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PKCθ expression in gastrointestinal stromal tumor

Kyoung-Mee Kim[1], Dong Wook Kang[2], Woo Sung Moon[3], Jae Bok Park[4], Cheol Keun Park[1], Jin Hee Sohn[1], Jin Sook Jeong[5], Mee-Yon Cho[6], So-Young Jin[7], Jong Sang Choi[8] and Dae Young Kang[9], Gastrointestinal Stromal Tumor Committee, The Korean Gastrointestinal Pathology Study Group

[1]*Department of Pathology, Sungkyunkwan University, Seoul, Korea;* [2]*Department of Pathology, Eulji University, Daejeon, Korea;* [3]*Department of Pathology, Chonbuk National University, Jeonju, Korea;* [4]*Department of Pathology, Daegu Catholic University, Daegu, Korea;* [5]*Department of Pathology, Dong-A University, Busan, Korea;* [6]*Department of Pathology, Yonsei Wonju University, Wonju, Korea;* [7]*Department of Pathology, Soonchunhyang University, Seoul, Korea;* [8]*Department of Pathology, Korea University, Seoul, Korea and* [9]*Department of Pathology, Chungnam National University, Daejeon, Korea*

Gastrointestinal stromal tumor is characterized by a gain of function mutation of *KIT* gene and the expression of c-kit protein, but in 5% of cases, c-kit expression is negative although histological findings of gastrointestinal stromal tumor are most suspicious. The existence of c-kit-negative gastrointestinal stromal tumors points to the need of additional markers for making the diagnosis. In this study, we studied the expression of PKCθ and correlated their expression with other immunohistochemical profiles of gastrointestinal stromal tumors and evaluated their usability as a diagnostic marker. For this purpose, 220 gastrointestinal stromal tumors were immunohistochemically stained for PKCθ, c-kit, CD34, α-smooth muscle actin and S-100 protein. Additionally, genetic studies of *KIT* and *PDGFRA* genes were performed using c-kit-negative or PKCθ-negative cases. All the 220 masses were either PKCθ-positive or c-kit-positive. PKCθ was positive in 212 (96%) cases and c-kit was positive in 216 (98%) cases in the cytoplasm of tumor cells with a diffuse staining pattern. Out of 212 PKCθ-positive GISTs, 208 (98%) cases were c-kit-positive, 174 (82%) cases were CD34-positive, 62 (29%) cases were SMA-positive and S-100 protein was positive in 54 cases (26%). Genetic analyses on eight PKCθ-negative cases showed exon 11 mutations of *KIT* gene in four cases. Two PKCθ-positive and c-kit-negative GISTs showed mutations of *PDGFRA* gene. Our study shows that PKCθ is a useful marker and it may play a role in the development of gastrointestinal stromal tumors. Together with c-kit, PKCθ immunostaining can be used as an important diagnostic tool in the pathologic diagnosis of gastrointestinal stromal tumors with its high specificity and sensitivity.
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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal neoplasm of the digestive tract. *KIT* proto-oncogene, located on chromosome 4q11–21, encodes a type III receptor tyrosine kinase protein (c-kit) of the immunoglobulin supergene family. Somatic gain of function mutation of *KIT* in GISTs is associated with activation mutations mostly in the juxtamembrane domain at exon 11, followed by exons 9, 13 and 17, which encodes the extracellular domain and phosphotransferase domain. A minority of GISTs lack demonstrable *KIT* mutations, but c-kit is nonetheless activated.[1] *KIT* gene product, c-kit, is expressed in the interstitial cells of Cajal (ICC) and in 81–100% of GISTs regardless of the site of origin, histologic appearance and biologic behavior, and is therefore considered to be best defining feature of GISTs.[2–7] However, c-kit expression as a diagnostic standards have several limitations. First, c-kit expression is negative in approximately 5% of GISTs with typical histologic features. These c-kit-negative GISTs are common in the stomach and frequent in the epitheliod or mixed epitheliod/spindle cell type.[8] Second, the proportion of c-kit-positive cells in GIST is usually at least 90%, but may be as low as 5–20% in occasional

