Protein Kinase C θ Is Highly Expressed in Gastrointestinal Stromal Tumors But Not in Other Mesenchymal Neoplasias

Pilar Blay,[1] Aurora Astudillo,[2] José M. Buesa,[1] Elías Campo,[4] Mar Abad,[5] Juan García-García,[2] Rosa Miquel,[4] Vicente Marco,[6] Marta Sierra,[1] Raquel Losa,[1] Angel Lacave,[1] Alejandro Braña,[3] Milagros Balbín,[7] and José M. P. Freije[7]

[1]Servicios de Oncología Médica, [2]Anatomía Patológica, and [3]Traumatología, Instituto Universitario de Oncología, Hospital Central de Asturias, Oviedo; [4]Laboratorio de Patología, Hospital Clinic, Institut d'Investigacions Biomediques August Pi i Sunyer, University of Barcelona, Barcelona; [5]Departamento de Biología Celular y Patología, Universidad de Salamanca, Salamanca; [6]Servicio de Anatomía Patológica, Hospital General de Catalunya, Barcelona; and [7]Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología, Universidad de Oviedo, Oviedo, Spain

ABSTRACT

Purpose: **Gastrointestinal stromal tumors (GIST) are a distinctive group of mesenchymal neoplasms of the gastrointestinal tract. The oncogene *KIT* has a central role in the pathogenesis of GIST, with c-kit receptor tyrosine kinase (KIT) protein expression being the gold standard in its diagnosis. The identification of GIST patients has become crucial, because the tyrosine kinase inhibitor Imatinib is effective in the treatment of this malignancy. However, a small set of GISTs remain unrecognized, because KIT protein expression is not always evident. The aim of this study was the identification of new markers for the differential diagnosis of GIST.**

Experimental Design: **By analyzing publicly available data from transcriptional profiling of sarcomas, we found that protein kinase C θ (PKC-θ), a novel PKC isotype involved in T-cell activation, is highly and specifically expressed in GIST. PKC-θ expression in GIST was confirmed by reverse transcription-PCR and Western blot. PKC-θ was analyzed by immunohistochemistry in a panel of 26 GIST, 12 non-GIST soft-tissue sarcomas, and 35 tumors from other histologies.**

Results: **We found that all of the GISTs expressed PKC-θ, whereas this protein was undetectable in other mesenchymal or epithelial tumors, including non-GIST KIT-positive tumors. PKC-θ immunoreactivity was also observed in interstitial cells of Cajal.**

Conclusions: **Our results show that PKC-θ is easily detected by immunohistochemistry in GIST specimens and that it could be a sensitive and specific marker for the diagnosis of this malignancy.**

INTRODUCTION

The term gastrointestinal stromal tumor (GIST) refers to a newly defined entity that is composed of a heterogeneous group of tumors of mesenchymal origin, most of them arising in the gastrointestinal tract. GISTs were designated previously as leiomyoblastoma, plexosarcoma, gastrointestinal autonomic tumor, or gastrointestinal pacemaker cell tumor, depending on its appearance. A general characteristic of these tumors is their resistance to treatment with conventional chemotherapy. GISTs are currently considered to be originated from interstitial cells of Cajal (ICC) or from a common undifferentiated cell and are immunohistochemically defined as c-kit receptor tyrosine kinase (KIT)-positive tumors. Thus, KIT positivity serves to differentiate GISTs from other gastrointestinal spindle cell tumors (1, 2).

A new drug, the protein kinase inhibitor Imatinib (STI571, Glivec), has been shown to be active against GISTs and is used currently in the clinic (3). This drug selectively inhibits the tyrosine signaling of a group of closely related tyrosine kinase receptors, including KIT, platelet-derived growth factor receptor (PDGFR), and ABL (4). Most GISTs carry specific *KIT* mutations leading to ligand-independent activation of the KIT tyrosine kinase activity, and it is now accepted that these mutations play a causal role in the development of this malignancy (5). However, there are GISTs in which KIT protein expression is not detectable, despite having activating KIT mutations (6). Moreover, alternative mutations in the tyrosine kinase receptor PDGFRα have been described recently (7). Thus, the existence of KIT-negative GISTs highlights the need of additional markers for the diagnosis of this neoplasia.

