

Expression and Mutational Status of PDGFR in Thymic Tumours

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Abstract. *Background:* There is an ongoing search for new therapeutic targets in invasive non-resectable thymic tumours because of the low response rates in current chemotherapeutic treatment modalities. In this study, the possibility that platelet-derived growth factor receptor A (PDGFRA) and/or PDGFRB may represent potential therapeutic targets in epithelial tumours of the thymus was investigated. *Patients and Methods:* Tissue samples were obtained by thymectomy from 36 different patients with epithelial tumours of the thymus (26 thymomas types A, AB, B1-3 and 10 thymic carcinomas). Normal thymi from three young children were used as controls. The PDGFRA and PDGFRB protein expressions as well as the mutational statuses of exons 12, 14 and 18 of the PDGFRA gene were analyzed. *Results:* All the subtypes of thymomas and the thymic carcinomas showed staining for PDGFRA, but no mutations in the known mutational hotspots were identified. Only about one third of the tumours stained for PDGFRB. PDGFRA and PDGFRB protein staining were slightly positively correlated. *Conclusion:* PDGFRA may represent a potential therapeutic target in thymic tumours.

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Thymomas and thymic carcinomas are rare epithelial tumours of the anterior mediastinum. Type A, type AB and type B1 thymomas have an excellent prognosis, while type B2 and type B3 thymoma as well as thymic carcinomas show a malignant behaviour with sometimes extended tumour disease, including infiltration of adjacent structures and metastasis (1-5). Surgery remains the gold standard in the treatment of these tumours (6).

Adjuvant therapy as well as postoperative radiotherapy or radiochemotherapy are proposed in invasive diseases. The relatively low response rates to chemotherapy demand the development of new therapeutic targets. For example, octreotide-based therapies in thymomas showed a response rate of 30% in a large study (7, 8), while response rates to epidermal growth factor receptor (EGFR) inhibitors were less than 60%, with progression-free survival of only a few months (9).

KIT, a receptor tyrosine kinase type III, has been extensively investigated concerning expression by immunohistochemistry and mutational status in thymomas and thymic carcinomas, but mutations in KIT in thymic carcinomas are very rare (10). Imatinib mesylate treatment resulted in a partial response for 9 months in one case of thymic carcinoma with a KIT mutation (11). In contrast, there is a lack of information concerning platelet derived growth factor (PDGF) receptors (PDGFR) in epithelial tumours of the thymus (10, 12). This prompted us to analyze PDGFRA and PDGFRB protein expressions as well as the mutational status in the mutational hotspots of exons 12, 14 and 18 of the PDGFRA gene in thymomas and thymic carcinomas.

Patients and Methods

Tissue and clinical data. Tissue samples were obtained by thymectomy from 36 patients with epithelial tumours of the thymus, the patient characteristics are shown in Table I. All the

samples were fixed in 4% formaldehyde and paraffin embedded by a routine procedure. Typing was performed according to the histological criteria of the WHO classification including immunohistochemistry. The 36 tumours encompassed the following subtypes: 3 type A thymomas, 7 type AB thymomas, 6 type B1 thymomas, 4 type B2 thymomas, 6 type B3 thymomas and 10 thymic carcinomas (former type C thymomas) of different stages (I-IV) according to Masaoka *et al.* (13) (Table I). The normal thymi from three young children were used as controls in order to see the expression in normal tissue.

