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ORIGINAL ARTICLE

Transforming growth factor β repressor, SnoN, is overexpressed in human gastrointestinal stromal tumors

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Summary

Purpose: *The transforming growth factor β (TGF-β)/Smad pathway is implicated in the development of interstitial cells of Cajal. The aim of this study was to examine the role of this pathway in human gastrointestinal stromal tumors (GISTs).*

Methods: *The expression of TGF-β receptor II (TβRII), phosphorylated Smad2 (p-Smad2), SnoN, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ was examined by immunohistochemistry in 30 human GISTs in relation to prognostic factors.*

Results: *TβRII was expressed in 76.9% of the cases. All cases were positive for p-Smad2 and SnoN, with significantly higher expression levels in small intestinal compared to gastric GISTs. Downregulation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ was found in 78.6% and 46.4% of the cases respectively, while cytoplasmic expression of $p27^{KIP1}$ was also noted in 50% of GISTs.*

Conclusions: *TGF-β/Smad pathway may contribute to GIST pathogenesis. SnoN overexpression and low levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ may be of importance in GISTs.*

Key words: GIST, $p27^{KIP1}$, $p21^{WAF1/CIP}$, Smad2, SnoN, TβRII

Introduction

GISTs are defined as specific, generally KIT (CD117)-positive and KIT or platelet derived growth factor receptor A (PDGFRA) mutation-driven mesenchymal tumors of the gastrointestinal tract, with a set of characteristic histological features including spindle, epithelioid, and rarely pleomorphic morphology [1]. They occur throughout the gastrointestinal tract from the lower esophagus to the anus, with the stomach followed by the small intestine being the most common sites involved [2]. GISTs are believed to originate from the interstitial cells of Cajal (ICCs) or their stem cell-like precursors [2-4].

TGF-β family of cytokines plays important roles in the regulation of mammalian cell growth, differentiation, and cancer [5]. Upon ligand binding, the type II receptor (TβRII) phosphorylates the type I receptor, which mediates downstream signaling through the family of Smad proteins [5]. The active TGF-β receptor complex phosphorylates Smad2 and Smad3 which then form heteromeric complexes with a common mediator, Smad4, and subsequently translocate into the nucleus where they activate or repress transcription of TGF-β target genes [5]. Smad complexes are subject to positive and negative regulation by several mechanisms and SnoN, a member of the Ski family of oncoproteins, was recently identified as a negative regulator of TGF-β signaling, via interacting with Smad proteins and repressing their transcriptional activity [6,7].

TGF-β signaling pathway exerts significant complex effects in carcinogenesis and has been considered as both a tumor suppressor pathway and promoter of tumor progression and invasion [8,9]. TGF-β is a potent inducer of growth inhibition in several cell types. Aberrations in components or downstream mediators of TGF-β signaling pathway such as TβRII, Smads and the cyclin-dependent kinase inhibitors (CDKIs) have been shown to contribute to the loss of TGF-β growth inhibitory function and are frequently observed in hu-

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man cancer [8,9]. However, TGF-β also functions as tumor promoter through its effects on tumor cell invasion and alterations in tumor microenvironment [8]. Further clarifying how specific alterations in TGF-β signaling pathway contribute to the development and progression of human cancer could provide novel opportunities for targeted anticancer therapies.

A fairly recent study showed that Smad3-null mice demonstrate a marked reduction, or even absence, of the ICCs in the colon together with a concomitant reduction of intestinal smooth muscle layer thickness, suggesting that the TGF-β/Smad signaling pathway is implicated in the development and differentiation of ICCs [10]. However, little is known about the effect of TGF-β signaling on GISTs. Taking the above into consideration, we investigated the expression of TβRII, phosphorylated Smad2 (p-Smad2) and SnoN in GISTs in relation to prognostic factors. We also evaluated the expression of the CDKIs, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, known downstream targets of the TGF-β /Smad signaling pathway [11].

