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Activating c-KIT mutations in a subset of thymic carcinoma and response to different c-KIT inhibitors

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Background: To analyze a multi-institutional series of type C thymic carcinomas (TCs) (including neuroendocrine tumors), focusing on the expression and mutations of c-KIT.

Materials and methods: Immunohistochemical expression of c-KIT/CD117, p63, CD5 and neuroendocrine markers, as well as mutational analysis of c-KIT exons 9, 11, 13, 14, 17 by direct sequencing of 48 cases of TCs. Immunohistochemical and molecular data were statistically crossed with clinicopathological features.

Results: Overall, 29 tumors (60%) expressed CD117, 69% were positive for CD5 and 85% (41 cases) for p63. Neuroendocrine markers stained all six atypical carcinoids and five poorly-differentiated thymic squamous cell carcinomas. Overall, six CD117-positive cases (12.5%) showed c-KIT mutation. No mutation was detected in CD117-negative tumors and carcinoids. All the mutations were found in poorly-differentiated thymic squamous cell carcinomas expressing CD117, CD5, p63 and lacking neuroendocrine markers (6 of 12 cases with these features). Mutations involved exon 11 (four cases: V559A, L576P, Y553N, W557R), exon 9 (E490K) and exon 17 (D820E).

Conclusions: All TCs need an immunohistochemical screening with CD117, while c-KIT mutation analysis is mandatory only in CD117-positive cases, particularly when coexpressing CD5 and p63, lacking neuroendocrine differentiation. The finding of c-KIT mutation can predict efficacy with different c-KIT inhibitors.

Key words: carcinoma, CD117, c-KIT, immunohistochemistry, mutation, thymus

introduction

Thymic carcinomas (TCs) (type C) are rare malignant neoplasms with poor prognosis and limited effective therapeutic options [1–4]. More than half of TCs express CD117, the product of the proto-oncogene c-KIT, while only a subset of TCs harbor c-KIT mutations [5–14]. Previous works have demonstrated the high frequency of CD117 expression in TC (~80%), whereas thymomas usually do not express CD117 and no c-KIT mutation has been detected in thymomas [5, 6, 8, 9, 11, 12]. Of note, different drugs acting as inhibitors of receptor tyrosine kinases (RTKs) including c-KIT have demonstrated a significant clinical benefit in selected patients with c-KIT-mutated TC [7, 10, 12, 13].

The prevalence and the role of c-KIT mutations in a multi-institutional series of consecutive CD117-positive and

CD117-negative TCs were here investigated. Based on our own experience and on a review of the pertinent literature, practical suggestions for a rationale use of RTK inhibitors in TC are also provided.

materials and methods

Forty-eight TCs and thymic neuroendocrine tumors were retrospectively collected from four different institutions (23 cases from Modena, 17 from Reggio Emilia, 6 from Padova and 2 from Cremona). All the cases were reviewed and reclassified as TCs (42 cases) and thymic neuroendocrine tumors (6 cases, all of them with the features of atypical carcinoid), according to the morphological criteria of the 2004-World Health Organisation classification of thymic tumors [2]. The material consisted of 31 mediastinal biopsies (64.5%) and 17 surgical resections. Two cases included in the present study have been previously published as case reports [10, 14].

Clinicopathological data were recorded from the pathological reports and the clinical charts. The following variables were registered in the

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present study: sex, age, histotype, immunohistochemical expression of CD117, CD5, p63, chromogranin, synaptophysin and the presence of *c-KIT* mutations. Patients' survival was not considered in the study because of the relatively limited number of cases with different tumor stage and with variable therapeutic approaches.

The study was conducted in accordance with the precepts of the Helsinki Declaration. According to the national laws, this study does not require any ethical committee approval since all data were handled anonymously.

immunohistochemical analysis

In each case, 4- μ thick sections were obtained from a representative block. Sections were air dried overnight at 37°C, then deparaffinized in xylene and rehydrated through a decreasing concentration of alcohol to water. Endogenous peroxidase activity was blocked by immersion for 10 min with 3% hydrogen peroxide (H₂O₂) in methanol. Incubation with primary antibodies was accomplished with a modified streptavidin–biotin–peroxidase technique using an automated immunostainer (Ventana Benchmark, Tucson, AZ); 3'-3diaminobenzidine was used as the chromogene and Harris's hematoxylin as the counterstain. The panel of antibodies used in the study and their technical characteristics are the following: CD117 (clone A4502, Dakopatts, Glostrup, Denmark; 1 : 200 dilution without antigen retrieval), CD5 (clone SP19, Ventana; prediluted with microwave antigen retrieval), p63 (clone 4A4, NeoMarkers, Fremont, CA; 1 : 400 dilution with microwave antigen retrieval), chromogranin (clone LK2H10, Ventana; prediluted with microwave antigen retrieval) and synaptophysin (polyclonal, Ventana; prediluted with microwave antigen retrieval).

Negative and positive controls were included in each batch. Negative controls for specificity of staining were carried out by immunostaining duplicate sections with nonimmune mouse IgG, at the same concentration as that of the corresponding primary antibody.