Gene expression studies using DNA microarrays have revealed that GISTs show a distinct and uniform gene expression profile, which allows distinguishing of these tumors from other malignancies of mesenchymal origin (8, 9). Among the transcripts identified as discriminatory in these studies, the gene encoding protein kinase C θ (PKC-θ) was overexpressed in GISTs samples. PKC-θ is a novel isotype of PKC involved in T-cell activation (10, 11), in skeletal muscle signal transduction (12), and in neuronal differentiation (13). This protein kinase has raised interest as a potential drug target due to its role in leukemia T-cell proliferation and survival (10, 11). Here we report that PKC-θ is expressed at the RNA and protein level in GISTs but not in other malignancies of epithelial or mesenchymal origin, including KIT-positive tumors. PKC-θ was easily

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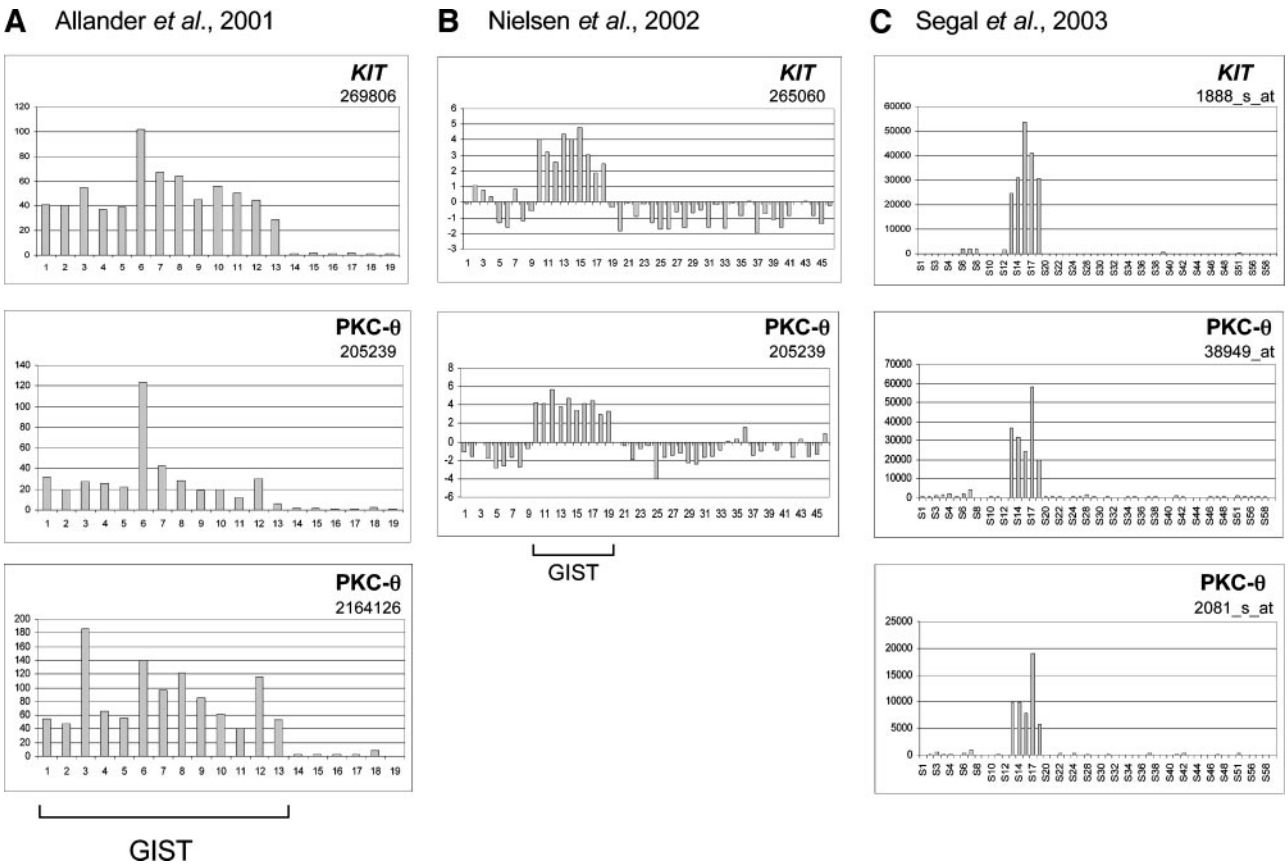