Correspondence: Dr DY Kang, MD, Department of Pathology, Chungnam National University, #6, Munwha-1-dong, Jung-gu, Daejeon, 301-131, Korea.
E-mail: dykang@cnu.ac.kr
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tumors.[2] This may account for the rare c-kit negativity in small biopsies of tumors with the morphology of a GIST. Third, c-kit is also expressed by a large number of other tumor types, such as leimyosarcoma, synovial sarcoma and Ewing sarcoma/primitive neuroectodermal tumor.[9,10] Fourth, c-kit positivity can be caused by technical artefacts such as improper dilution and immoderate antigen retrieval.[2]

Recently, a gene expression study on GISTs revealed constant overexpression of protein kinase C theta (PKCθ), a novel PKC isotype involved in T-cell activation,[11,12] in skeletal muscle signal transduction,[13] and in neuronal differentiation.[14] Although PKCθ expression in GISTs was confirmed in previous studies,[15–17] the number of GISTs used for these studies was small and the potential diagnostic relevance for paraffin-embedded sections was not completely evaluated. In this study, we studied the expression of PKCθ in 220 GISTs and correlated their expression with the other immunohistochemical profiles of GISTs and further evaluated their usability as an additional diagnostic marker.

Materials and methods

The cases analyzed in this study were 220 GISTs, 25 leiomyomas, 19 inflammatory fibroid polyps, 17 leiomyosarcomas, 15 schwannomas, 10 metastatic melanomas, four inflammatory myofibroblastic tumors, four desmoid-type fibromatosis, three poorly differentiated carcinomas and one metastatic endometrial stromal sarcoma of the gastrointestinal tract. All the cases consisted of surgical specimens obtained from Samsung Seoul Hospital, Chungnam National University Hospital, Korea University Hospital, Kangbuk Samsung Hospital, the affiliated hospitals of The Catholic University of Korea, Soonchunhyang University Hospital, Inje University Pusan Paik Hospital, Pusan National University Hospital, Dong-A University Hospital, Kosin University Hospital, Pusan Marinol General Hospital, Pusan Wallace Memorial Baptist Hospital, Kyungpook National University Hospital, Daegu Catholic University Hospital and Chonbuk National University Hospital from 2001 to 2005. Clinical information concerning the sites of the lesion and the sizes of the tumor were obtained from reviewing the medical records. The pathologic findings, including the histopathologic tumor types and mitotic counts per 50 high power fields were evaluated in each tumor after reviewing the H&E stained slides. The diagnostic criteria for the risk of aggressive behavior of GISTs were assessed by the criteria of the NIH GIST workshop.[2]

All the immunohistochemical stainings were performed using primary antibodies, including rabbit polyclonal anti-human c-kit (Dako), PKCθ (clone 27, BD Transduction Laboratories, 1:100), CD34 (clone Qbend10, Dako, 1:50), α-smooth muscle actin (SMA) (clone 1A4, Dako, 1:50) and S-100 protein (polyclonal, Dako, 1:1000) with DAKO EnVision™ + System according to the manufacturer's instructions and diaminobenzidine (DAB) was used as a chromogen. For PKCθ, antigen retrieval was performed using a pressure cooker and microwaves for 20 min. For the antigen retrieval of CD34, boiling the slides in citrate buffer for 10 min was performed with microwaves. For the staining of c-kit, a 1:800 dilution of the anti-c-kit antibody without antigen retrieval was selected with using a *KIT* mutation positive GIST as a positive control and normal smooth muscle was used as a negative control.

