Thymic epithelial tumors express vascular endothelial growth factors and their receptors as potential targets of antiangiogenic therapy: A tissue micro array-based multicenter study.

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Thymic epithelial tumors express vascular endothelial growth factors and their receptors as potential targets of antiangiogenic therapy: A tissue micro array-based multicenter study

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Abstract

Objectives: Tumor angiogenesis is an essential and complex process necessary for the growth of all tumors which represents a potential therapeutic target. Angiogenesis inhibitors targeting vascular endothelial growth factor (VEGF) or their receptor tyrosine kinases have been approved by the FDA. In thymic epithelial tumors (TET), targeted therapies have been sporadically applied due to their rarity. To ascertain the presence of potential therapeutic targets, we analyzed by immunohistochemistry the expression of angiogenesis-related biomarkers in a large series of TET arranged in Tissue Micro Arrays (TMA).

Materials and methods: We assessed by immunohistochemistry the expression of the possible molecular target of anti-angiogenic therapy, *i.e.* VEGFA, VEGFC, VEGFD, VEGFR1, VEGFR2, VEGFR3, and PDGFR β , in a TMA series of 200 TET collected in the framework of a multi-institutional collaborative project for Rare Diseases.

Results: When compared to the low-risk tumors, high-risk TET (B2, B3, carcinomas) contained higher proportion of cancer cells expressing VEGFA, VEGFC and VEGFD ($P < 0.001$, $P < 0.001$, and $P < 0.001$) growth factors, and their receptors VEGFR1 ($P = 0.002$), VEGFR2 ($P = 0.013$), and VEGFR3 ($P = 0.041$). No differences were observed in terms of PDGFR β expression.

Conclusions: According to our data, it is possible to hypothesize the existence of multiple paracrine and/or autocrine loops in TET, particularly in the high-risk ones, involved in TET growth and progression. Anti-angiogenic agents, directed to inhibit these loops, are therefore to be considered as potential tools in advanced TET therapy.

Keywords: Thymoma; Thymic epithelial tumors; Biomarkers; Tissue Micro Array; Vascular endothelial growth factor; Vascular endothelial growth factor receptor

Introduction

Although thymic epithelial tumors (TET) are rare, with an overall incidence of 0.15 per 100,000 person-years in the US, they represent the most common primary adult neoplasia in the anterior mediastinum [1] and [2]. TET usually exhibit indolent behavior, but do have the capacity to invade locally, to present multiple relapses and to metastasize to distant sites. The most relevant prognostic factors in TET are completeness of resection, tumor stage, and WHO histologic type [2] and [3]. Currently, when compared with A, AB and B1 TET (low risk tumors), the B2, B3 histotypes, often presenting as stage II and III tumors, and thymic carcinomas are to be considered high-risk tumors with a potential to metastasize [4], [5], [6], [7], [8] and [9]. Due to both their heterogeneity and infrequency, TET still represent a diagnostic as well as a therapeutic challenge.

Angiogenesis has been recognized as a complex and dynamic process necessary for the growth, invasion and progression of all solid tumors [10]. The vascular endothelial growth factor (VEGF) pathway plays a critical role in angiogenesis. Much attention has been focused on this pathway, with the development of the first Food and Drug Administration (FDA) approved antiangiogenic drug, the humanized antibody bevacizumab (Avastin®, Genentech, San Francisco, CA) targeted against VEGF [11]. The mammalian VEGF family consists of 5 glycoproteins referred to as VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor

(PGF). The best characterized VEGFA, commonly referred to as VEGF, is the most powerful angiogenic factor known to date. The VEGF ligands bind to and activate 3 structurally similar type III receptor tyrosine kinases (RTKs), designated VEGFR1/FLT1, VEGFR2/KDR, and VEGFR3/FLT4. The assortment of VEGF ligands has distinctive binding specificities for each of these RTKs, which contributes to their diversity of function [10] and [11]. In addition to neutralizing antibodies to VEGF or VEGFRs and to soluble VEGF receptors or receptor hybrids, several TK inhibitors with selectivity for VEGFRs have been developed. Owing to their mode of action at the adenosine triphosphate binding pocket, TK inhibitors are selective rather than specific for a particular kinase(s). Thus, TK inhibitors designed to target VEGF receptors are actually considered multi-kinase inhibitors. For example, sorafenib and sunitinib, both FDA approved drugs, also have a significant activity against platelet-derived growth factor receptor beta (PDGFR β) and fibroblast growth factor receptor (FGFR), KIT, Raf, and FMS receptors [12]. Therefore, VEGFs and RTKs can be considered pharmacodynamic markers (*i.e.* markers that reflect antiangiogenic drug mechanisms of action or effects), and potential predictive markers (*i.e.* markers that predict drug efficacy) [11], [13] and [14]. In TET, the relevance of angiogenesis has been suggested by several approaches, as tumor angiogenesis was correlated with invasiveness [15] and VEGF expression was associated with increased microvessel density [16]. In the present study, we assessed the expression of the possible molecular targets of anti-angiogenic therapy, *i.e.* VEGFA, VEGFC, VEGFD, VEGFR1, VEGFR2, VEGFR3, and PDGFR β , in a series of 200 TET cases, arranged in tissue microarrays (TMA), collected in the framework of a multi-institutional collaborative project.

Materials and methods

Patient and case series

A total of 200 TET cases, surgically removed between January 1996 and December 2008, were included in this multicenter retrospective study, performed in accordance with the Declaration of Helsinki and approved by the institutional Ethical Review Boards. The need for individual patient consent was waived because individuals were not identified in the study. All cases derived from surgical resections. Original hematoxylin and eosin stained slides from 200 TET were reviewed by two pathologists (MM and MP) according to the 2004 WHO classification [2]. In our series, the age of the patients ranged from 8 to 85 years (median age 59.5 years). There were 101 females and 99 males. There were 10 type A, 37 type AB, 31 type B1, 67 type B2, 33 type B3, 6 micronodular thymomas (MNT) and 16 thymic carcinomas. Fourteen carcinomas were of squamous type and two were carcinoma not otherwise specified (NOS). There were no neuroendocrine thymic tumors in our series. According to Masaoka staging system [3], there were 61 stage I, 74 stage II, 44 stage III, and 21 stage IV tumors.

TMA construction

TMA were constructed by extracting 2-mm diameter cores of histologically confirmed representative TET areas from each original paraffin block according to a previously reported procedure [17].

Immunohistochemistry

Immunohistochemistry (IHC) was carried out on 5-micrometer tissue sections stained with primary antibodies as reported in Table 1 The Dako EnVision kits (K4001 and K4003, Glostrup, Denmark) were used for signal amplification, as appropriate. Immunohistochemical analysis was performed by three authors (RL,

RLS and MM) by consensus without knowledge of the clinicopathologic information. All samples were anonymous. The expression of VEGFs and VEGFRs, defined by the presence of distinct specific cytoplasmic staining in tumor epithelial cells, was quantitatively assessed according to the percentage of positive tumor cells. In control sections the specific primary antibodies were omitted or replaced with non-immune serum or isotype-matched immunoglobulins.

Statistical methods

Marker expression was reported as mean $\pm$ SE values, and these values were compared by Student's *t*-test (Table 2 and Table 3). In addition, marker status was dichotomized in high expression and low expression following the box-and-whisker diagram (see Supplemental Fig. 1). A cut-off corresponding to the 50th percentile was chosen for VEGFA, VEGFC, VEGFR1, VEGFR2. In the case of VEGFD, VEGFR3 and PDGFR β the cut-off was the 75th percentile. After dichotomization, the relationships between marker expressions were assessed by chi-square test (contingency table, Table 4). High- and low-expressing cases were also compared by Kaplan‑Meier plots and univariate analyses. Disease free survival (DFS) was defined as the time from surgery to the first of the following events: tumor recurrence at local site or at distant sites. SPSS Version 15.0 (SPSS, Chicago, IL) was used throughout, and $P < 0.05$ was considered statistically significant.

