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Somatostatin receptor expression in thymic tumors

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1. ABSTRACT

Detection of thymic tumors by [^{111}In-DTPA^{0}]octreotide scintigraphy and the antitumor effect exerted by somatostatin (SS) analogs suggest significant expression of SS receptors (SSRs) in these tumors. We measured SSR subtype $(sst)_{2A}$ and sst_3 expression by immunohistochemistry (IHC) in 14 thymic tumors previously studied by SSR scintigraphy (SRS). Scintigraphy showed significant [^{111}In-DTPA^{0}]octreotide uptake in 13/14 tumors (tumor-to-background ratios: 1.4- to 6-fold). By IHC, 4 tumors were positive for sst_{2A} and sst_3; two for sst_{2A} and five tumors for sst_3. Three tumors were completely negative. Overall, 11/14 (approximately 78% of cases) expressed at least one SSR subtype. Staining was highly heterogeneous: sst_{2A} was confined to malignant epithelial cells or within stromal structures, and sst_3 was predominantly associated with thymocytes. SRS provides immediate visualization of primary and metastatic lesions, while IHC reveals SSR subtype expression. This joined approach could help to select SS analogs beneficial for therapy.

2. INTRODUCTION

High somatostatin receptors (SSRs) expression have great relevance in neuroendocrine human tumors (1-3). Imaging by [^{111}In-DTPA^{0}]octreotide scintigraphy is a well established *in vivo* method used to detect SSR-expressing tumors (4), whereas therapy with SS analogs (SSAs) is effective in selected SSR-positive neuroendocrine tumors (3,5). Thus, *in vivo* and *in vitro* identification of SSR provides the rationale for the therapeutic use of SSAs. Five different SSRs have been identified so far, of which sst_2 is the most widely expressed. Moreover, sst_2 has the highest affinity for the SSA octreotide compared with the other SSAs, and high tissue sst_2 expression is mostly responsible for *in vivo* visualization of SSRs with SSR scintigraphy (SRS) and for the clinical response to octreotide. Therefore, *in vitro* identification of SSRs is clinically relevant. After *in vitro* identification of SSR in tumor tissue by ligand-binding techniques and RT-PCR, SSA can be used for diagnosis and therapy (6-8). Thanks to the development of antibodies specific for the different SSR subtypes, SSR expression can

Table 1. Comprehensive view of patients' characteristics and related somatostatin receptor scintigraphy and immunohistochemical findings

N.	Sex/Age	Surgery	Histotype	Stage	Associated syndrome	SRS		IHC	
						Sites of uptake	T-to-B ratios	sst_{2A}	sst_3
1	f/57	B	B2	IVB	PRCA	Med	3.1	Scattered TE	+ TH, + TE
2	m/27	Thy	B2	II	MG	Med	1.4	+ TE	+ TH, ± TE
3	f/48	Thy	C	IVA		Med, lung	6.3	+ TE	+ TH, ± TE
4	M/59	B	B2	IVB		Med, pleura	2.1/1.4	+ TE	+ TH, ± TE
5	f/56	Thy	B2+B3	IVA		Med	6.2.	-	+ TH, ± TE
6	m/52	Thy	B2	IVA	MG	Med	3.3	-	+ TH, ± TE
7	m/53	Thy	B2	IVA		Med	3.2/1.7	-	+ TH, ± TE
8	m/23	B	SCNC	IVB		Med, cervical lymph	1.5/1.5	-	+ TH, ± TE
9	m/72	Thy	B2	III		Med	1.4	n.e.	+ TH, + TE
10	f/68	B	C	III	My	Med, lung	4.2/3.3	Scattered TE	-
11	f/67	B	SCNC	IVA		Med	3.4	+ TE	n.e.
12	m/48	Thy	B3	IVA		None	-	-	-
13	f/74	Thy	B1	IVA	MG	Med	2.6	-	-
14	f/60	B	C	IVB		Med	2.1	-	-