For the genetic analyses, 12 GISTs negative for either PKCθ or c-kit were analyzed for exons 9, 11, 13 and 17 of *KIT* gene and for exons 10, 14 and 18 of *PDGFRA* gene. Genomic DNAs from both the tumors and normal tissues were extracted from the paraffin-embedded tissue by standard proteinase K digestion and phenol/chloroform extraction as described previously.[18] DNA sequences of the primers and PCR conditions are described in Table 1. PCR products were electrophoresed through 8% polyacrylamide gel with ethidium bromide to confirm the correct amplification. The amplified PCR products were diluted 1:1 in loading buffer (94% formamide, 10 mg bromophenol blue and 0.05%

Table 1 The sequences of primer and PCR conditions for *KIT* and *PDGFRA* mutations

	Forward (5′–3′)	*Reverse (5′–3′)*	*Product size (bp)*	*Annealing temp (°C)*
KIT				
Exon 9	AGCCAGGGCTTTTGTTTTCT	GCCTAAACATCCCCTTAAATTGG	263	60
Exon 11	CTCTCCAGAGTGCTCTAATGAC	AGCCCCTGTTTCATACTGACC	219	58
Exon 13	CGGCCATGACTGTCGCTGTAA	CTCCAATGGTGCAGGCTCCAA	227	65
Exon 17	TCCTCCAACCTAATAGTGTATTCACAG	TTTGCAGGACTGTCAAGCAGAGAATG	174	65
PDGFRA				
Exon 10	GGCCCTATACTTAGGCCCTTT	TCAGCTGATGAGTTGTCCTGA	262	62
Exon 14	TGGTAGCTCAGCTGGACTGAT	GGGATGGAGAGTGGAGGATT	246	65
Exon 18	GCTACAGATGGCTTGATCCTGAGT	AGCCTGACCAGTGAGGGAAGT	200	60

xylene cyanol), and denatured by heating them at 98°C for 5 min. The products were next put on ice and then loaded onto a 5% SequaGel sequencing system (National Diagnostics, Atlanta, GA, USA). Electrophoresis was carried out for 90 min at a constant power of 1800 V with electrophoresis apparatus (BioRad, USA). After electrophoresis, the gels were stained with DNA silver staining kit (Bioneer, Korea). The samples showing either normal or abnormal migration bands on PCR-SSCP were purified, and both strands were directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit and analyzed with an Applied Biosystems 3700 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis was carried out using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA). The Pearson correlation test was used for analysis of the results. A *P*-value of less than 0.05 was considered statistically significant.

Results

Clinicopathologic and Immunohistochemical Staining Results

The ages of patients ranged from 18 to 84 years (mean age: 57.4 years). Among 220 patients, 108 were males and 112 were females. The clinical stages at presentation included tumor localized within an organ in 204 cases, tumor invasion into adjacent organs in 10 cases and distant tumor metastasis in six cases. GISTs occurred in the stomach in 130 cases (59%), the jejunum and ileum in 57 cases (26%), the duodenum in 21 cases (10%), the colorectum in nine cases (4%) and the esophagus in three cases (1%). The histologic types of GISTs included the spindle cell type in 166 cases (75%), the epitheliod cell type in 20 cases (9%) and the mixed type in 34 cases (16%). The pathologic risk of aggressive behavior was very low risk in 23 cases (11%), low risk in 66 cases (30%), intermediate risk in 49 cases (22%) and high risk in 82 cases (37%).

PKCθ was positive in the 212 cases (96%) having the typical histologic features of GISTs (Figure 1). According to the staining pattern, diffuse cytoplasmic staining was observed in 203 cases (cytoplasmic dot-like staining was found in one case), focal cytoplasmic staining was detected in two cases and weak staining was observed in seven cases. Relationship between the expression of PKCθ and the other immunohistochemical markers of GISTs are described in Table 2. Expression of PKCθ according to the risk of aggressive behavior included 91% for the very low risk, 100% for the low risk, 98% for the intermediate risk and 94% for the high risk. PKCθ expression was not related to the clinical stages, histologic types, degrees of cellularity and cellular atypia, the presence of necrosis, the presence of mucosal invasion, the locations of GISTs (Table 3) and the risks of aggressive behavior ($P > 0.05$) (Table 4). All PKCθ-negative GISTs were S-100 protein negative.

