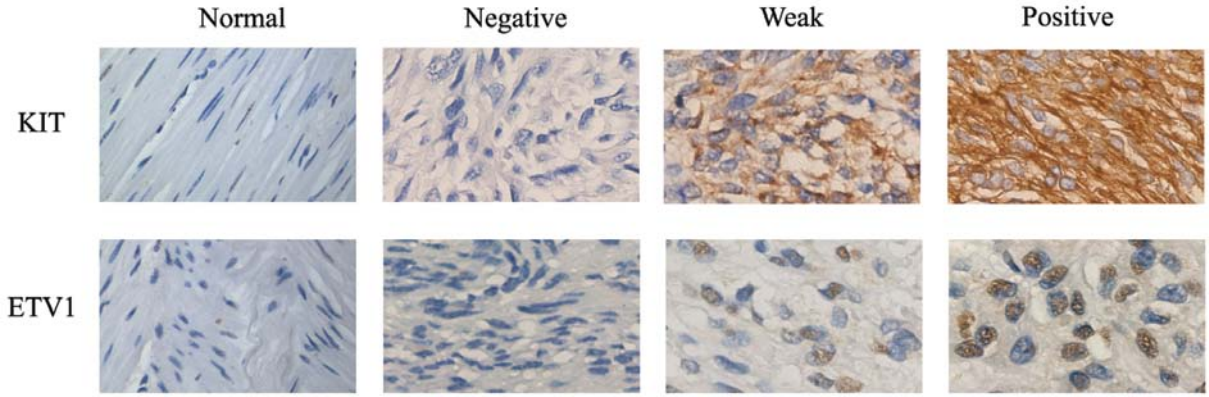

Figure 2. Immunochemical analysis of KIT and ETV1 expression compared with the normal tissues. Original magnification, x400.

Next, we determined the ETV1 protein expression in the same 72 patient tissue samples and found that ETV1 was upregulated in 65.3% (47/72) of these cases (P<0.05). No expression of ETV1 was detected in the other sarcomas. The ratio of the protein expression levels in the image was measured by densitometry using Quantity One software, which revealed that the pattern of expression of the ETV1 protein was similar to the semi-quantitative RT-PCR results (Fig. 1B).

In addition, immunohistochemical staining revealed that ETV1 was strongly expressed in the GIST TMA sections which contained 156 cases. ETV1 was diffusely positive in the nuclei of the tumor cells. Among the cases examined, 108 patients (69.2%) were considered to be positive for ETV1. As shown in Fig. 2, no expression of ETV1 was observed in the adjacent normal tissues. We analyzed the relationship between the expression of ETV1 and patient age, gender and tumor location (Table I). The data showed a strong correlation between ETV1 and KIT expression (P<0.05). Moreover, GISTs were prone to localize in the stomach (P<0.05). Table II summarizes the levels of ETV1 and KIT and their frequencies in the subtypes of the tumor samples. First, high frequencies of ETV1 and KIT were mainly found in the GIST tumors and were significantly higher in the GIST tumor samples with low risk or intermediate risk. ETV1-positive cases were identified

Table I. Clinical characteristics of the 156 patients.

Factor	No.	ETV1 (+)	ETV1 (-)	P-value
KIT (+)	133	88	45	<0.05
KIT (-)	23	20	3	
Age (years)	50.9±11.9	48.6±10.0	50.2±10.8	0.534
Gender				
Male	89	63	26	0.628
Female	67	45	22	
Location				
Stomach (gastric cardia)	65	37	28	<0.05
Small intestine	48	36	12	0.298
Colon	11	10	1	0.174
Rectum	17	12	5	0.898
Other location	15	13	2	0.151
Risk classification				
Low	107	72	35	0.438
Intermediate	31	22	9	0.815
High	18	14	4	0.404

Table II. Frequency of oncogenic ETV1 expression in comparison with KIT expression in the GIST tumor samples with different risk levels.