Abbreviations: f, female; m, male; B, biopsy, Thy, thymectomy; SCNC, small cell neuroendocrine carcinoma; PRCA, Pure Red Cell Aplasia; MG, Myasthenia Gravis; My, Myeloradiculopathy; Med, mediastinum lymph lymphnode; T-to-B, Tumor to Background ratio; n.e., not evaluated; TE, tumor epitheliali cells; TH, thymocytes.

be studied by immunohistochemistry (IHC) on paraffin-embedded tissues (9,10). Preliminary reports indicated that IHC is a reliable method with which to evaluate SSR expression in carcinoids and other SSR-positive tumors (11-14). IHC is easier to perform and more practical for clinical use than RT-PCR or *in situ* hybridization methods. Furthermore, IHC reveals the exact cell localization of a given antigen-protein and can be applied on fixed material.

SSRs are differentially, but widely, expressed in normal and abnormal lymphoid tissue and immune cells, including thymic tumors (15,16). Moreover, SSR expression in thymic epithelial tumors could indicate a neoplasm originating from an organ characterized by neuroendocrine cells.

We previously demonstrated that thymic tumors can be visualized *in vivo* by SRS and have very high tumor-to-background (T-to-B) ratios (17). Moreover, patients with thymic tumors have been reported to respond to SSA-based therapies (18-20). SSR expression was not found in thymic tumors by *in vitro* autoradiographic techniques using [^{125}I-Tyr^3]octreotide and ^{125}I-[Leu^8, D-Trp^{22}, Tyr^{25}]-SS-28, although high expression was found in thymic carcinoids and in normal thymuses (21). However, more recently, evaluation with a combination of *in vitro* techniques demonstrated the expression of sst_1, sst_2 and sst_3 in a B1 (cortical) thymoma, which was previously detected by SRS (22), and of sst_2 and sst_3 in an A (medullary) thymoma also previously imaged with SRS (23).

The aim of the present study was to verify sst_{2A} and sst_3 expression in a series of 14 thymic tumors previously imaged with SRS to verify the concordance, if any, between *in vitro* and *in vivo* results.

3. MATERIALS AND METHODS

3.1 Patients

Fourteen patients (7 f and 7 m, age-range: 23-74 yrs) with a diagnosis of thymic tumors were evaluated. According to the Masaoka *et al* classification (24), 1 patient was at stage II, 2 patients at stage III, 7 patients were at stage IVA and 4 patients were at stage IVB. Myasthenia gravis was the most common associated paraneoplastic syndrome in this series, whereas pure red cell aplasia was present in only one patient. One patient had also myeloradiculopathy. Six patients underwent only biopsy because of unresectable primitive tumor at presentation, whereas the others underwent thymectomy. Tumors were classified according to the 1999 WHO classification (25): 1 B1 thymoma, 6 B2 thymomas, 1 B3 thymoma, 1 combined B2+B3 thymoma, 3 C thymoma, 2 small cell neuroendocrine carcinoma (SCNC). The patients' profiles are shown in Table 1. Ethical committee approved the study and all patients gave their informed consent.

3.2. Scintigraphic studies

Scintigraphy with [^{111}In-DTPA^0]octreotide (Mallinkrodt, Petten, The Netherlands) was performed as previously reported (17). Briefly, planar and tomographic images were collected 24 hrs after the i.v. injection of 180-222 MBq of radioligand. Labeling was performed according to the manufacturer's instructions; injected preparations had <2% of uncoupled ^{111}In. Images were obtained with a γ-camera (Siemens, Erlangen, Germany) equipped with a medium energy collimator (Photopeaks were set at 172 and 242 keV, 10% window). Tumor-to-background ratios were obtained with the regions of interest (ROIs) method. A ROI was manually drawn around the area/s of radioligand uptake; a similar ROI was placed on the patient's chest where no uptake was detectable. In each patient, the T-to-B ratio was obtained in the planar or tomographic images of the chest 24 hrs after the injection of the radioligand by dividing the counts in the thymic tumor by those in the background area.