Mutational analysis of exons 12, 14 and 18 of the PDGFRA gene. Mutational analysis was performed for the type A thymomas, the A component of type AB thymomas, type B3 thymomas and thymic carcinomas due to the low number of lymphocytes, so that all mutations could be detected in the malignant epithelial cell component. For these cases, the haematoxylin and eosin stained slides were reviewed to localize tumour regions with less than 10% lymphocyte-infiltration and $\geq 90\%$ tumour cell content. From each case one paraffin block was selected and a 16 gauge needle for bone marrow aspiration was used to obtain the tissue cores (2 mm diameter, 3 mm height) for DNA extraction. Genomic DNA was isolated from the paraffin-embedded tissue samples after deparaffinization with xylene using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The DNA was eluted twice with 50 μ l 10 mM Tris HCl pH 8.0. Exons 12, 14 and 18 of the human *PDGFRA* gene were amplified by nested polymerase chain reaction (PCR) using the following oligonucleotides: exon 12 (first PCR, forward 5'-GGACTCTACTGTGTCCAGTC-3' and reverse 5'-GATCTC TATTCTGCCAAGGCC -3'; nested PCR forward 5'-CTC TGTTGCACTGGGACTTT-3' and reverse 5'-GCAAGGGAA AAGGGAGTCTT-3'); exon 14 (first PCR, forward 5'-GAGGCCA AGTAGCTATCTGC-3' and reverse 5'-CACAACCACATGTGTC CAGTG-3'; nested PCR forward 5'-TCTGAGAACAGGAAG TTGGTAGC-3' and reverse 5'-CCAGTGAAAATCCTCACTCCA-3'); exon 18 (first PCR, forward 5'-GTGCCACCATGGA TCAGCCAG-3' and reverse 5'-GGCACCGAATCTCTAGAACG-3'; nested PCR forward 5'-CTTGCAGGGGTGATGCTATT-3' and reverse 5'-AGAAGCAACACCTGACTTTAGAGATTA-3'). The PCR reactions were carried out in a volume of 20 μ l containing 2 μ l of genomic DNA from the second eluate using HotStar Taq DNA polymerase (Qiagen). The incubations were conducted at 95°C for 15 min, then 10 cycles of 94°C for 20 s, 60°C for 1 min, 72°C for 1 min, followed by 30 cycles of 94°C for 20 s, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were purified using a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and sequenced in both directions (Eurofins Medigenomix, Martinsried, Germany). The retrieved sequences were subjected to an internet-based (BLAST) analysis (14) and the electropherograms were further inspected visually for double peaks.

Immunohistochemistry. Tissue microarrays (TMA) were constructed from the paraffin wax-embedded blocks of the 39 specimens of the thymus. A tissue arrayer device (Beecher Instruments, Sun Prairie, WI, USA) was used. All the investigated cases were reviewed and representative tumour areas were marked in the corresponding paraffin wax blocks and at least two areas were sampled. The diameter of the cylinders was 1.2 mm.

For PDGFRA staining (SC-338, Santa Cruz, CA, USA, dilution 1: 50), the antigen retrieval was performed in sodium citrate buffer, pH 6.0 (10 mmol/L) in the microwave for 15 min. Detection was performed with a Dako REAL™ Detection System using peroxidase. A gastrointestinal stromal tumour with an activated *KIT* mutation was used as the positive control and negative controls were performed without the primary antibody, showing no signals. Incubation with the PDGFRB antibody (BD Bioscience, CA, USA, dilution 1:50) was performed as described for PDGFRA. Normal testis (Score 3) was used as the positive control.

The cytoplasmic intensity of PDGFRA and PDGFRB was scored for each specimen on a scale of 0 to 3: 0=negative, 1=weak, 2=moderate and 3=strong (8). The staining intensity was evaluated for the maximum intensity among the positive cells ("maximum intensity of staining") (8). The slides were evaluated by two independent observers (M.M., R.R.)

Statistical analysis. With respect to the stage and histological subtype, the differences in the frequency distribution of the intensity were analyzed using the Kruskal-Wallis Test. A difference was considered statistically significant if the *p*-value did not exceed 5% (*p*≤0.05).

Results

Immunohistochemistry. In Table I, the results of the PDGFRA immunohistochemistry are given for the 39 investigated cases (36 tumours and 3 normal controls). All the types of thymic tumours showed positive staining (Table I); in particular the thymic carcinomas showed strong positive staining, while the normal thymi showed weak to moderate positive staining (see Figure 1). PDGFRB showed weak staining in all the normal thymi, and in about 1/3 of the thymic carcinomas and some of the thymoma cases (see Table I and Figure 1).

Association of PDGFRA and PDGFRB staining with histologic subtype and stage. Due to the fact that PDGFRA was expressed especially strongly in the thymic carcinomas, there was a significant difference between them with the thymomas subtypes (*p*<0.0024) as well as with stage (*p*<0.0040), since higher stages had a stronger expression. Since PDGFRB was especially expressed in about 1/3 of the thymic carcinomas, there was a significant difference for histology (*p*<0.0140) in comparison to the thymoma subtypes, and for stage (*p*<0.0120) since there was a expression in higher stages. No significant difference was found for either protein concerning gender (*p*>0.8116 for PDGFRA and *p*>0.4667 for PDGFRB). There was a slight positive association between these two proteins (Spearman test: 0.1121).

Mutational status of the PDGFRA gene. The mutational status of exons 12, 14, and 18 of the *PDGFRA* gene was evaluated in 26 samples. No mutations were found in the sequenced exons of the *PDGFRA* gene.