Results

Clinicopathological variables

Cases were categorized into high- and low-risk groups: eighty-four cases were regrouped as low-risk (A, AB, B1, and MNT), and 116 (B2, B3, carcinomas) as high-risk TET [2], [4], [6], [7], [8] and [9]. Follow-up data were available for 133 patients (median follow-up = 60.0 months; range 3‑216 months), and among them, 13 cases, all high risk TET, relapsed. In 9 cases, (two B2, four B3, two Carcinomas, NOS and one Squamous Cell Carcinoma), relapsed tumor tissue was available for the TMA study. Among these 9 cases, four patients had one relapse, three patients had two subsequent relapses; one B3 TET and one case of thymic carcinoma relapsed three and four times, respectively.

Expression of angiogenesis-related biomarkers

By immunohistochemical staining of TMA, distinct proportions of TET cells showed a positive cytoplasmic staining for VEGFA, VEGFC, VEGFD, VEGFR1, VEGFR2, VEGFR3 and PDGFR β with granular and heterogeneous staining in some specimens. The distribution of marker expression for all cases and in high- and low-risk groups are reported as box-and-whisker plots in Supplemental Fig. 1. Based on median values, the percentage of TET cells expressing VEGFA, VEGFC, VEGFR1 and VEGFR2 was higher with respect to VEGFD, VEGFR3 and PDGFR β expression (Supplemental Fig. 1A). Higher numbers of positive cells were found in high-risk than in low-risk TET (Supplemental Fig. 1B and C). Examples of specific positive immunohistochemical staining for VEGFs, and VEGFRs are reported in Supplemental Fig. 2 The mean ($\pm$ standard error, SE) expression of these markers in all cases and in high-risk ($n = 116$) and low-risk TET ($n = 84$) are shown in Table 2. When compared to the low-risk counterpart, high-risk tumors contained not only significantly higher proportion of VEGFA ($P < 0.001$), VEGFC ($P < 0.001$), and VEGFD ($P < 0.001$), but

also of VEGFR1 (*P* = 0.002), VEGFR2 (*P* = 0.013), and VEGFR3 (*P* = 0.041) expressing cells. No differences were observed in terms of PDGFR β expression frequency among low- and high risk tumors.

The mean percentages of expression showed in Table 2 for each marker were then compared by independent-Samples *t*-test. The results of these comparisons (Table 3) showed that in high-risk TET the expression rates of VEGFA (58.4 $\pm$ 3.5 SE) and VEGFC (56.5 $\pm$ 3.4 SE) were significantly higher than those of VEGFD (44.7 $\pm$ 3.4 SE) (*P* = 0.006 and *P* = 0.015, respectively). Among receptors, VEGFR1 (57.5 $\pm$ 3.9 SE) and VEGFR2 (61.9 $\pm$ 3.5 SE) were more expressed then VEGFR3 (38.7 $\pm$ 3.8 SE) (*P* = 0.001 and *P* < 0.001, respectively), and then PDGFR β (30.1 $\pm$ 3.3 SE) (*P* < 0.001 and *P* < 0.001).

In high-risk TET, the correlations existing among expression rates of VEGFs and their receptors were also investigated by chi-square test (contingency table). In these tumors, among significant correlations, positive correlations were found between the expression of VEGFA and its receptor VEGFR1 (*P* = 0.020) and between VEGFA and PDGFR β (*P* = 0.033). A positive correlation was also found between VEGFD and its receptor VEGFR3 (*P* = 0.024), and between VEGFD and PDGFR β expression (*P* = 0.021) (Table 4).

We have also performed subgroup analyses by histologic type and Masaoka stage. As reported in Supplemental Table I, B3 TET and thymic carcinoma expressed significantly higher levels of both VEGFA and VEGFC when compared to A, AB, and B1 tumors. In addition, stage IV tumors expressed higher percentage of VEGFA and VEGFC positive cells than stage I and stage II tumors.