Fig. 1 Microarray data on *KIT* and protein kinase C θ (*PKC*θ) expression levels in human sarcomas. Arbitrary expression units (*Y* axis) for *KIT* and PKC-θ are shown for different sarcoma samples (*X* axis). *A*, cDNA microarray data from Allander *et al.* (8);[8] the panel labeled as *KIT* corresponds to IMAGE clone 269806, whereas the two panels labeled as PKC-θ correspond to IMAGE clones 205239 and 2164126. *B*, cDNA microarray data from Nielsen *et al.* (9).[9] The panel labeled as *KIT* corresponds to the expression data for IMAGE clone 265060 and the panel labeled as PKC-θ to IMAGE clone 205239. *C*, expression analysis with Affymetrix GeneChips, published by Segal *et al.* (14).[10] The panel labeled as *KIT* represents the signals for the probe 1888_s_at, whereas the panels labeled as PKC-θ represent the signals for probes 38949_at and 2081_s_at. All of the samples identified as gastrointestinal stromal tumor (*GIST*) in *A* and *B* express PKC-θ, but 1 GIST samples in B (*sample 19*) does not express *KIT*. All of the samples in *C* that express *KIT* also express PKC-θ.

detected by immunohistochemistry in all of the samples from a panel of GISTs, suggesting its potential as a molecular marker for the diagnosis of this tumor type. Immunoreactivity was also observed in ICCs, supporting the common lineage of these cell types.

MATERIALS AND METHODS

Microarray Data. Microarray data on gene expression profiles of human sarcomas have been reported previously and are publicly available (8, 9, 14).[8,9,10]

The relative hybridization values from the cDNA microarrays or the signal intensities from the oligonucleotide chip were imported in Microsoft Excel spreadsheets to compose the graphs shown in Fig. 1.

Tumor Samples. Formalin-fixed, paraffin-embedded tissue blocks and freshly frozen tumor samples were collected from the Hospital Central de Asturias, the Hospital Clínic de Barcelona, and the Hospital Universitario de Salamanca. Tumors included 26 GIST from different locations, 12 non-GIST soft-tissue sarcomas, and 35 benign and malignant tumors from different histologies (Table 1). GISTs were histologically classified as predominantly spindled, epithelioid, or mixed, as well as benign-appearing, borderline, or malignant based on cellularity, pleomorphism, mitotic activity, necrosis, and growth pattern.

Reverse Transcription-PCR. Total RNA from tumor samples and cell lines was isolated as described (15). One μg each of total RNA was reverse-transcribed using the Termoscript Reverse Transcription-PCR kit (Life Technologies, Inc., Rockville, MD). PCR primers for PKC-θ were 5′-TGGACAATCCCTTTTACCCACG (sense) and 5′-GTCTCTG-

[8] Internet address: http://research.nhgri.nih.gov/microarray/gist_data.txt.
[9] Internet address: http://genome-www.stanford.edu/sarcoma/data/74252filtered(SAM_GIST)final.xls/.
[10] Internet address: http://www.mskcc.org/genomic/ccsmsp.

Table 1 Histological and immunohistochemical features of the tumors included in this study

Tumor	KIT[a]	PKC-θ
GIST (n = 26)		
Esophagus	+	+
Stomach (n = 14)	+	+
Small intestine (n = 7)	+	+
Peritoneum	+	+
Retroperitoneum	+	+
Peritoneum metastasis	+	+
Liver metastasis	+	+
Soft tissue sarcomas (n = 12)		
Leiomyosarcoma (n = 4)	−	−
Leio-rhabdomyosarcoma	−	−
Endometrial stromal sarcoma	−	−
Alveolar rhabdomyosarcoma	−	−
Rhabdoid tumor	−	−
Dermatofibrosarcoma protuberans	−	−
Malignant fibrous histiocytoma	−	−
Malignant schwannoma	−	−
Angiosarcoma	−	−
Other tumors (n = 35)		
Adenocarcinoma[b] (n = 10)	−	−
Squamous cell carcinoma[c] (n = 7)	−	−
Oat cell carcinoma (n = 2)	+	−
Melanoma (n = 5)	+	−
Seminoma (n = 4)	+	−
Ewing sarcoma	+	−
Leiomyoma (n = 3)	−	−
Neurofibroma	−	−
Schwanoma	−	−
Fibroma	−	−

[a] KIT, c-kit receptor tyrosine kinase; PKC-θ, protein kinase C θ; GIST, gastrointestinal stromal tumor.
[b] Four colon, 3 stomach, 1 pancreas, 2 lung.
[c] Four lung, 2 larynx, 1 vulva.