3.3. Tissue samples

The samples of 14 histologically proven thymomas were collected and used for IHC. All samples were formalin-fixed, paraffin-embedded. Fixation was performed for 24-36 hours.

3.4. Immunohistochemical localization of sst_2 and sst_3 receptors

IHC was performed on 5-μm paraffin-embedded sections as previously reported (22). Negative controls for

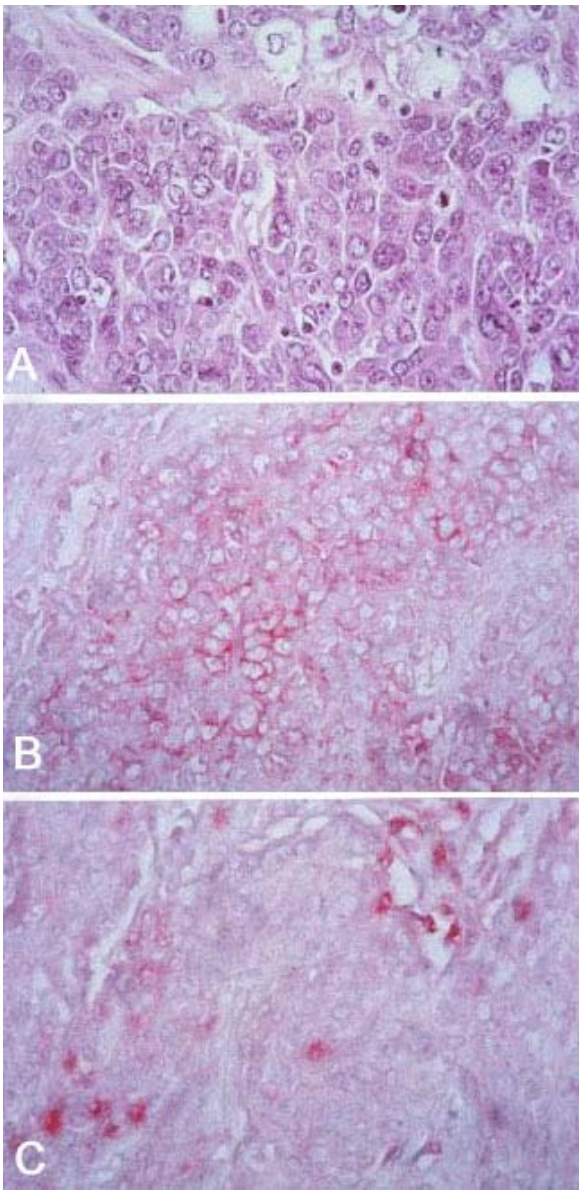

Figure 1. *In vitro* expression of somatostatin receptors in the same patient (n. 3, Table 1): a) hematoxylin-eosin staining of tumor section (magnification 400X); b) immunohistochemistry for sst_2 shows clear membrane staining (magnification 200X); c) immunohistochemistry for sst_3 shows intense focal staining (magnification 200X). Sections developed with New Fucsine/Naphtol AS-MX. The sections are slightly counterstained with haematoxylin.

IHC included omission of the primary antibody and preabsorption of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nm). A tissue was considered positive when the immunostaining was abolished by preabsorption of the antibody with the respective peptide antigen. A standard streptavidin-biotinylated-alkaline phosphatase (ABC kit, Biogenic, San Ramon, CA, USA) was used according to the manufacturer's recommendations to visualize the bound antibodies. Sections from normal human pancreas (islets) served as positive controls. Stained slides were evaluated by three different observers, including a pathologist, unaware of the clinical study.

4. RESULTS

4.1. Somatostatin receptor scintigraphy

SRS was positive in 13 out of 14 patients. In 4 cases, there were at least two sites of abnormal radioligand uptake in the chest, whereas in the other 9 patients there was a single area of uptake. The degree of [^{111}In-DTPA^0]octreotide uptake varied greatly among the patients. The SRS results and T-to-B ratios are shown in Table 1.