Primary tumor and tumor tissue from the first relapse (9 patients) did not differ in the expression of angiogenic markers (Supplemental Table II). The variable time-course of angiogenic marker expressions, as detected in TMA, in primary tumors *versus* the subsequent relapse/s is shown in Supplemental Fig. 3 A cut-off value of 24 months was chosen to separate short- from long-term relapses. As reported in Supplemental Table III, no significant differences in terms of VEGFs and VEGFRs were found in these two groups.

Kaplan-Meyer plot (Supplemental Fig. 4) and univariate analysis (Supplemental Table IV) did not show any significant association between marker expression and DFS. However, the relationships between high VEGFC expression and shorter DFS showed a trend toward statistical significance (Kaplan‑Meier plot: *P* = 0.146; Univariate analysis: Hazard ratio 2.8, 95% Confidence Interval, 0.7‑13.9; *P* = 0.165).

Discussion

Neovascularization is important in neoplastic development and progression because both tumor growth and metastatic dissemination of tumor cells depend on vascular support. In thymomas, a significant increase in the frequency of immature and intermediate blood vessels has been reported compared to normal thymus and to hyperplastic myasthenic thymus [18], suggesting a possible role for antivascular therapy. VEGF and VEGFR1 & 2 were found to be expressed in normal thymus and in 7 thymomas [19]. As with other epithelial tumors, in TET there appears to be an association between tumor angiogenesis and the invasiveness, particularly based on the correlation between high microvessel density and VEGF expression and advanced clinical stage [15]. In addition, increased circulating levels of VEGF have been found in TET patients [20].

In biomarker discovery and validation, TMA based studies are particular relevant. Immunohistochemistry on TMA yields a high throughput of data to correlate with clinicopathological variables [21] and [22] and to

establish potentially predictive biomarkers in different tumor systems [23]. Due to the TET's rarity, TMA have been rarely arranged: multitumor TMA studies exploring KIT distribution [24] and [25] as well as an EGFR expression study [26] were reported. A TMA study [27] described the histotype distribution of p63 in 66 TET cases. Moreover, Kojika et al. [28] reported the Glucose Transporter 1 (GLUT-1) distribution in TMA constructed with 87 TET cases. Our data, derived from the largest – to our knowledge – retrospective TMA TET series so far established (200 cases), indicated that these tumors expressed statistically significant proportions of endothelial growth factors and their receptors. It is worthy to note that two recent papers [29] and [30] dealing with the biological correlation and the diagnostic/clinical usefulness of FDC-PET/CT in two TET case series (49 and 33 cases, respectively), reported increasing VEGFA expression which immunohistochemically scored low- (A, AB and B1) to high-risk thymomas (B2, B3) and thymic carcinomas. In our series, high-risk TET, defined by regrouping B2-B3 histotypes with thymic carcinomas [4], [5], [6], [7], [8] and [9], when compared to low-risk ones (A, AB, B1 and MNT), were found to contain higher proportion of cells expressing VEGFA, VEGFC, VEGFD, and their RTKs VEGFR1, VEGFR2, VEGFR3. Thus, it was possible to hypothesize the existence of multiple paracrine and/or autocrine loops in TET, particularly in the high-risk ones, involved in TET growth and progression. Anti-angiogenic agents able to inhibit these loops are to be then considered as possible useful tools in advanced/relapsing or metastatizing TET.