GAGGGGCAAGATTCA (antisense). A 363-bp PCR product was then amplified according to the following conditions: 94°C for 5 min; 30 cycles of amplification (94°C for 30 s, 62°C for 30 s, and 72°C for 60 s); and 7 min at 72°C. For amplification of the housekeeping gene β-*2 microglobulin*, the primers used were 5′-CCAGCAGAGAATGGAAAGTC (sense) and 5′-GATGCTGCTTACATGTCTCG (antisense) that amplify a 267-bp fragment. Amplification reaction was performed at 95°C for 5 min followed by a total of 40 cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 60 s) and a final extension at 72°C for 5 min. The PCR reaction products were resolved by electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Western Blot Analysis. A small piece of frozen GIST (~5 mg) and 10^6 cell of the cell line K562 were lysed in 500 μl and 200 μl, respectively, of 50 mM EDTA containing 2 mM sodium orthovanadate and protease inhibitors. Aliquots (45 μl) of each sample were mixed with 15 μl of 4× SDS-PAGE loading buffer, sonicated, and boiled before loading the gel. Tissue and cell extracts were electrophoresed on 12% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose filters. The filters were blocked for 1 h with 5% skim milk in PBS (pH 7.4) and 0.1% Tween 20 and then incubated for 1 h with a 1:200 dilution of PKC-θ monoclonal antibody (BD Transduction Laboratories, San Diego, CA) in PBS (pH 7.4) and 0.1% Tween 20 containing 1% BSA plus 5% fetal bovine serum. The filters were washed three times for 5 min each with PBS (pH 7.4) and 0.1% Tween 20 and then incubated for 1 h with a horseradish peroxidase-conjugated goat antimouse antibody at a 1:20,000 dilution (Pierce Biotechnology, Rockford, IL). The secondary antibody was detected using the PicoSignal chemiluminescent reagent from Pierce Biotechnology.

Immunohistochemical Analysis. Immunohistochemical staining was performed according to standard procedures. Sections (4-μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized by treatment with xylene, rehydrated by sequential immersion in graded alcohol, and autoclaved in citrate buffer for epitope retrieval. After endogen peroxidase blocking, the slides were incubated with the primary antibodies anti-PKC-θ or anti-KIT (DAKO Laboratories, Carpinteria, CA) for 1 h and developed with the DAKO EnVision+ System following the manufacturer's instructions. The optimal dilutions of the antibodies were 1:200 for anti-KIT and 1:50 for anti-PKC-θ. The slides were counterstained with hematoxylin, thoroughly rinsed in distilled water, then immersed in 37 mmol/liter of ammonia water, dehydrated, and mounted with permanent mounting medium. Tumors were scored as positive for KIT or PKC-θ if there was either a diffuse staining or a focal expression in several clusters of cells. Cases with a minimal expression of KIT or PKC-θ in a few single cells were scored as negative.

RESULTS

In an attempt to gain a better understanding of the molecular biology of GISTs and to identify new markers for the differential diagnosis of this malignancy, we reviewed the publicly available data from two different transcriptional studies of sarcomas performed with cDNA microarrays (8, 9). This approach revealed that *PRKCQ*, the gene encoding the serine-threonine protein kinase PKC-θ, was consistently overexpressed in GISTs (Fig. 1, *A* and *B*). The first of these studies included 13 GISTs with *KIT* mutations and 6 other tumors (1 sarcomatoid mesothelioma, 4 spindle cell sarcomas, and 1 fibromatosis). As can be seen in Fig. 1*A*, all of the GISTs expressed high levels of *KIT* and PKC-θ mRNA, which were absent in the non-GIST tumors. The second study provided transcriptional data from 8 GISTs and 33 non-GIST samples (8 monophasic synovial sarcomas, 4 liposarcomas, 11 leiomyosarcomas, 8 malignant fibrous histiocytomas, and 2 schwannomas). It is interesting to note that 1 of the GISTs in this study does not shown KIT mRNA expression (Fig. 1*B, sample 19*), whereas high levels of PKC-θ mRNA are observed in all of the cases. To confirm these observations, we also reviewed the gene expression data from a recent study of sarcomas using Affymetrix high-density oligonucleotide microarrays (14). As shown in Fig. 1*C*, high levels of PKC-θ mRNA were detected in 5 sarcoma samples, whereas none of the remaining specimens analyzed in this study expressed significant levels of this molecule. Although the online data from this study do not include information on sarcoma type, the fact that the PKC-θ-positive samples also overexpressed KIT indicates that these 5 sarcomas were probably GISTs (Fig. 1*C*). Remarkably, these studies were performed using three different gene expression profiling platforms, which reinforces