GIST	Average age	Case no.	Immunoreactive to ETV1					Immunoreactive to KIT				
			Ngt (0-2)	Wk/Md (3-4)	OEPT (5-6)	Expression rate (%)	Overexpression rate (%)	Ngt (0-2)	Wk/Md (3-4)	OEPT (5-6)	Expression rate (%)	Overexpression rate (%)
Low-risk	48.3±9.8	107	35 (0.3±0.4)	36 (3.7±0.5)	36 (5.7±0.5)	67.3% (72/107)	33.6% (36/107)	14 (0.8±0.5)	16 (3.6±0.5)	77 (5.6±0.5)	87.0% (93/107)	72.0% (77/107)
Intermediate risk	54.3±12.3	31	9 (0.2±0.4)	6 (3.3±0.5)	16 (5.3±0.4)	71.0% (22/31)	51.6% (16/31)	3 (0.7±0.6)	3 (3.3±0.6)	25 (5.4±0.5)	90.3% (28/31)	80.6% (25/31)
High-risk	48.0±9.2	18	4 (0)	5 (3.8±0.4)	9 (5.6±0.5)	77.8% (14/18)	50.0% (9/18)	6 (0.7±0.5)	5 (3.6±0.5)	7 (5.3±0.4)	66.7% (12/18)	38.9% (7/18)
Total	50.9±11.9	156	48 (0.2±0.4)	47 (3.7±0.5)	61 (5.5±0.5)	69.2% (108/156)	39.1% (61/156)	23 (0.7±0.5)	24 (3.5±0.5)	109 (5.7±0.5)	85.2% (133/156)	69.9% (109/156)

Average ETV1 and KIT expression scores are provided. Positive-staining was semi-quantitatively determined with a combined score 5-6 as overexpression. Expression rate (%) was calculated as: Total number of positive cases/total number of tested samples x 100%. Overexpression rate (%) was calculated as: Total number of overexpressed cases/total number of tested samples x 100%. Ngt, negative; Wk, weak; Md, moderate; OEPT, overexpression. GIST, gastrointestinal stromal tumor.

Table III. ETV1 is associated with KIT expression in GIST tumor samples with high-risk.

Levels of ETV1 expression	Immunoreactive to KIT		
	Negative n (%)	Positive n (%)	Total n (%)
Low-risk			
Negative	4 (3.7)	31 (29.0)	35 (32.7)
Weak/moderate	3 (2.8)	33 (30.8)	36 (33.6)
Overexpression	7 (6.5)	29 (27.1)	36 (33.6)
Total	14 (13.1)	93 (87.0)	107 (100)
Intermediate risk			
Negative	0 (0.0)	9 (29.0)	9 (29.0)
Weak/moderate	0 (0.0)	6 (19.4)	6 (19.4)
Overexpression	3 (9.7)	13 (41.9)	16 (51.6)
Total	3 (9.7)	28 (90.3)	31 (100)
High-risk			
Negative	0 (0.0)	4 (22.2)	4 (22.2)
Weak/moderate	2 (11.1)	3 (16.7)	5 (27.8)
Overexpression	4 (22.2)	5 (27.8)	9 (50.0)
Total	6 (21.4)	12 (83.3)[a]	18 (100)

Chi-square test and Fisher's exact test were used to determine the statistical significances among groups. [a]The percentage of KIT expression showed significant difference in the high-risk level in corresponding samples ($P<0.05$). GIST, gastrointestinal stromal tumor.

in 67.3% (72/107 cases) of the low-risk group and in 71.0% (22/31 cases) of the intermediate-risk group, respectively. The frequencies were still lower than that noted for KIT, which was identified in 87.0% (93/107) and 90.3% (28/31) of the cases in the corresponding groups. Second, in the GIST with high-risk, the frequency of ETV1 positivity was higher when compared to that for KIT expression. ETV1 positivity was identified in 50.0% (9/18) of the cases and only 38.9% (7/18) of the cases demonstrated KIT positivity. We then analyzed the relationship between ETV1 and KIT (Table III). Similarly, ETV1 was associated with KIT expression in the GISTs.