Discussion

The expression of PDGFRA was detected in the normal thymi and all the subtypes of thymomas and was interestingly more pronounced in the thymic carcinomas. However, no mutations were found at the hotspots of the *PDGFRA* gene in a subset of these tissue samples.

The absence of activating mutations in *PDGFRA* does not exclude the possible treatment efficiency of selective tyrosine kinase inhibitors such as imatinib mesylate. Imatinib mesylate was the first clinically useful synthetic inhibitor that blocks the adenosine triphosphate binding site of the subclass III group of tyrosine kinases, which includes KIT, PDGFRA, PDGFRB and Abelson protooncogene (ABL). When this drug binds to the kinase domain, it prevents phosphate from being transferred from adenosine triphosphate to tyrosine residues. This results in an inhibition of proliferation and cell mortality (15-20). Activating *PDGFRA* mutations affecting exons 12 and 18 were detected by two independent groups in 14 of 40 and in 5 of 8 *KIT* wild-type gastrointestinal stromal tumors (GISTs), while being absent from any of 36 and of 10 *KIT* mutant GISTs examined (21, 22). The authors of these studies concluded that activating mutations of *KIT* or *PDGFRA* are mutually exclusive oncogenic events and that these mutations have similar biological consequences. Autocrine expression of both PDGF ligands and PDGFRA and B receptors is thought to play a role in cancer development, as shown in sarcomas, glial tumours and gynaecological malignancies for example (17). As PDGFRA signalling is required for the recruitment of vascular endothelial growth factor-producing stromal fibroblasts for tumour angiogenesis and growth, it is an interesting therapeutic target (23) even when no mutations exist.

Imatinib mesylate has an inhibitory activity against PDGFRA and KIT, but also not only against PDGFRB. *In vivo* studies of PDGFR signalling indicated that PDGFRB signal transduction could replace that of PDGFRA (24). Additionally PDGFRB activated ABL influenced downstream signalling which contributed to tumour growth (25). Several studies have shown that PDGFRB played an important role in tumour progression in gastric cancer, osteosarcomas and prostate cancer (26-29). Therefore, imatinib mesylate may well be a potential therapeutic target in some cases of thymomas and thymic carcinomas.

In summary, thymomas as well as thymic carcinomas express PDGFRA and sometimes PDGFRB. Thus PDGFR inhibitors may be potential therapeutic agents in these tumours. The lack of long-term toxicity data limits their clinical use as an option where established local and systemic treatment regimen has failed to control the disease.

Table I. Patient characteristics and *PDGFRA* and *PDGFRB* expression.

#	Age (years)	Gender	Hist	Stage	pA	pB
1	71	m	A	2	2	1
2	67	m	A	1	2	.
3	68	m	A	2	2	1
4	58	f	A	3	2	1
5	56	m	AB	2	1	0
6	68	m	AB	2	2	0
7	49	f	AB	2	1	0
8	74	f	AB	2	1	0
9	48	f	AB	2	2	0
10	48	f	AB	2	1	0
11	58	f	B1	1	1	0
12	66	f	B1	2	2	0
13	52	m	B1	2	3	0
14	41	f	B1	1	1	0
15	71	m	B1	1	1	0
16	34	m	B1	2	2	0
17	55	m	B2	3	2	0
18	43	m	B2	4	3	1
19	49	f	B2	.	1	0
20	76	f	B2	2	2	0
21	62	f	B3	2	1	0
22	58	m	B3	3	1	0
23	66	m	B3	3	3	1
24	62	f	B3	4	2	0
25	73	m	B3	2	1	0
26	88	f	B3	3	2	1
27	68	f	C	3	3	1
28	57	f	C	4	3	1
29	58	f	C	2	3	0
30	66	m	C	3	3	0
31	63	f	C	3	3	1
32	63	f	C	4	3	2
33	51	m	C	4	3	0
34	76	f	C	3	3	0
35	62	f	C	4	2	1
36	44	f	C	4	3	2
37	0.8	m	N	.	1	1
38	0.1	m	N	.	2	1
39	0.2	m	N	.	1	1

pA, PDGFRA; pB, PDGFRB; gender: m, male; f, female; hist, histological subtype according to WHO (A, AB, B1, B2, B3, C = thymic carcinoma), N, normal thymus; pA/B score, 0, negative, 1, weak, 2, moderate, 3, strong stain intensity.