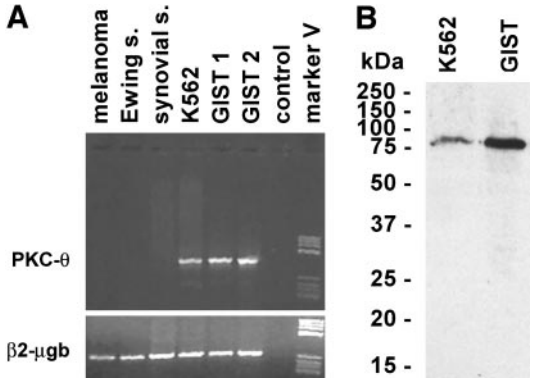

Fig. 2 Expression analysis of protein kinase C θ (*PKC*θ) in frozen samples. *A*, reverse transcription-PCR analysis. Total RNA was isolated from the indicated samples, reverse-transcribed using random hexamers as primers, and the product used for PCR with PKC-θ-specific primers. In parallel, a fragment of β2-microglobulin was amplified from the same reverse-transcription products, as control. A PKC-θ cDNA fragment of the expected size was obtained in the gastrointestinal stromal tumor (*GIST*) samples as well as in the K562 leukemia cells used as control, but not in other tumors. *B*, Western blot analysis of PKC-θ. Protein extracts were prepared from the indicated samples, separated by SDS-PAGE, blotted onto nitrocellulose and incubated with an anti-PKC-θ mouse monoclonal antibody. Immunoreactive bands were detected by enzymatic chemiluminescence.

the conclusion that PKC-θ is highly expressed in GIST and not in other soft tissue tumors.

To analyze PKC-θ expression at the transcriptional level, we performed reverse transcription-PCR with total RNA from GIST samples and from a set of other different tumors (Ewing sarcoma, synovial sarcoma, and melanoma). As shown in Fig. 2*A*, PKC-θ mRNA was expressed in the GIST samples, as well as in the K562 human leukemia cell line used as positive control. In contrast, PKC-θ mRNA was not detected by this method in any of the other analyzed tumors, confirming the differential expression of this protein kinase in GIST at the RNA level.

Then we investigated whether PKC-θ was also expressed at the protein level. For this purpose, we prepared protein extracts from fresh-frozen tissue samples, and we analyzed the presence of this protein by Western blot using a mouse monoclonal antibody. As shown in Fig. 2*B*, the antibody recognizes a single protein band in extracts from GIST and K562 leukemia cells used as positive control. This band corresponds to a molecular mass, which is in good agreement with the expected size of human PKC-θ (81.9 kDa). This result confirms the expression of PKC-θ in this neoplasm at the protein level and demonstrates the specificity of the used monoclonal antibody, which did not cross-react with other human proteins present in the analyzed extracts.

To identify PKC-θ-expressing cells in a larger number of samples, we next explored the possibility of analyzing the expression of this protein in paraffin-embedded tissues by immunohistochemistry. This type of analysis was performed on GISTs ($n = 26$), on a variety of soft tissue sarcomas ($n = 12$), and on other tumors from different histologies ($n = 35$; Table 1). Among GISTs, 9 tumors were benign, 13 were malignant with different grade of differentiation, and 4 were borderline. The site of origin was predominantly the gastrointestinal track, except in 2 cases that were retroperitoneal and peritoneal. Two tumor samples correspond to metastasis, 1 in the liver and 1 in the peritoneum. The tumor size was <5 cm in 12 patients and ≥ 5 cm in 5 patients (not determined in 9 patients). Immunostaining with anti-PKC-θ was clearly and reproducibly positive in tumor cells from all of the GISTs, whereas no signal was observed in the accompanying normal tissue (Fig. 3). Immunostaining was cytoplasmic and diffusely granular, with occasional paranuclear dots. Different grade of intensity was observed among the samples, but we did not find a correlation with any of the parameters described above (site of origin, tumor size, grade of malignancy, or histological appearance).