ETV1 was found to be expressed at a significantly higher level in GISTs than that in the normal tissues and acts as a unique transcription factor which is essential to the growth of GIST cells. However, to date, the mechanism of how it works is not clear. To identify which signaling pathway may be associated with ETV1 driving GIST oncogenesis, we detected the relative levels of phosphorylation of kinases and their protein substrates including Raf/MEK/extracellular signal-regulated kinase (ERK1/2), Bcl-2/Bax/caspase-3 and cadherin/β-catenin signaling cascades in the 72 paired tissue samples.

The Raf/MEK/ERK pathway often takes part in controlling cell survival, cell cycle progression and differentiation. Notably, the present study showed opposite results. The phosphorylation levels of ERK1/2 and MEK in the GISTs were significantly downregulated whereas total protein levels were unchanged. Phosphorylation of MEK and ERK was significantly higher in the normal group than that in the tumor

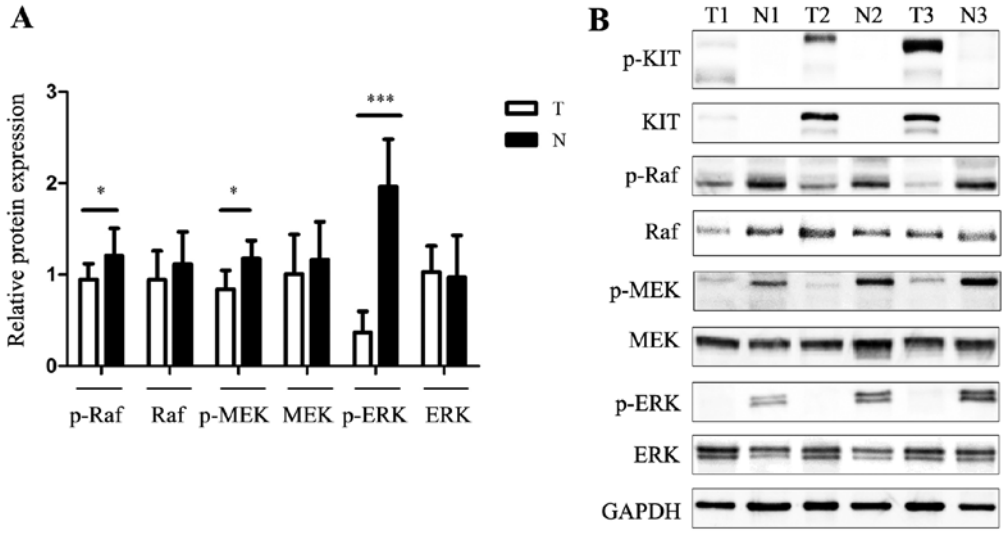

Figure 3. (A) Bar graph shows the significant difference in protein expression of Raf, MEK and ERK between the two groups in the 72 paired tissues (***P<0.01, *P<0.05). (B) Western blotting of three representative paired cases of GIST. The expression of phosphorylated isoforms of Raf, MEK, ERK was decreased in tumors compared with the normal tissues. The membranes were also reprobed for GAPDH, Raf, MEK and ERK as the loading control. T1-3 denote tumor tissue samples and N1-3 denote adjacent normal tissues.