c-kit positivity was found in 216 cases (98%). Both PKCθ and c-kit were diffusely stained in the cytoplasm and along the membrane of the tumor cells. Of the 18 cases showing prominent perinuclear dot-like staining for c-kit, one case showed the same Golgi zone-like staining for PKCθ. Among the 20 epithelioid GISTs, one case was negative for PKCθ and one was negative for c-kit, respectively. For the mesenchymal tumors of the gastrointestinal tract other than GIST, all the leiomyomas, inflammatory fibroid polyps, leiomyosarcomas, melanomas, inflammatory myofibroblastic tumors, desmoid-type fibromatosis, poorly differentiated carcinomas and endometrial stromal sarcoma were completely negative for PKCθ while four (40%) melanomas involving the colon were positive for

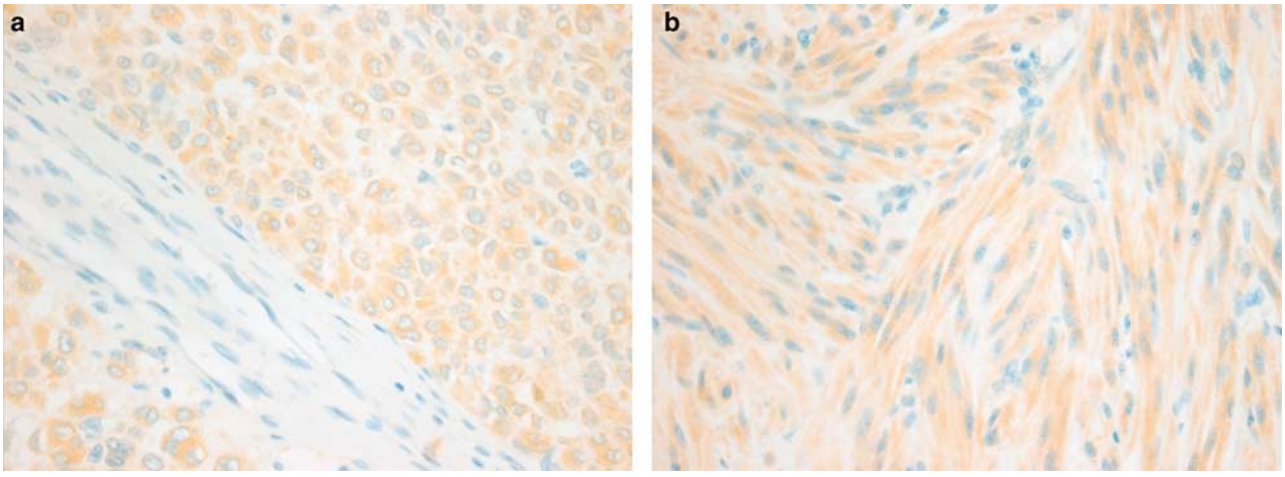

Figure 1 Immunohistochemistry of PKCθ in the gastrointestinal stromal tumor. Both epithelioid cell type (**a**) and spindle cell type (**b**) gastrointestinal stromal tumor showed diffuse cytoplasmic staining.

Table 2 Relationship between expression of PKC-θ and other immunohistochemical markers of GISTs

		CD117		*CD34*		*SMA*		*S-100*	
		Negative	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*
PKC-θ	(–)	0	8	2	6	5	3	8	0
	(+)	4	208	37	175	152	60	158	54
Total	220	4 (2%)	216 (98%)	39 (18%)	181 (82%)	157 (71%)	63 (29%)	166 (76%)	54 (24%)

Table 3 Expression of PKC-θ according to the locations of GISTs ($P = 0.85$)

		Stomach	*Jejunum and ileum*	*Duodenum*	*Colorectum*	*Esophagus*
PKC-θ	(–)	5 (3%)	1 (2%)	1 (5%)	1 (11%)	0 (0%)
	(+)	125 (97%)	56 (98%)	20 (95%)	8 (89%)	3 (100%)
Total (220)		130	57	21	9	3