4.2. Immunohistochemical detection of sst_2 and sst_3 receptors

Expression of sst_{2A} and sst_3 or their co-expression was clearly detectable by IHC in 11 out of 14 thymic tumors, whereas 3 tumors were completely negative. Immunostaining was possible for both SSR subtypes in 12 cases, and for only one subtype in 2 cases because of a limited amount of tissue and antibody. sst_3 was expressed in more than half the patients studied (9/14) while sst_{2A} was clearly detectable in 4 cases and on scattered cells in other 2 cases. The results are reported in Table 1. The B1 tumor had no SSR staining; 3/5 B2 tumors were positive for sst_{2A} and all were positive for sst_3. The B2/3 tumor was positive only for sst_3, whereas the B3 tumor was positive only for sst_{2A}. One C tumor was negative for both receptors, one was positive only for sst_{2A}, one was positive for both, and one SCNC was positive for sst_{2A} whereas the other was positive for sst_3. The immunostaining was weak to moderate. However, the distribution of both subtypes was highly heterogeneous.

IHC revealed the exact location the cellular distribution of SSR within these thymic tumors. The sst_{2A} was homogenously expressed throughout the tumor in only 2 cases (n. 3 and 11) whereas immunoreactivity was highly heterogeneous in the remaining sections and was confined to limited areas in the tissue samples (n. 2 and 4), or on scattered cells (n. 1 and 10). sst_3 was expressed throughout the tumor in both cell types, but was mainly associated with thymocytes. After absorption with specific peptide antigen, the binding of these antisera was completely prevented on adjacent sections taken from all patients, which demonstrates the specificity of sst_{2A} and sst_3 immunostaining.

The expression of sst_{2A} was predominantly associated to neoplastic epithelial cells, whereas the expression of sst_3 was found mainly associated to normal reactive thymocytes. In 4 thymic tumor biopsies (n. 1-4), both sst_{2A} and sst_3 were localized. In detail, in 2 cases (n. 3 and 4) sst_{2A} immunoreactivity was present on tumor cells, whereas in the other 2 (n. 1 and *in vivo* 2) immunoreactivity was detected on few scattered cells in 1 (n. 1), and on the stromal compartment of the medullary region in the remaining case (n. 2). One of the 2 cases with immunohistochemical co-expression of sst_{2A} and sst_3 (case

3) had the highest level of [^{111}In-DTPA^0]octreotide uptake *in vivo*. Figure 1 shows the co-expression of sst_2 and sst_3 in case 3. In 5 cases (n. 5-9), only sst_3 was detectable. The sst_{2A} receptor was not co-expressed in 4 of these biopsies and not measurable in the remaining one (n. 9) due to the limited amount of sample available. In 2 cases (n. 10 and 11), only sst_{2A} was expressed. sst_3 was undetectable in one of these cases and not measurable in the other case (see above). In 3 cases (n.12-14) neither sst_{2A} nor sst_3 were detectable.

4.3. Comparative analysis of *in vitro* and *in vivo* results

We next compared the results of immunostaining with the T-to-B ratios measured at SRS. The higher T-to-B ratios *in vivo* corresponded to the expression of at least one of the two SSR subtypes *in vitro*. In particular, the highest ratio was found in a patient with widespread sst_{2A} and sst_3 co-expression. The expression of both subtypes and sst_{2A} alone occurred within tumors with a high T-to-B ratio.

5. DISCUSSION

In normal thymuses, sst_1 and sst_{2A} mRNAs have been identified in thymic tissue and isolated thymic epithelial cells, whereas sst_3 mRNA has been demonstrated in thymic tissue only and in mature isolated thymocytes (26,27). However, the evidence that thymic tumors may express SSRs is poor (23). Reubi *et al* demonstrated SSRs in normal thymuses using autoradiography, and a lack of binding sites in 4 epithelial thymic tumors using autoradiography with radiolabeled SSAs (21). However, this finding appeared to be in contrast with the evidence that tumors originating from SSR-positive tissues generally express SSRs. The identification by IHC of sst_{2A} and/or sst_3 in most of our 14 thymic tumors provides the first confirmation, and in a larger series, that SSRs are expressed in this rare human malignancy.