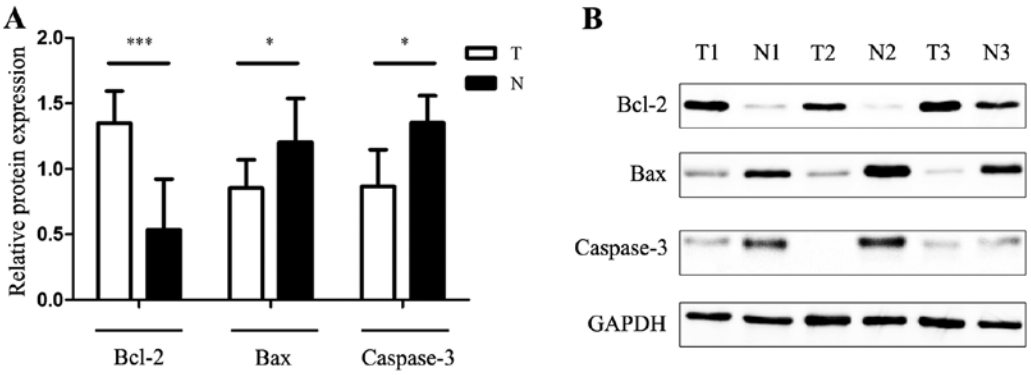

Figure 4. (A) Bar graph shows the significant difference in protein expression of Bcl-2, Bax and caspase-3 between the two groups in 72 paired tissues (***P<0.01, *P<0.05). (B) Western blotting of three representative paired cases of GIST. The expression of Bcl-2 was increased whereas the expression of Bax and caspase-3 was decreased in tumors when compared with the normal tissues. The membranes were also reprobed for GAPDH as the loading control. GIST, gastrointestinal stromal tumor. T1-3 denote tumor tissue samples and N1-3 denote adjacent normal tissues.

group (Fig. 3) We hypothesized that this phenomenon was due to innate tumor-suppressive responses, which were elicited as a protection against cancer.

As shown in Fig. 4, Bax/Bcl-2 and caspase-3 protein were detected in a matching approach. Prominent expression of Bcl-2 in the GIST tissues was investigated, and the expression of Bax and caspase-3 was much higher in the uninvolved normal group when compared with the tumor group. Accumulating evidence suggests that overexpression of anti-apoptotic protein Bcl-2 and underexpression of pro-apoptotic protein Bax prevented tumor cells from undergoing apoptosis in response to a variety of stimuli and promoted tumorigenesis.

A previous study revealed that convergence of Wnt, β-catenin and cadherin pathways may control cell-cell adhesion and determine the cell fate. Cadherin may also act as a negative regulator of β-catenin signaling as cadherin binds β-catenin forming a cadherin-catenin complex and thereby sequesters it from the nucleus (15). A recent study investigated the tight junction between E-cadherin and the prostate tumor suppressor SPDEF, the latest discovered transcription factor of the ETS family (16). In the present study, we investigated whether activated ETS family member ETV1 was correlated with the wnt-β-catenin-cadherin signaling pathway in the tumor progression of GISTs. In a similar experiment, we observed that rarely was E-cadherin expression found in the GIST group when compared with the non-tumor group. Nevertheless, significant high levels of β-catenin and Dvl2 were detected (Fig. 5). These results point to a potential role of the wnt-β-catenin-E-cadherin pathway in ETV1-regulated GIST tumorigenesis.

Discussion

GISTs are clinically and histologically heterogeneous neoplasms that are driven by oncogenic KIT or PDGFRA mutations (17). Our findings demonstrated that prominent ETV1 expression was noted in the majority of GISTs both at the mRNA and protein levels. IHC analysis confirmed