Table 4 Expression of PKC-θ according to the risk of aggressive behavior of GISTs ($P = 0.12$)

		Risk of aggressive behavior				*Total*
		Very low	*Low*	*Intermediate*	*High*	
PKC-θ	(–)	2 (9%)	0 (0%)	1 (2%)	5 (6%)	8
	(+)	21 (91%)	66 (100%)	48 (98%)	77 (94%)	212
Total		23	66	49	82	220

c-kit (Figure 2). In four cases out of 15 schwannomas (27%), PKCθ was weakly positive in the palisading Verocay body, which is characteristic to identify these tumors as schwannoma (Figure 3).

Genetic Analysis

Among the 12 GISTs negative for PKCθ or c-kit, two c-kit-negative and PKCθ-positive gastric GISTs showed mutations of *PDGFRA* gene at exons 14 and 18. These gastric GISTs with *PDGFRA* mutations were SMA and S-100 negative and one of them was CD34-positive. In seven c-kit-positive and PKCθ-negative GISTs, *KIT* mutations were found in four out of five of the entirely sequenced cases. For one case, because of PCR failure, complete sequencing of PCR products was not available. All *KIT* mutations found in this study were located in exon 11 and they included WKKVV557-561C, 11 duplication 575–591 + intron, deletion WK557-558, and point mutation of L576P. The c-kit-positive and PKCθ-negative GISTs harboring *KIT* mutations were located in the stomach (three cases) and rectum (one case). The precise results of mutation tests for *KIT* and *PDGFRA* genes and their clinicopathologic features are described in Table 5.

Discussion

Numerous polyclonal and monoclonal anti-KIT antibodies have been produced and used in the pathology laboratories to diagnose GISTs. Yet the KIT expression in GISTs depends on the antibodies and the staining conditions, such as antigen retrieval, dilution and the detection methods. It has often been difficult to interpret the significance of weak or focal positive c-kit staining. Moreover, there remains a small problematic group of tumors with the morphological features of GISTs, but they are without a c-kit expression.[19] Some pathologists have designated these as GIST-like tumors. In these c-kit-negative GIST cases, the pathologic diagnosis is difficult without genetic analyses. On the contrary, c-kit is also expressed by a large number of other tumor types such as synovial sarcoma, malignant fibrous histiocytoma, leiomyosarcoma and endometrial stromal sarcoma.[2,9,10] To overcome these problems, we studied the expression of a newly developed protein, PKCθ, in 220 paraffin blocks of GISTs and we correlated their expression with the other immunohistochemical profiles and evaluated PKCθ's utility as a diagnostic marker.

PKC is a serine/threonine kinase involved in the control of cell proliferation, differentiation, and motility.[20] KIT is a 145-kDa transmembrane glycoprotein that serves as the receptor for stem cell factor (SCF) and has tyrosine kinase activity.[21] Binding of SCF to KIT results in receptor homodimerization, activation of tyrosine kinase activity and the resultant phosphorylation of a variety of substrates.[22] In many GISTs, the mutant KIT isoforms demonstrated constitutive kinase activity and their kinase domains were active even in the absence of SCF. PKC controls KIT/SCFR tyrosine kinase activity and modulates the cellular responses to SCF. Recently, it has been shown that PKCθ is also