Methods

Tissue samples

Paraffin-embedded tissue samples from 30 primary human GIST cases were retrieved from the archives of the Department of Pathology, General Hospital "Agios Andreas" Patras, Greece. Hematoxylin & eosin (H&E) sections were reviewed by 2 independent reviewers. The tumors' locations were the stomach (12 cases; 40%) small intestine (14 cases; 46.7%), rectum-anus (1 case; 3.3%), while some of them presented as mesenteric deposits/intraabdominal mass (3 cases; 10%). Tumors were sub-classified according to their mitotic count and size. Based on Fletcher's et al. consensus criteria [1], 8 cases were of low-risk, 8 of intermediate risk and 14 of high risk. Based on Miettinen's et al. criteria [2], separating the biological behavior of gastric and small intestinal GISTs, 2 (6.7%) cases were of very low malignant potential, 9 (30%) cases of low malignant potential, 6 (20%) cases of intermediate malignant potential and 13 (43.3%) of high malignant potential. All of the cases were strongly positive for KIT (CD117). None of the patients had received treatment with imatinib mesylate. All research was conducted according to the institutional ethical standards.

Immunohistochemistry

Immunohistochemistry for SnoN was performed using a rabbit polyclonal anti-SnoN antibody (1:80, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as previously described [10]. Immunostaining for TβRII (1:100, Santa Cruz Biotechnology), p-Smad2 (1:1000, Chemicon, Millipore, Billerica, MA, USA), $p21^{WAF1/CIP1}$ (1:25, DAKO, Glostrup, Denmark) and $p27^{KIP1}$ (1:180, Thermo Fisher Scientific, Fremont, CA, USA) was performed using an automatic staining system (DAKOautostainer, DAKO). Briefly, for deparaffinization, rehydration and antigen retrieval, representative 4 μm tissue sections were treated with DAKO target retrieval solution PH 9 in a DAKO pressure cooker (PT, DAKO) for 15 min at 90° C. Detection was performed using the Envision detection kit or the CSAII biotin-free tyramide amplification system (DAKO) according to the manufacturer's instructions. Diaminobenzidine (DAB) was used as the chromogen for visualization. Slides were counterstained with hematoxylin, subsequently dehydrated and mounted. As negative control, blocking solution was added instead of the primary antibody. Cases of colorectal carcinoma were used as positive control.

Immunohistochemical evaluation

All slides were assessed by two independent pathologists who were blinded to the case. Cytoplasmic and nuclear staining, where observed, were evaluated separately. Immunoreactivity for TβRII, p-Smad2 and SnoN was scored on a scale of 0-3, depending on the intensity of staining and the percentage of positive cells. Staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The percentage of positive cells was scored as 0 (<1%), 1 (1-25%), 2 (25-50%) and 3 (50-75%) and 4 (75-100%). The two scores were multiplied and the immunoreactivity score (values from 0-12) was classified as follows: score 0 as negative; score 1 (multiplication values 1,2) as weakly positive; score 2 (multiplication values 3,4,6) as moderately positive and score 3 (multiplication values 8,9,12) as strongly positive. Nuclear staining for $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ were evaluated based on the percentage of positive cells. According to previously published reports, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression was considered positive when more than 10% of tumor cells showed nuclear immunoreactivity [12,13]. Cytoplasmic expression of $p27^{KIP1}$ was considered positive when more than 10% of tumor cells showed cytoplasmic immunoreactivity

Statistical analysis

Statistical analysis was performed with the SPSS for Windows, release 12.0 (SPSS Inc, Chicago, IL, USA). The significance of differences among groups of clinicopathological parameters (tumor location, Fletcher and Miettinen criteria of malignant behavior, mitotic count, tumor size) was evaluated using the non-parametric Kruskal-Wallis or Mann-Whitney tests. Correlations between expression of proteins were investigated using the Spearman rank-order correlation coefficient. All ranking tests were performed with correction for ties. The significance level was defined as $p < 0.05$.