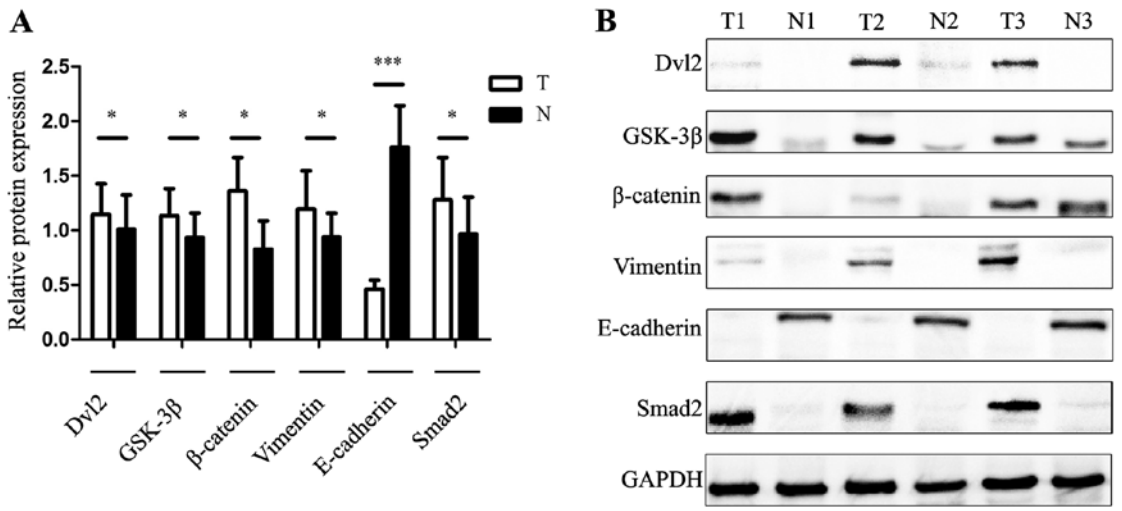

Figure 5. (A) Bar graph shows the significant difference between the protein expression of Dvl2, GSK-3β, β-catenin, vimentin, E-cadherin and Smad2 in the two groups in 72 paired tissues ($^{***}P<0.01$, $^{*}P<0.05$). (B) Western blotting of three representative paired cases of GIST. The expression of Dvl2, GSK-3β, β-catenin, vimentin, Smad2 was increased whereas the expression of E-cadherin was decreased in the tumors when compared with the normal tissues. The membranes were also reprobed for GAPDH as the loading control. T1-3 denote tumor tissue samples and N1-3 denote adjacent normal tissues.

that ETV1 was strongly expressed in the GISTs and revealed that ETV1 was correlated with KIT expression. Fig. 2 and Tables II and III show that the frequencies of ETV1 and KIT were high in the GISTs. ETV1-positive cases were identified in 67.3% (72/107) of the cases in the low-risk group and 71.0% (22/31) of the cases in the intermediate-risk group, respectively. The frequency of KIT was higher in 87.0% (93/107) of the low-risk cases and in 90.3% (28/31) of the cases in the intermediate-risk group, respectively. As to the GIST tumor samples with high-risk, ETV1 expression was significantly higher. ETV1 positivity was identified in 50.0% (9/18) of the cases and only 38.9% (7/18) of the cases showed KIT positivity. Taken together, ETV1 was strongly expressed in the GISTs which can aid in the diagnosis of GISTs particularly when KIT is negative in the high-risk group.

Novel prognostic markers have been successively found in GISTs, yet the action mode of relevant factors has received little attention. Thus, we aimed to explore how ETV1 integrates with cellular upstream or downstream signaling molecules in the tumorigenesis of GIST. In light of our findings from ChiP (12), we inferred that ETV1 can be stabilized through KIT-independent MAPK signaling. Furthermore, the ERK pathway induced by Ras/Raf mutations participates in cell proliferation, differentiation and cell migration in a variety of cancer types (18). However, in our cohort, lower expression of the phosphorylation of the ERK pathway was noted in tumor tissues than that in the tumor-adjacent normal tissues, since the total protein levels were not significantly altered. Immunohistochemical results reported by Schoppmann *et al* (19) demonstrated that only 29.1% of patients showed pMEK1/2 overexpression and 86.3% of patients showed upregulation of PEBP1, known as a Raf kinase inhibitory protein in GIST. Downregulation of p-ERK expression was also reported in colorectal cancer in a previous study (20). Aberrant Raf/MEK/ERK signaling can be involved as follows. First, ERK signaling is rarely detected, suggesting that the Raf/MEK/ERK pathway does not provide a growth advantage or significant inflammation reaction. It should be noted that ERK inactivation promotes a less differentiated phenotype (20). Our studies did not investigate the expression at different stages, thus we cannot exclude that possibility. However, normal tissues, which can be referred as premalignant lesions to some extent (21,22), sustained activation of the Raf/MEK/ERK pathway eliciting senescence-like growth arrest responses compared with the tumor tissues. This phenomenon can be interpreted as a physiologic fail-safe anti-tumorigenic mechanism (23). Furthermore, it is noteworthy that Hollenhorst *et al* (9) observed that ETS proteins activated a MEK/ERK-regulated gene expression program in the absence of ERK action. ETS/AP-1-binding sites in the PLAU enhancers can act as response elements for the Ras/MAPK signaling pathway. In RWPE-1 cells in the presence of the MEK inhibitor U0126 or lacking supplements, PLAU expression was elevated by oncogenic ETS proteins ETV1 and ERG. Thus, overexpression of oncogenic ETS proteins can replace Ras/MAPK pathway activation in prostate cells. ETV1 can activate targeted gene expression when the Ras/MAPK pathway is off and superactivates when the pathway is on. We hypothesized that ETV1 may serve the same function in GIST, promoting cell proliferation in the absence of the Ras/Raf/MEK/ERK pathway. Based on the findings, the activity of the system was dynamic so that we could not draw a definite conclusion or take this phenomenon as direct proof that the signaling was downregulated. Further study on the specific mechanism is warranted.