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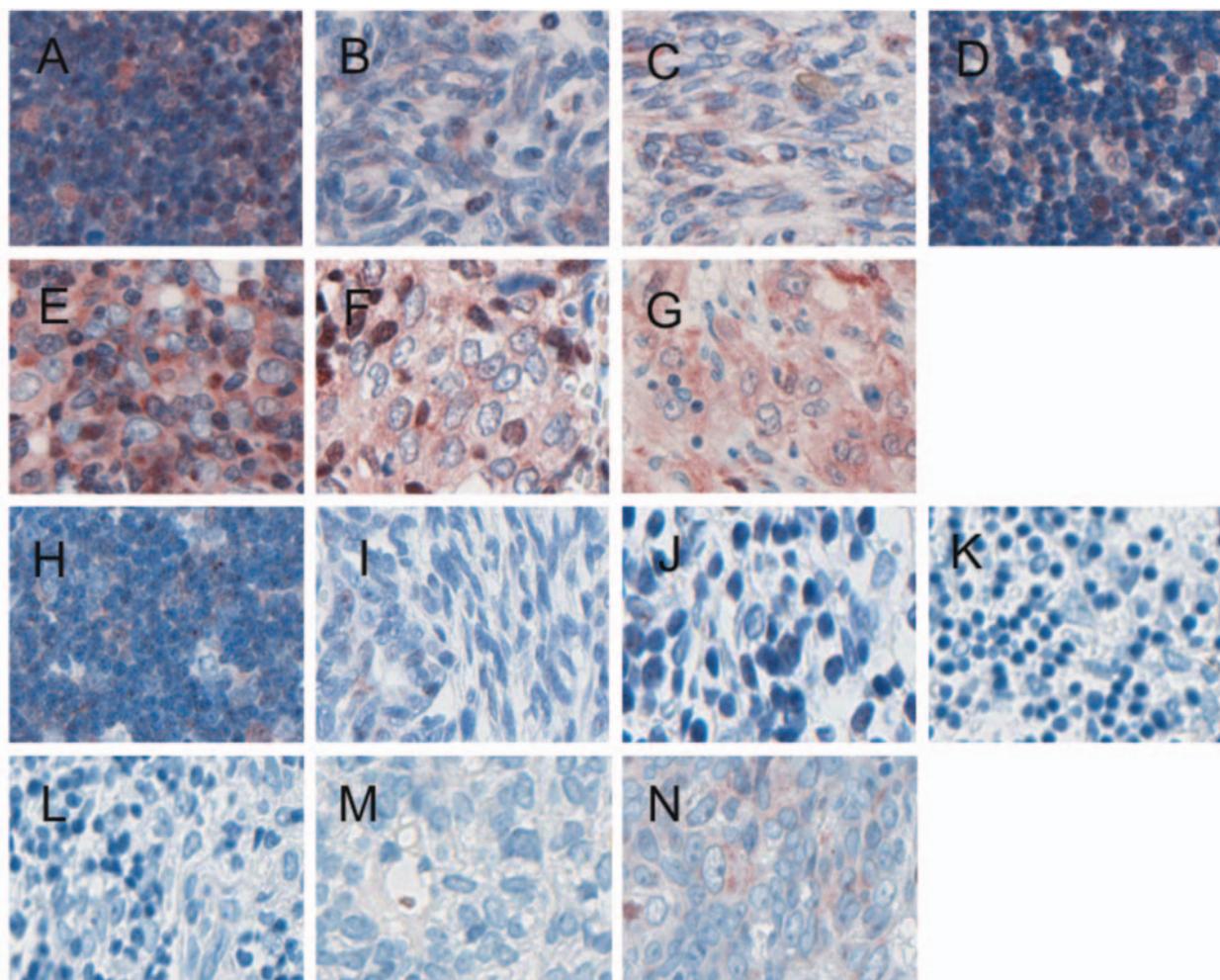


Figure 1. A-G. Immunohistochemical staining for PDGFRA. A, Normal thymus (case no. 38, score: 2); B, type A thymoma (case no. 3, score: 2); C, type AB thymoma (case no. 10, score: 1); D, type B1 thymoma (case no. 14, score: 1); E, type B2 thymoma (case no. 18, score: 3); F, type B3 thymoma (case no. 23, score: 3); G, thymic carcinoma (case no. 33, score: 3); H-M, immunohistochemical staining for PDGFRB; H, normal thymus (case no. 38, score: 1); I, type A thymoma (case no. 3, score: 1); J, type AB thymoma (case no. 7, score: 0); K, type B1 thymoma (case no. 11, score: 0); L, type B2 thymoma (case no. 19, score: 0); M, type B3 thymoma (case no. 24, score: 0); N, thymic carcinoma (case no. 28, score: 1).

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