Results

TβRII is frequently expressed in GISTs

In adjacent non-tumoral areas, expression of TβRII was observed in gastrointestinal epithelial cells, endothelial cells, nerves and scattered stromal cells (Figure 1A, 1B). TβRII expression was positive in 20 out of 26 (76.9%) GISTs. The staining of tumor cells was cytoplasmic and granular, as previously reported in other types of tumors (Figure 1C). Weak immunoreactivity (score 1) was found in 6/26 of the cases (23.1%), moderate immunoreactivity (score 2) was observed in 6/26 (23.1%) of the cases studied, and strong staining (score 3) was found in 8/26 (30.8%) of the cases. There was no significant correlation of TβRII expression with

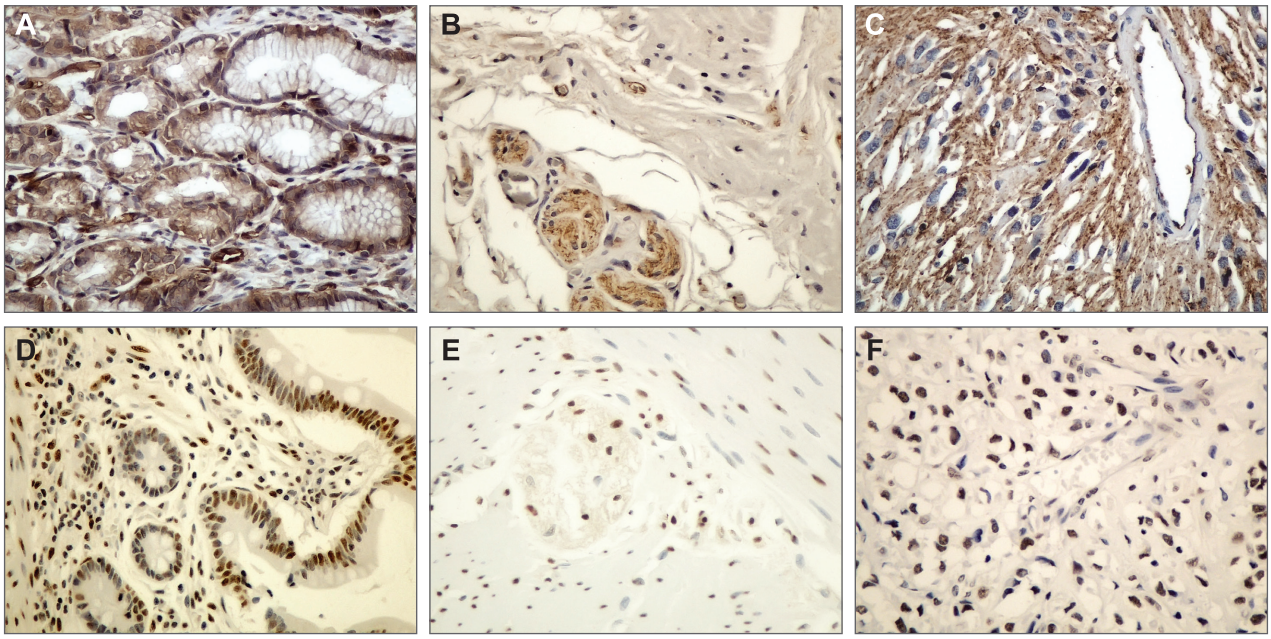

Figure 1. Expression of TβRII and p-Smad2 in human GISTs. **A:** Positive expression of TβRII in epithelial and endothelial cells of normal small intestinal mucosa. **B:** Section through adjacent non-tumoral small intestinal muscularis externa showing positive staining of TβRII in nerves and endothelial cells. **C:** Cytoplasmic granular immunostaining of TβRII in a representative case of human GIST. **D:** Positive expression of p-Smad2 in epithelial and stromal cells of normal small intestinal mucosa. **E:** Section through normal small intestinal muscularis externa showing positive staining of pSmad-2 in nerves and smooth muscle cells. **F:** Nuclear expression of p-Smad2 in case of a small intestinal GIST (×400).

tumor location, Fletcher or Miettinen grading system, tumor size and mitotic count.