Cancer is both a consequence of uncontrolled proliferation, as well as disturbed differentiation (24,25). Thus, we detected Bcl-2/Bax/caspase-3 expression levels. Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria (26). Previous studies have found the involvement of Bcl-2 expression in GIST (27,28). In the pre-imatinib era, Bcl-2 expression was associated with worse prognosis. In contrast, Steinert *et al* (27) analyzed data from 81 GIST patients and demonstrated that higher levels of Bcl-2 were associated with improved PFS in patients who were subsequently treated with imatinib. In the present study, Bcl-2 expression was found in the majority of GISTs. The caspase family also plays a key

role in the initiation and execution of programmed cell death through both the intrinsic and extrinsic signaling pathways. Western blot analysis indicated that GIST tissues antagonized apoptosis by elevating anti-apoptotic Bcl-2 activity, whereas the normal tissues were prone to prominent apoptosis. Although its ability to promote proliferation and tumorigenesis, inflammatory factors such as Akt and ERK could not protect against ROS-mediated cell death but rather sensitized cells to this cell death (29). ROS are natural by-products of oxidative energy metabolism which play a critical role in determining the lifespan of mammalian cells and induce severe damage such as DNA lesions and protein oxidation. Bax is phosphorylated and translocated to the mitochondrial outer membrane. Subsequently release of cytochrome *c* from the mitochondrial inter-membrane space into the cytosol occurs. The mitochondrial caspase cascade is activated (30,31). Collectively, these data suggest that an anti-apoptotic effect is obvious in GIST.

Importantly, our results also indicated that the wnt-β-catenin-E-cadherin signaling pathway may be involved in GIST progression. ETS family member, SPDEF, is overexpressed in aggressive PC3 prostate cancer cells, leading to increased E-cadherin expression, an adhesion molecule with a crucial role in preventing metastatic spread (16). Loss of E-cadherin has been regarded as a central event in the switch to epithelial-mesenchymal transition and tumor metastasis (32). In the GIST group, we detected vimentin instead of E-cadherin and confirmed the component of the tumor arising from mesenchymal tissue. Loss of E-cadherin makes it difficult for β-catenin to combine with. In addition, our data suggest that Wnt/β-catenin also existed in the GISTs. Activation of Wnt/β-catenin signaling triggers Dvl2 activity and GSK-3β inhibition, thereby phosphorylation of β-catenin is reduced. On the basis of our research, we hypothesized that loss of E-cadherin and reduced phosphorylation of β-catenin resulted in accumulation of cytoplasmic β-catenin, which became available to bind the TCF/LEF family of transcription factors, induce target gene expression and promote GIST pathogenesis. ETV1 and SPDEF appear to be two mutual antagonistic transcription factors in the ETS family demonstrated in prostate cancer (9). It is plausible that a similar mechanism may also operate in GISTs and whether ETV1 interacts with Wnt/β-catenin/E-cadherin remains unclear.

In conclusion, our investigation revealed that ETV1 was upregulated at a higher rate in GISTs and was correlated with KIT expression, which is consistent with previous research. Limited by the patient number and follow-up time, we could not obtain the long-term outcome data of patients and analyze the prognostic value of ETV1 in GIST. Several studies have carried out relevant research, demonstrating that there is no correlation with clinical outcome (19,33,34). The prognostic significance is still controversial and warrants detailed study. Novel molecular pathways were found activated in GISTs, raising the possibility of an interaction between them in the tumorigenesis in GISTs. Nevertheless, more specific mechanisms still need to be explored.

Acknowledgements

This study was supported by the Scientific Research Foundation of the Ministry of Health of China (WKJ2011-2-002). We also thank the patients of the Department of Oncology and Gastrointestinal Surgery at The First Affiliated Hospital of Zhejiang University.

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