To determine the specificity of PKC-θ as a marker for GISTs, a set of other types of mesenchymal tumors as well as other malignant and benign tumors from other histologies were analyzed (tumor types are summarized in Table 1). Immunoreactivity was not observed in any of the different types of soft tissue tumors nor in tumors from other histologies (Fig. 3 shows a leiomyosarcoma as an example). We have also included in our series non-GIST tumors that express KIT, such as 2 oat cell lung carcinomas, 1 Ewing sarcoma, 5 melanomas, and 4 seminomas. These tumor samples show strong signal for KIT, whereas immunostaining for PKC-θ was completely negative (Fig. 3 shows a Ewing sarcoma as en example). Taken together, these results indicate that PKC-θ can be used as a sensitive and specific molecular marker for the differential diagnosis of GIST.

In tissue sections containing Auerbach's plexus we observed PKC-θ-positive cells located among and around the nervous ganglions of this plexus. These cells were assumed to be ICCs based on their morphology and location and on the fact that similar cells on parallel sections were KIT positive (Fig. 3). These results are in agreement with recent reports (16–18) describing the expression of PKC-θ by guinea pig ICCs. Other PKC-θ-immunoreactive scattered cells could also be ICCs, although we cannot exclude the possibility that some of them are mast cells, in which the expression of this protein kinase has also been reported (19).

DISCUSSION

The remarkable antitumor activity of the molecular inhibitor Imatinib (Glivec) in metastatic GIST has required accurate diagnosis of this neoplasia and its distinction from other gastrointestinal tumors. Expression of KIT is seen in almost all of the GISTs regardless of the site of origin, histological appearance, and biological behavior and is, therefore, considered the best defining feature of this entity. However, there is a small proportion of tumors with morphological features of GIST that are essentially KIT negative, having low to undetectable KIT protein expression by immunohistochemistry and Western blotting evaluation. Although some of these cases can be explained by sampling error or clonal evolution, there is a group of patients where an alternative (non-KIT) mechanism can be responsible for malignancy (2). Heinrich *et al.* (7) have shown that a proportion of GISTs lacking KIT mutations have activating mutations in the related receptor tyrosine kinase PDGFRα and that KIT and PDGFRα mutations appear to be alternative and