Nuclear p-Smad2 is highly expressed in GISTs and correlates with tumor location

Nuclear p-Smad2 expression was positive in gastrointestinal epithelial cells, stromal cells, smooth muscle cells and nerves of adjacent non tumoral areas (Figure 1D, 1E). Expression of p-Smad2 was also positive in all of the cases of GISTs studied, while immunoreactivity was confined to the nucleus of tumor cells (Figure 1F). Expression of p-Smad2 was significantly higher in tumors located in the small intestine compared to gastric GISTs (p=0.04), while there was no correlation of p-Smad2 expression with other clinicopathological parameters (Table 1).

SnoN is overexpressed in GISTs and correlates with tumor location

Expression of SnoN in adjacent normal tissue was weak or absent (Figure 2A, 2B). In contrast, SnoN was positive in all of the tumors examined. Immunoreactivity for SnoN was cytoplasmic or cytoplasmic and nuclear (Figure 2C). All of the cases showed cytoplasmic immunoreactivity for SnoN, while 18 out of 28 cases (64.3%) demonstrated both cytoplasmic and nuclear

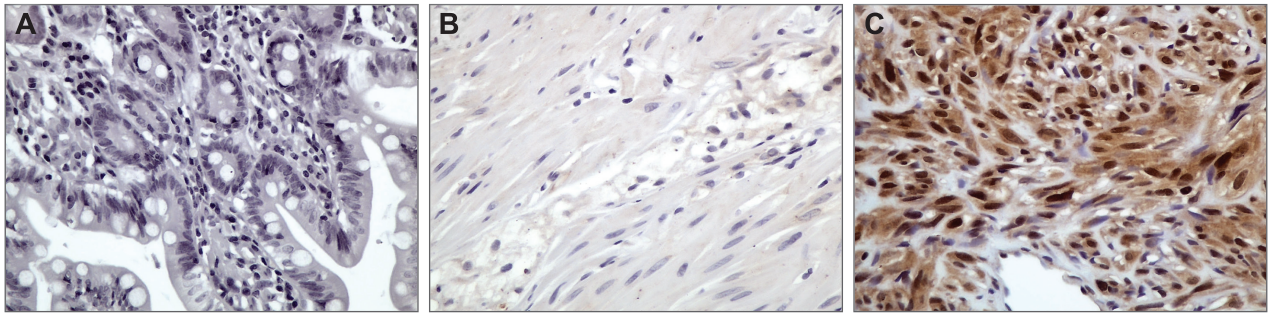

Figure 2. SnoN is overexpressed in human GISTs. **A:** Negative expression of SnoN in epithelial cells and lamina propria of small intestinal mucosa. **B:** Section through small intestinal muscularis externa showing negative staining of SnoN. **C:** Strong cytoplasmic and nuclear expression of SnoN in a case of a small intestinal GIST (×400).

Table 1. Expression of p-Smad2 and SnoN in GISTs. Correlation with clinicopathological parameters