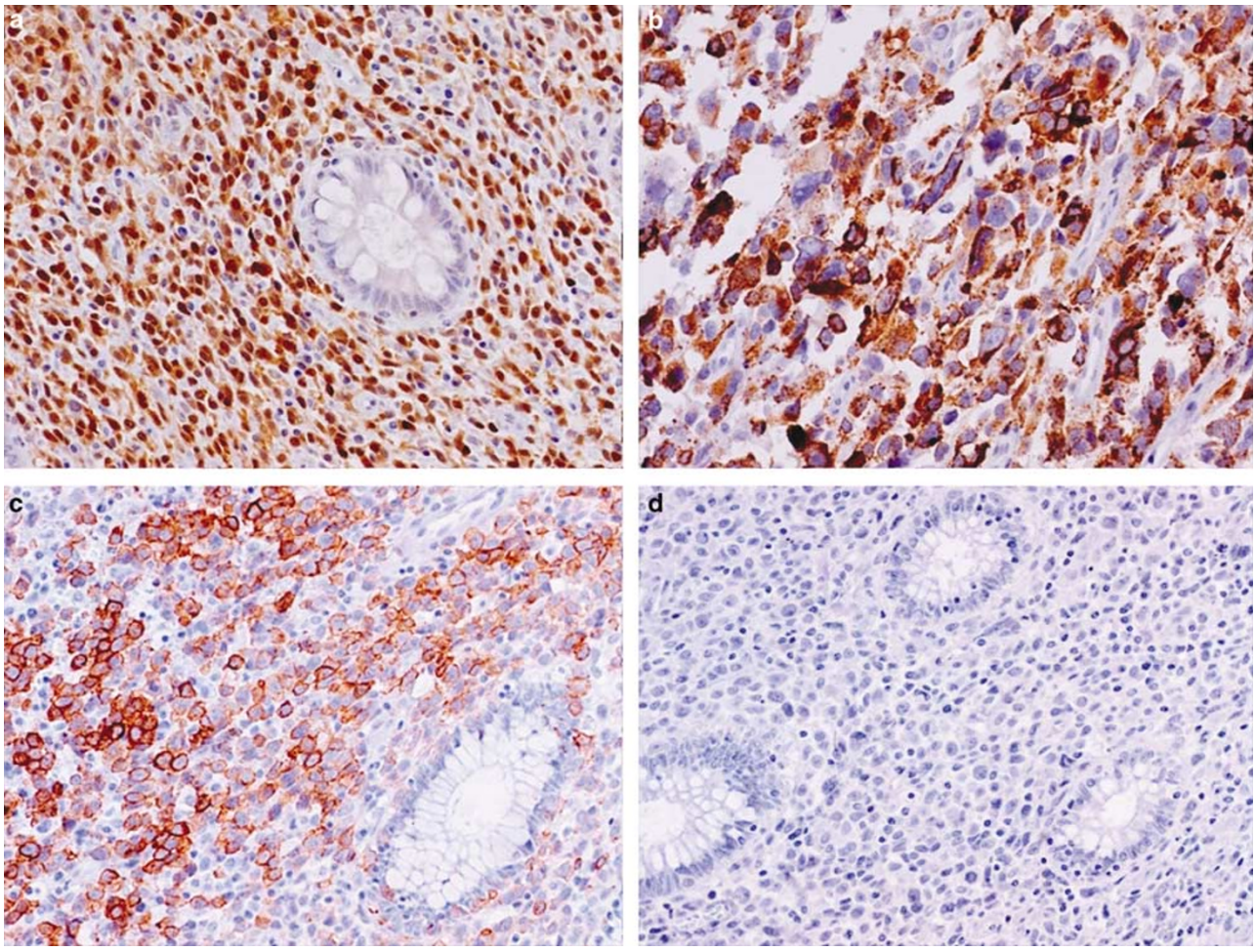

Figure 2 Immunohistochemistry of metastatic melanoma in the colon. The tumor cells were strongly positive for S-100 protein (**a**), HMB-45 (**b**) and c-kit (**c**), but negative for PKCθ (**d**). Note strong membranous and cytoplasmic staining of c-kit in the tumor cells.

constitutively activated in GISTs including those which are c-kit-negative, although immunoblotting PKCθ expression in these is 50% lower than in c-kit-positive GISTs.[15] Southwell *et al*[23] found localization of this protein in the ICCs of guinea-pig gastrointestinal tract. In the present study, we could detect weak and scattered positivity in the ICCs of human intermyenteric plexus and expression of PKCθ in GISTs was 96%. Although our expression frequency of PKCθ was slightly higher than that of a previous report,[17] this was related to a higher frequency of c-kit positivity and to our selection of GISTs with using strict criteria. These findings strongly support potential use of PKCθ inhibitors in the treatment of GISTs.