Even though numerous angiogenesis inhibitors have been developed and several of them have been FDA approved, not much data are available regarding the use of angiogenesis inhibitors in thymic malignancies. Combined treatment with bevacizumab and erlotinib was evaluated in a phase II trial of 18 patients with recurrent TET. No patients achieved a response, but 11 out of 18 (60%) had stable disease [31]. A partial response has been reported in a phase I study combining docetaxel with aflibercept, a soluble VEGF-A binding receptor [32]. It is interesting to note that no hemorrhagic side-effect has been reported to occur with these drugs, although thymic tumors have large tumor burden and frequently alongside mediastinal vascular structures. Beyond the inhibition of KIT, sunitinib and sorafenib also inhibit VEGFR1, VEGFR2, VEGFR3 at the nanomolar range. An antiangiogenic effect has been attributed to these drugs in thymic carcinoma [33], [34] and [35]. In a recently reported phase II study, sunitinib demonstrated antitumor activity in KIT mutation negative, pretreated thymic carcinoma patients, but not in thymomas [36]. In a phase IB study, motesanib (AMG-706; Amgen), an oral VEGFR1, 2, 3, KIT and PDGFR inhibitor, was reported to control the growth of a thymic carcinoma refractory to chemotherapy [37]. However, in most TET studies antiangiogenetic drugs have been employed without previously evaluating tumor specimen for the distribution and level of putative responsive receptors, thus accounting for heterogeneity and partiality of responses. Immunohistochemistry has also recently been performed in novel clinical trials evaluating VEGF isoform distribution in association with progression free survival in metastatic colorectal cancer patients [38]. Targeted therapies in TET have been occasionally associated with immunohistochemical [39] and [40], and molecular genetic [9], [41], [42] and [43] tumor tissue based characterization, but only on single or on limited TET case series. However, a recent study by the Eastern Cooperative Oncology Group (ECOG) in advanced Thymoma, correlating a tissue based characterization for a variety of biomarkers with outcome indicators, has pointed out to the role that immunohistochemistry plays in the identification of specific treatment targets in TET [44]. Molecularly targeted therapies aim to interfere with molecular mechanisms selectively involved in carcinogenesis and tumor growth in order to optimize the efficacy and minimize the side effects of treatment. Pre-treatment target assessment in primary tumors and in recurrences are prerequisites for therapy with all targeted agents [13], [14] and [41].

However, as for other possible detection methods (ELISA, Western and Northern blots, RT-PCR, *etc.*), methods, reagents, and positivity criteria are still to be defined [13] and [14]. According to reported preliminary data, it appears that by FDG/PET comparison, multiple different metastatic lesions in malignant

thymomas and thymic carcinomas in a single patient might be heterogeneous, thus accounting for a mixed response to targeted treatments and to partial sorafenib response [45].

Conclusions

We showed here that high-risk TET expressed multiple potential targets of already available antiangiogenic treatments. Even though there is no "gold standard" test to select patient for antiangiogenic therapy to date, IHC constitutes a useful tool to this end because it is a simple, common but cheap detection method. It can facilitate to differentiate between tumoral and nontumoral marker expression and to identify pathways potentially responsive to therapeutic approaches. However, in order to transform pharmacodynamic markers into predictive ones, prospective validation studies involving adequate numbers of patients are required. To this end, due to the rarity of high-risk TET, an international, multicentric effort is mandatory.

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Conflict of interest statement

All authors, including the contributors to the "Thymic Epithelial Tumor Working Group", declare no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence their work. Particularly the authors declare no conflict of interests regarding employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications and registrations and grants and any other funding.

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Tablee 1
List of antibodies.

Antibody	Company/catalog No.	Type	Dilution (incubation)	Antigen retrieval/buffer
VEGFA	Neomarkers, CA/MS-350-P	Monoclonal, mouse	1: 50 (90')	Microwave (10')/EDTA pH 8.5
VEGFC	Invitrogen, CA/Z-CVC7	Polyclonal, rabbit	1: 100 (60')	Microwave (10')/citrate pH 6.0
VEGFD	R&D, MN/MAB286	Monoclonal, mouse	1: 50 (90')	Microwave (10')/citrate pH 6.0
VEGFR1	S.Cruz, CA/sc-316	Polyclonal, rabbit	1: 100 (30')	Microwave (10')/citrate pH 6.0
VEGFR2	S.Cruz, CA/sc-504	Polyclonal, rabbit	1: 50 (60')	Microwave (10')/citrate pH 6.0
VEGFR3	Monosan, NED/Monx11065	Monoclonal, mouse	1: 25 (60')	–
PDGFR(3	S.Cruz, CA/sc-339	Polyclonal, rabbit	1: 150 (40')	Microwave (10')/citrate pH 6.0

Table 2
High-risk TET, when compared to low-risk ones, express higher proportions of VEGF and VEGFR positive cells.