	p-Smad2[a]					*Cytoplasmic SnoN*[a]					*Nuclear SnoN*[a]				
	0	*1*	*2*	*3*	*p-value*[c]	*0*	*1*	*2*	*3*	*p-value*[c]	*0*	*1*	*2*	*3*	*p-value*[c]
	n (%)	*n (%)*	*n (%)*	*n (%)*		*n (%)*	*n (%)*	*n (%)*	*n (%)*		*n (%)*	*n (%)*	*n (%)*	*n (%)*	
GISTs Total	0/30	1/30	14/30	15/30		0/28	4/28	9/28	15/28		10/28	8/28	4/28	6/28	
	(0)	(3.3)	(46.7)	(50)		(0)	(14.3)	(32.1)	(53.6)		(35.7)	(28.6)	(14.3)	(21.4)	
Tumor location					0.04					0.015					0.520
Stomach	0/12	1/12	8/12	3/12		0/11	2/11	5/11	4/11		5/11	4/11	0/11	2/11	
	(0)	(8.3)	(66.7)	(25)		(0)	(18.2)	(45.5)	(36.4)		(45.5)	(36.4)	(0)	(18.2)	
Small intestine	0/14	0/14	5/14	9/14		0/13	2/13	2/13	9/13		4/13	3/13	3/13	3/13	
	(0)	(0)	(37.5)	(64.3)		(0)	(15.4)	(15.4)	(69.2)		(30.8)	(23.1)	(23.1)	(23.1)	
Other	0/4	0/4	1/4	3/4		0/4	0/4	2/4	2/4		1/4	1/4	1/4	1/4	
	(0)	(0)	(25)	(75)		(0)	(0)	(50)	(50)		(25)	(25)	(25)	(25)	
Risk grade[b]					0.541					0.174					0.506
Low risk	0/8	0/8	4/8	4/8		0/7	2/7	2/7	3/7		3/7	3/7	1/7	0/7	
	(0)	(0)	(50)	(50)		(0)	(28.6)	(28.6)	(42.9)		(42.9)	(42.9)	(14.3)	(0)	
Intermediate risk	0/8	1/8	4/8	3/8		0/8	2/8	3/8	3/8		2/8	3/8	1/8	2/8	
	(0)	(12.5)	(50)	(37.5)		(0)	(25)	(37.5)	(37.5)		(25)	(37.5)	(12.5)	(25)	
High risk	0/14	0/14	6/14	8/14		0/13	0/13	4/13	9/13		5/13	2/13	2/13	4/13	
	(0)	(0)	(42.9)	(57.1)		(0)	(0)	(30.8)	(69.2)		(38.5)	(15.4)	(15.4)	(30.8)	
Size (cm)					0.255					0.126					0.240
2-5	0/8	0/8	5/8	3/8		0/8	2/8	3/8	3/8		3/8	4/8	1/8	0/8	
	(0)	(0)	(62.5)	(37.5)		(0)	(25)	(37.5)	(37.5)		(37.5)	(50)	(12.5)	(0)	
6-10	0/14	1/14	7/14	6/14		0/13	2/13	5/13	6/13		3/13	3/13	3/13	4/13	
	(0)	(7.1)	(50)	(42.9)		(0)	(15.4)	(38.5)	(46.2)		(23.1)	(23.1)	(23.1)	(30.8)	
>10	0/8	0/8	2/8	6/8		0/7	0/7	1/7	6/7		4/7	1/7	0/7	2/7	
	(0)	(0)	(25)	(75)		(0)	(0)	(14.3)	(85.7)		(57.1)	(14.3)	(0)	(28.6)	
Mitotic count					0.560					0.648					0.509
≤5/50HPF	0/19	1/19	9/19	9/19		0/18	4/18	5/18	9/18		7/18	6/18	2/18	3/18	
	(0)	(5.3)	(47.4)	(47.4)		(0)	(22.2)	(27.8)	(50)		(38.9)	(33.2)	(11.1)	(16.7)	
6-10/50HPF	0/4	0/4	1/4	3/4		0/3	0/3	1/3	2/3		1/3	1/3	1/3	0/3	
	(0)	(0)	(25)	(75)		(0)	(0)	(33.3)	(66.7)		(33.3)	(33.3)	(33.3)	(0)	
>10/50HPF	0/7	0/7	4/7	3/7		0/7	0/7	3/7	4/7		2/7	1/7	1/7	3/7	
	(0)	(0)	(57.1)	(42.9)		(0)	(0)	(42.9)	(57.1)		(28.6)	(14.3)	(14.3)	(42.9)	

[a]Expression was scored as described in methods. [b]Risk grade according to Fletcher et al. [1]. [c]Kruskal-Wallis test