We were unable to identify correlation between tumor histotype and SSR pattern probably because of the relatively small number of cases and of their considerable heterogeneity. However, B2 thymomas, which was the most represented in this series of tumors, expressed at least one SSR subtype. Despite their "classical" neuroendocrine phenotype, SCNC expressed only one SSR. This pattern of SSR expression supports the concept of that thymic tumors share a common neuroendocrine phenotype (28,29).

Expression of sst_3 was predominantly associated to thymocytes, whereas sst_{2A} expression was confined to malignant epithelial cells and/or within the thymic stroma (endothelium, accessory bone marrow-derived cells). Indeed, thymomas are prevalently constituted by thymic epithelial cells mixed with benign thymocytes (T lymphocyte precursors) and stromal cells (30). There are architectural differences, i.e. prevalence of cortical areas and deficiency of medullary areas, between thymic tumors and normal thymus (31). There is a strong compartmentalization in the distribution of SSRs within the normal thymus (21,26). The same was reported for other neuropeptide receptors within thymus (32). This not casually determined distribution of receptors could be particularly important in modulating the physiological processes occurring in this organ. Different SSRs are selectively expressed in different regions and perhaps on specific epithelial cell types in the human thymus (26). Our results indicate that this complex distribution of SSR is preserved also in thymic tumors. If confirmed, this differential expression might indicate that the SS/SSR system plays a role in thymic disturbances and their relative clinical syndromes.

Although we did not find a clear correlation between histotype and SSR expression, a comparison of the scintigraphic results and immunohistochemical findings indicates that the higher T-to-B ratios *in vivo* correspond to the expression of at least one of the two SSR subtypes *in vitro*. In particular, the highest ratio occurred in one of the two patients with widespread co-expression of sst_{2A} and sst_3. sst_3 has recently been implicated in the uptake of [^{111}In-DTPA^0]octreotide *in vivo* in pheocromocytomas not expressing sst_{2A} (33). We found a link between histotype and SSR expression also in one of our cases with negative SRS, in which IHC failed to detect SSR in the tumor tissue. However, T-to-B ratios were high in other two patients with tumor lacking SSR expression *in vitro*. Although the sensitivity of this technique or the sample fixation procedures might account for this discrepancy, the presence of another SSR subtype, namely sst_3, cannot be ruled out. Moreover, the *in vivo* visualization of cases found to be sst_2/sst_3-negative might also result from aspecific or specific uptake in other adjacent tissues (i.e. inflammatory tissues).

[^{111}In-DTPA^0]octreotide binds with high affinity to sst_2 and with lower affinity to sst_3 and sst_5. We have evaluated 2 of these SSRs and both receptors appear to be involved in the uptake of the radiopharmaceutical. In 3 cases a high uptake *in vivo* corresponded to the selective expression of sst_3 within the tumor, confirming that, despite the lower affinity for [^{111}In-DTPA^0]octreotide, sst_3 may play a role or be directly involved in the uptake of the radiotracer (26,33). Apart from the affinity, additional biological events related to membrane receptor turnover or receptor cross-talk might play a role in determining the *in vivo* uptake of radiolabeled SSAs. In fact, given the high rate of agonist-dependent internalization described in cells transfected with this receptor subtype (34), sst_3 may be involved in this process. This hypothesis is in line with recent data about neuropeptide receptor trafficking and cross talk in cells co-expressing different SSR subtypes that derive from receptor dimerization (35,36).

In conclusion, sst_2 and sst_3 appear to be highly and heterogeneously expressed in thymic tumors, without correlation with epithelial rather than neuroendocrine histotype. The immunohistochemical detection of these SSR subtypes concords with scintigraphic results obtained with [^{111}In-DTPA^0]octreotide. Thus, SRS provides immediate visualization of primary and metastatic sites of tumor, whereas IHC can be used to define more precisely the pattern of SSR subtype expression. This combined approach provides diagnostic information and the results

may be useful in deciding which SSA is the most appropriate for the treatment of a given patient.

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