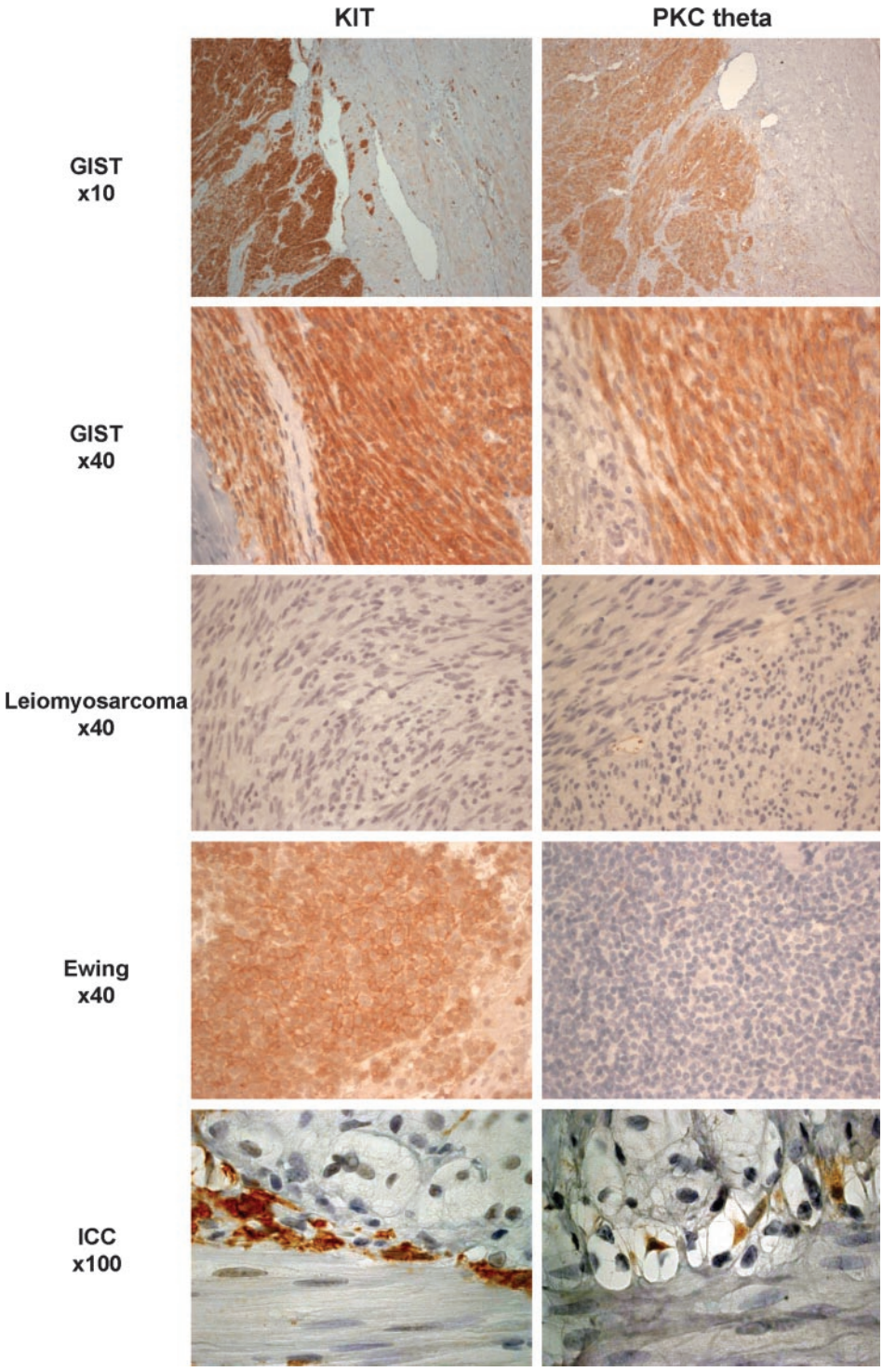

Fig. 3 C-kit receptor tyrosine kinase (*KIT*) and protein kinase C θ (*PKC theta*) expression determined by immunohistochemistry in a representative set of tumors. *Left* and *right columns* show immunostaining with anti-KIT and anti-PKC-θ, respectively. The magnification of each image is indicated. GIST cells show strong diffuse positive immunostaining with anti-KIT and anti-PKC-θ, whereas no signal was detected in the adjacent normal tissue. Leiomyosarcoma show complete absence of immunoreactivity with anti-KIT or with anti-PKC-θ. A Ewing sarcoma shows positive immunostaining with anti-KIT but complete absence of signal with anti-PKC-θ. Interstitial cells of Cajal show positive immunostaining with both anti-KIT and anti-PKC-θ.

mutually exclusive oncogenic mechanisms in these tumors. The same study shows that neoplastic cells from some GISTs bearing PDGFRα mutations do not express KIT by immunohistochemistry analysis, whereas PKC-θ expression in those GISTs, analyzed by Western blot, seems to be independent of the type of mutation. Interestingly, Bauer *et al.* (6) have reported the existence of GIST patients who responded well to Imatinib mesylate despite having near complete absence of KIT expression. Although KIT positivity is the gold standard in the diagnosis of GIST, these findings indicate that additional markers are required for the accurate identification of this tumor type.