Marker	All cases n: 200 Mean ± SE[a]	High-risk n: 116 Mean ± SE	Low-risk n: 84 Mean ± SE	High- vs low-risk P values[b]
VEGFA	49.6 ± 2.7	58.4 ± 3.5	38.0 ± 3.8	<**0.001**
VEGFC	47.8 ± 2.7	56.5 ± 3.4	36.0 ± 4.2	<**0.001**
VEGFD	36.0 ± 2.6	44.7 ± 3.4	24.4 ± 3.7	<**0.001**
VEGFR1	49.7 ± 3.0	57.5 ± 3.9	39.5 ± 4.4	**0.002**
VEGFR2	56.1 ± 2.7	61.9 ± 3.5	48.5 ± 4.1	**0.013**
VEGFR3	34.0 ± 2.7	38.7 ± 3.8	27.8 ± 3.8	**0.041**
PDGFR(3	27.4 ± 2.4	30.1 ± 3.3	23.9 ± 3.3	0.190

[a] Percent of positive tumor cells ± Standard Error (SE).
[b] High-risk vs low-risk TET (independent-Samples t-test). The significant P values are reported in bold.

Table 3
Correlations between marker expressions (means ±SE) in TET.[a]

Comparisons	All cases	High-risk	Low-risk
VEGFA vs VEGFC	0.646	0.707	0.721
VEGFA vs VEGFD	<**0.001**	**0.006**	**0.012**
VEGFC vs VEGFD	**0.002**	**0.015**	**0.039**
VEGFR1 vs VEGFR2	0.110	0.401	0.135
VEGFR1 vs VEGFR3	<**0.001**	**0.001**	0.050
VEGFR1 vs PDGFR{3	<**0.001**	<**0.001**	**0.005**
VEGFR2 vs VEGFR3	<**0.001**	<**0.001**	<**0.001**
VEGFR2 vs PDGFR{3	<**0.001**	<**0.001**	<**0.001**
VEGFR3 vs PDGFR{3	0.067	0.086	0.436

[a] The mean percentages of expression showed in Table 2 for each marker were compared by independent-Samples t-test. P values < 0.05 (in bold), indicate that the first marker is significantly more expressed than the second one.

Table 4
Correlations between marker expressions in high-risk TET (contingency table).

		VEGFA High low	VEGFC High low	VEGFD High low	VEGFR1 High low	VEGFR2 High low	VEGFR3 High low	PDGFR β High low
VEGFA	High		25 19		24 23			35 47
	Low		18 54		20 49			7 27
	P		**<0.001**	NS	**0.020**	NS	NS	**0.033**
VEGFC	High	25 18		36 41				
	Low	19 54		10 29				
	P	**<0.001**		**0.044**	NS	NS	NS	NS
VEGFD	High		36 10				56 23	58 23
	Low		41 29				18 19	17 18
	P	NS	**0.044**		NS	NS	**0.024**	**0.021**
VEGFR1	High	24 20					39 41	38 43
	Low	23 49					8 28	8 27
	P	**0.020**	NS	NS		NS	**0.008**	**0.022**
VEGFR2	High							
	Low	NS	NS	NS	NS		NS	NS
	P							
VEGFR3	High			56 18	39 8			60 19
	Low			23 19	41 28			17 20
	P	NS	NS	**0.024**	**0.008**	NS		**0.003**
PDGFRβ	High	35 7		58 17	38 8		60 17	
	Low	47 27		23 18	43 27		19 20	
	P	**0.033**	NS	**0.021**	**0.022**	NS	**0.003**	

For each marker, high and low expressing tumors were defined on the basis of the cut-off values reported in Section 2. After this dichotomization, possible correlations between marker expressions were investigated by chi-square test (contingency table). P values in bold indicate significant positive correlations between markers. NS: not significant.