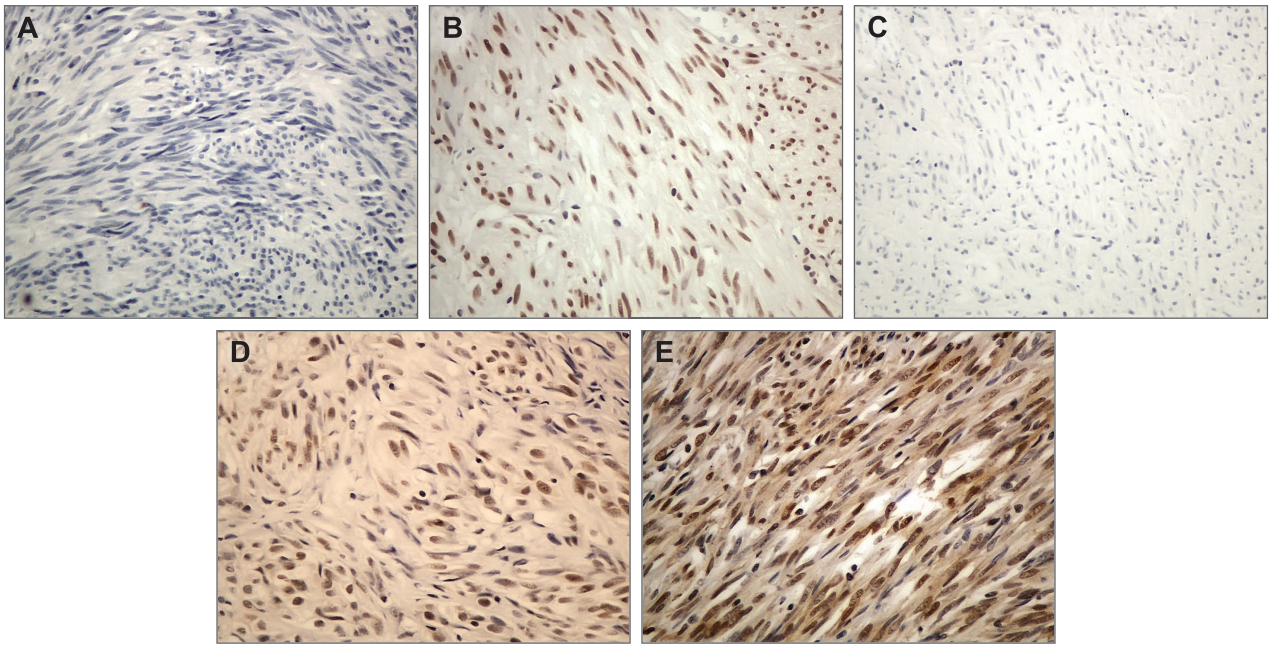

Figure 3. Representative cases of human GISTs with negative $p21^{WAF1/CIP1}$ **(A)** and $p27^{KIP1}$ **(C)** expression, positive nuclear expression of $p21^{WAF1/CIP1}$ **(B)** and $p27^{KIP1}$ **(D)** and positive cytoplasmic $p27^{KIP1}$ immunostaining **(E)** (×400).

immunoreactivity. Cytoplasmic expression of SnoN was significantly higher in tumors located in the small intestine compared to gastric GISTs (p=0.015). However, there was no correlation of SnoN expression with Fletcher or Miettinen risk grade, tumor size or mitotic count (Table 1). Moreover there was no correlation of SnoN expression with p-Smad2. Finally, there was a significant positive correlation between cytoplasmic SnoN and TβRII expression (r=0.427, p=0.037).

Expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in GISTs

$p21^{WAF1/CIP1}$ nuclear expression was negative in 22/28 cases (78.6%) (Figure 3A, B). The loss of $p21^{WAF1/CIP1}$ expression showed no correlation with any of the clinicopathological parameters examined. Nuclear expression of $p27^{KIP1}$ was negative in 13 out of 28 cases (46.4%) (Figure 3C, D). Furthermore, 14 out of 28 cases (50%) showed cytoplasmic expression of $p27^{KIP1}$ (Figure 3E). There was no correlation between nuclear or cytoplasmic $p27^{KIP1}$ expression and clinicopathological parameters. Nuclear $p27^{KIP1}$ expression significantly correlated with expression of TβRII (r=0.577, p=0.003).