DNA microarrays are currently the most powerful tool to

analyze the molecular complexity of tumors. Genomic studies using microarray-based technologies are expected to identify new diagnostic and prognostic markers and to lead to the discovery of new potential therapeutic targets. However, potential markers identified by these approaches need to be validated by other techniques to be introduced in routine tests in hospital settings (20). In an attempt to identify genes differentially expressed in GIST, we analyzed publicly available data from two different microarray studies of soft tissue tumors (8, 9). In the first of these studies, Allander *et al.* (8) identified an expression signature that differentiates GIST from other spindle cell tumors. Independently, Nielsen *et al.* (9) have identified a set of genes that distinguish GIST from synovial sarcoma, neural tumors, or leiomyosarcomas. Although both studies used different cDNA microarrays, several genes were found to be specifically expressed by GISTs in both studies. One of these genes encodes PKC-θ, a serine-threonine protein kinase, which had not been related previously to mesenchymal tumors.

We have used a monoclonal antibody against human PKC-θ to examine by immunohistochemistry a number of GISTs and other mesenchymal tumors that are considered in its differential diagnosis. All of the GISTs in our series expressed PKC-θ, regardless of site of origin, morphology, and grade of malignancy, indicating that this protein is a highly sensitive marker for these tumors. Our series does not include any KIT-negative GIST, because they are rare, but microarray data from Nielsen *et al.* (9) and the above-mentioned report by Heinrich *et al.* (7) demonstrate the existence of a number of GISTs that are PKC-θ+/KIT−, suggesting that PKC-θ might be a marker even more sensitive than KIT. Our series includes a representative sample of tumor types of mesenchymal and epithelial origin. We could not find PKC-θ expression at protein level in any of those tumors samples with the exception of GIST, which is in agreement with the results obtained at the RNA level in the above discussed transcriptomic analyses of mesenchymal tumors (8, 9). Expression of KIT is not specific of GIST, and a number of other malignancies have been reported to be KIT positive (21). Consequently, we investigated whether PKC-θ is also expressed in non-GIST KIT-positive malignancies, and we found that PKC-θ immunostaining was negative in tumors showing positivity for KIT such as oat-cell lung carcinoma, Ewing sarcoma, melanoma, and seminoma. Together, these findings indicate that PKC-θ is a highly sensitive and specific marker for GIST.

PKC-θ is a serine/threonine protein kinase of the Ca^{2+}-independent novel PKC subfamily (22, 23). It has been shown to be expressed in a relatively selective manner in skeletal muscle, lymphoid organs, and in certain parts of the nervous system (24). PKC-θ is highly expressed in T cells, where it is an essential component of the immunological synapse, playing an important role in T-cell activation and survival (11). The importance of PKC-θ signaling in this process has been substantiated by the recent analysis of two independent PKC-θ knockout mice, which display a selective T-cell activation defect (25, 26). PKC-θ has also been implicated in pathways protecting T cells from apoptosis and in the acquisition of a multidrug resistance phenotype (27–29). In muscle fibers, PKC-θ is localized at the neuromuscular junction where it might play a specific role in mediating nerve-muscle interaction and signal transduction. Besides, the expression of this protein is prevalent in white glycolytic muscle fibers, where it could participate in regulating the signaling cascade of insulin receptor activation (30). PKC-θ mRNA is also expressed in mouse brain and in the peripheral nervous system, but its role in these tissues remains unknown (24). We observed that ICCs were also positive for PKC-θ. This finding agrees with recent reports in which PKC-θ immunoreactivity was found in ICCs from guinea-pig gastrointestinal tract (16–18) and supports the current hypothesis according to which GISTs are derived from ICCs or from a common precursor (31, 32).

The coexpression of KIT and PKC-θ in GISTs suggests that both molecules could be functionally related. Blume-Jensen *et al.* (33) have reported that classical isoforms of PKC are involved in modulating KIT signaling by phosphorylating specific serine residues in this molecule, but the possible participation of the −θ isoform remains unexplored. Investigating in depth the roles of PKC-θ in the signaling networks underlying the biology of GIST cells could eventually facilitate the development of additional therapies against this malignancy. In this regard, it is interesting to note that PKC-θ is also expressed in several leukemia types, and it has been proposed that it could represent a potential target of therapeutic intervention (10, 11).

In summary, our results indicate that PKC-θ protein is highly expressed in GIST by immunohistochemistry and that it is a sensitive and specific marker for these tumors. We also show PKC-θ immunostaining in ICC, supporting the current hypothesis that ICC and GIST derive from a common precursor. We finally remark on the usefulness of the hypothesis generated by the analysis of DNA microarray data for the discovery of new molecular markers.

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