In this study, we could find that PKCθ was strongly expressed in GIST-like tumors showing typical histologic features of GISTs with negative expression of c-kit. The expression of PKCθ in various locations of GISTs and their relation to the risk of aggressive behavior has not been studied. PKCθ expression was not related to the locations and the pathologic risks of GISTs. Although PKCθ showed focal weak reactivity in schwannomas due to its role in neuronal differentiation, PKCθ expression was not present in other mesenchymal tumors of the gastrointestinal tract other than GISTs. Even in schwannomas, the expression of PKCθ was limited to the Verocay body and it also served as a definite clue for the diagnosis of this tumor. In this study, 40% of the melanomas showed strong cytoplasmic staining for c-kit protein while they were completely negative for PKCθ. PKCθ was strongly positive in two gastric c-kit-negative GISTs with *PDGFRA* mutations. With its high specificity and sensitivity, PKCθ immunostaining can be used as an important diagnostic tool for making the pathologic diagnosis of GISTs in the routine pathology laboratory, in addition to determining the c-kit protein expression.

For the immunohistochemistry of c-kit, most investigators have used heat retrieval with acid (citrate, pH 6.0) or alkaline buffer (EDTA, 372 mg/l, pH 8.0). We tried various conditions during setting up the staining conditions of c-kit. Epitope retrieval with citrate buffer at pH 6.0 and a dilution at 1:400 was first used. As we found a weak positive reaction in a negative control, various dilution rates ranging