Discussion

Alterations in TGF-β signal transduction pathway are common in human cancer, indicating an important role of the above pathway in carcinogenesis. In this study, we showed that TβRII and nuclear p-Smad2 are frequently expressed in GISTs, suggesting that the TGF-β/Smad signaling pathway is active in these tumors. In addition, we demonstrated that p-Smad2 expression was significantly higher in tumors located in the small intestine, which are well known to have a worse prognosis compared to gastric GISTs [2]. TGF-β has been previously shown to transform fibroblasts and to promote cell proliferation of rhabdomyosarcoma and osteosarcoma cell lines [14,15]. A significant, albeit complex, role of TGF-β in malignant progression has also been constantly demonstrated in several human cancers [5,8,9,16-18]. Furthermore, TGF-β/Smad signaling has been shown to affect the development and differentiation of ICCs, as Smad3 null mice showed a marked reduction or absence of ICCs in the colon [10]. Taking the above into consideration, it is strongly suggested that functional TGF-β/Smad signaling may contribute to GIST pathogenesis.

We also demonstrated increased expression of SnoN in GISTs. SnoN is an important negative regulator of TGF-β signalling, as it can interact with Smad proteins and repress their transcriptional activity, while overexpression of SnoN has been shown to inhibit TGF-β-induced growth arrest [6,7]. Consistent with its role as an oncoprotein and in agreement with our findings, overexpression of SnoN has been previously shown to induce oncogenic transformation of chicken and quail embryo fibroblasts [19], while upregulation of SnoN has been demonstrated in several human malignancies including colorectal cancer, breast cancer and mela-

noma [17,20-22]. Therefore, increased levels of SnoN may contribute to GIST tumorigenesis through loss of TGF-β induced growth arrest. Although a Smad2 dependent upregulation of SnoN transcription has been demonstrated in fibroblasts [23], we found no significant correlation of SnoN with nuclear p-Smad2 expression, suggesting that other mechanisms, such as gene amplification or increased protein stability may account for SnoN overexpression in GISTs [20,23].

Furthermore, in our study SnoN was localized in both the cytoplasm and nucleus of tumor cells, with a predominance of cytoplasmic localization, and it was only cytoplasmic SnoN expression that correlated with tumor location (small intestine GISTs, demonstrating increased aggressiveness). Consistently, distinct localization patterns (nuclear and cytoplasmic) in relation to different tumor characteristics have been reported for SnoN in the literature [17,21,24]. It is therefore a reasonable assumption that not only the protein levels, but also the intracellular localization of SnoN may be of importance in GIST pathogenesis.

In accordance with previous studies, we showed that the expression of cyclin-dependent kinase inhibitors, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, is frequently negative in GISTs [13,25]. Cip/Kip inhibitors are known to mediate TGF-β induced cell cycle arrest in a variety of cell types [9,11]. Significant evidence suggests that aberrations of the TGF-β signaling components or downstream effectors, including CDKIs, have been shown to confer resistance to the growth inhibitory effect of TGF-β and contribute to tumorigenesis [5-9]. Therefore, it seems likely that events initiated after the ligand dependent activation of Smads, such as low levels of nuclear $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ may result in GIST growth promotion.

Notably, we also demonstrated frequent cytoplasmic localization of $p27^{KIP1}$ in GISTs. Consistent with our findings, $p27^{KIP1}$ is often found either absent in the nucleus or re-localized in the cytoplasm in aggressive malignancies, while cytoplasmic $p27^{KIP1}$ expression is correlated with poor patient prognosis in several human tumors [26,27]. Tumor cell relocalization of $p27^{KIP1}$ into the cytoplasm has been shown to result not only in the loss of nuclear tumor suppressor function, but also in a gain of function in terms of promoting cell motility, invasion, and metastasis [28]. However, cytoplasmic $p27^{KIP1}$ in our study showed no correlation with risk grade, and further investigation is required to evaluate its prognostic significance in GISTs.

In conclusion, our results suggest a potential involvement of TGF-β/Smad signaling in human GIST pathogenesis, with the expression of p-Smad2 and SnoN being higher in small intestinal GISTs. TGF-β pathway alterations downstream to receptor mediated activation of p-Smad2, such as SnoN overexpression and low nuclear levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, may be of importance in human GISTs.

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