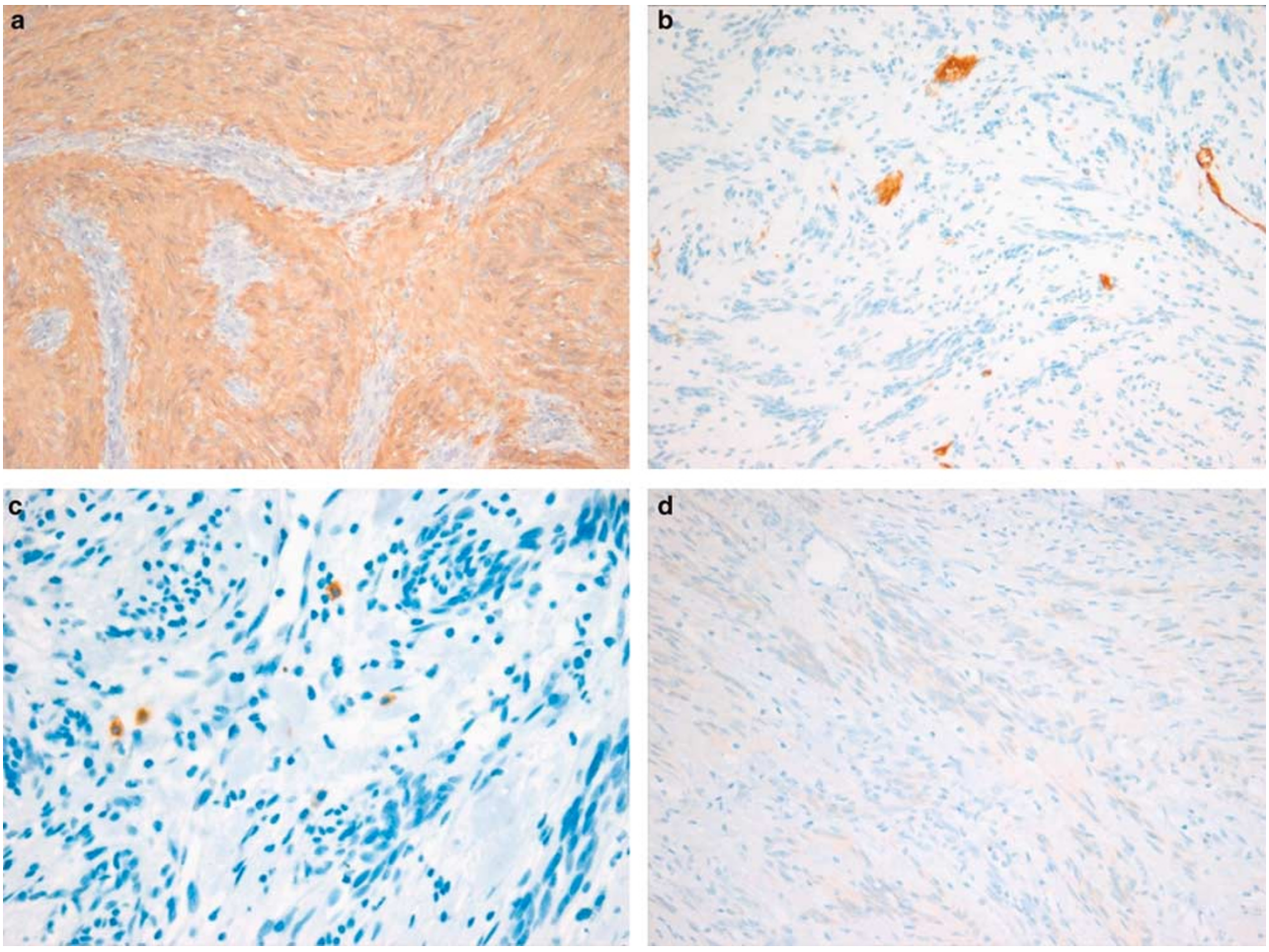

Figure 3 Immunohistochemistry of schwannoma. Strong expression of S-100 protein (**a**) and no expression of CD34 (**b**) and c-kit (**c**) were observed. For PKCθ (**d**), focal weak positivity was observed in the Verocay bodies.

Table 5 Results of mutation tests for *KIT* and *PDGFRA* genes and their clinicopathologic features

Sex/ages	*Mutation*	*PKC*θ	*c-kit*	*Location*	*Histologic type*	*Pathologic risk*
M/55	PCR fail	–	+	Duodenum	Spindle	High risk
M/52	KIT exon 11	–	+	Rectum	Spindle	Very low risk
M/52	PCR fail	–	+	Jejunum and ileum	Mixed	High risk
M/76	KIT exon 11	–	+	Stomach	Spindle	High risk
M/59	KIT exon 11	–	+	Stomach	Spindle	High risk
F/72	No mutation	–	+	Stomach	Epithelioid	Intermediate risk
M/79	KIT exon 11	–	+	Stomach	Spindle	Very low risk
M/40	PCR fail	–	+	Stomach	Spindle	High risk
M/62	PDGFRA exon 14	+	–	Stomach	Epithelioid	Low risk
M/22	No mutation	+	–	Rectum	Mixed	High risk
M/54	No mutaion	+	–	Stomach	Spindle	Low risk
M/55	PDGFRA exon 18	+	–	Stomach	Mixed	High risk

from 1:50 to 1:1600 without antigen retrieval was tried. Finally, a 1:800 dilution without antigen retrieval was selected.

In our immunohistochemistry results, we found very interesting results on the expression of CD34 in the small intestine. Unlike the relatively higher expression of CD34 in the stomach (93%), esophagus (100%) and colorectum (89%), the small intestinal GISTs showed a lower expression, that is, 57% for duodenum tumor and 47% for jejunum and ileum tumor. Miettinen *et al*[24] also found that among c-kit-positive GISTs, CD34 was positive in only 47% of the small intestinal GISTs. In our study, all but one CD34-positive GISTs were c-kit-positive and among 39 CD34-negative GISTs, 36 cases were c-kit-positive. These results are compatible with

previous reports that CD34-positive GISTs are almost always c-kit-positive and most CD34-negative GISTs are c-kit-positive.[25]

In conclusion, our results indicate that PKCθ is easily detected by immunohistochemistry in paraffin blocks of GISTs and it is a very sensitive and specific supplementary marker for the diagnosis of GISTs.

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