A normal tonsil tissue was used as a positive control for CD117 (mast cells), CD5 (T lymphocytes) and p63 (squamous epithelium), while a pulmonary typical carcinoid serve as positive control for chromogranin and synaptophysin. The percentage of positive cells and the intensity of staining (0: negative; 1+: weak; 2+: moderate; 3+: strong) were recorded, and a lesion was considered positive when at least 10% of the cells reacted with a moderate or strong intensity on the relevant subcellular localization (nuclear for p63, cytoplasmic for chromogranin and synaptophysin and cytoplasmic/membranous for CD117 and CD5).

mutational analysis

Molecular analysis was carried out on formalin-fixed paraffin-embedded tissues in 43 cases and on frozen samples in 5 cases. DNA content was extracted from tumor cells. PCR was carried out in 20 μ l reactions containing 50–200 ng DNA, 2 μ l of commercial PCR buffer at final concentration 1X (Applied Biosystems, Foster City, CA), 1.0–2.0 mM MgCl₂, 400 μ M of each dNTP, 40 pmol of each primer and 3 units of AmpliTaq gold polymerase (Applied Biosystems). PCR reaction was carried out on Uno II Thermoblock (Biometra, Gottingen, Germany). Initial denaturation at 94°C for 10 min was followed by 41 cycles and by a final extension step (7 min at 72°C). The cycles included denaturation at 95°C for 1 min, annealing at 53°C–66°C for 1 min and extension at 72°C for 2 min. The amplified DNA was electrophoresed on 2% agarose gel for 1 h at 110 V. The amplification products were then purified by using MinElute PCR purification Kit (Qiagen, Hilden, Germany) as indicated by the manufacturer's instructions. PCR products were then sequenced in both directions with ABI Prism BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems), using the same primers as those employed for PCR. Cycle sequencing products were finally purified by Centri-Sep Spin Columns (Applied Biosystems) and subsequently run on the ABI Prism 310 automatic sequencer (Applied Biosystems). The data were analyzed with the Sequencing Analysis 5.2 Software (Applied Biosystems).

The forward and reverse oligonucleotide primers used to amplify *c-KIT* exons 9, 11, 13, 14 and 17 are listed in supplemental Table S1 (available at *Annals of Oncology* online).

statistical analysis

The correlation between clinicopathological variables, immunohistochemical and molecular results was carried out using contingency table methods and tested for significance using the Pearson's χ^2 test (SPSS version 16.0; Chicago Inc., Chicago, IL). A difference with probability (*P*) values <0.05 was considered as significant.

results

Clinicopathological features are summarized in Table 1. Briefly, the case series consisted of 26 males (54%) and 22 females with a mean age of 61 years. Apart from 6 atypical carcinoids, non-neuroendocrine TCs mainly consisted of squamous cell histotype (38 cases) with poorly-differentiated morphology (24 cases). Overall, 42 cases (87%) of the tumors were in stage III or IV at diagnosis. Overall survival was available in 38 patients, ranging from 8 to 89 months (mean, 25 months).

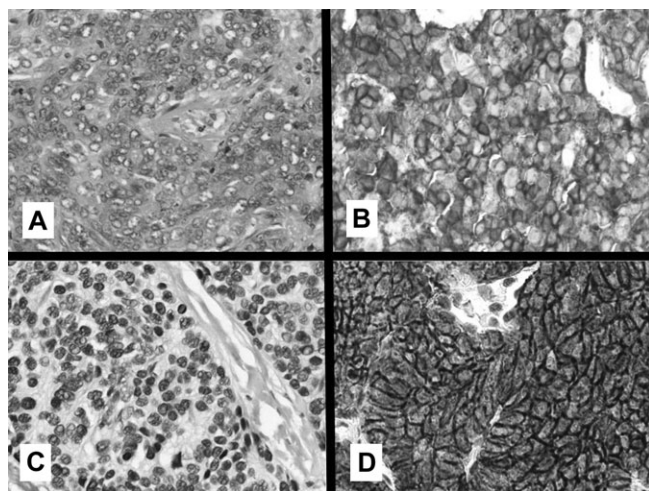
Immunohistochemical and molecular characteristics are summarized in Table 2. Of note, 29 cases (60%) showed immunostaining for CD117 (all carcinoids and 23%/60.5% of squamous cell carcinomas), while 33 cases (69%) were positive with CD5 (32%/84% squamous cell carcinomas and lymphoepithelioma-like carcinoma) and 41 cases (85%) immunoreacted with p63 (all squamous cell carcinomas, mucoepidermoid carcinoma, lymphoepithelioma-like carcinoma and myoepithelial carcinoma) (Figure 1). Positive staining with chromogranin and synaptophysin was observed in all six atypical carcinoids and in one and four poorly-differentiated thymic squamous cell carcinomas, respectively.

Table 1. Clinicopathologic characteristics of thymic neoplasms

Characteristics	Cases (N = 48) n (%)
Age	Mean, 61 years; median 63.5 years (range, 34–84 years)
Sex	
Male	26 (54)
Female	22 (46)
Type of material	
Biopsy	31 (64.5)
Resection	17 (33.5)
Histotype	
Squamous cell	38 (79)
Adenocarcinoma	1 (2)
Mucoepidermoid	1 (2)
Atypical carcinoid	6 (13)
Lymphoepithelioma like	1 (2)
Myoepithelial	1 (2)
Stage	
II	6 (13)
III	11 (23)
IVA	20 (41)
IVB	11 (23)

Table 2. Immunohistochemical and molecular features of 48 thymic carcinomas

Feature	Cases (N = 48) n (%)
CD117	
Positive	29 (60)
Negative	19 (40)
CD5	
Positive	33 (69)
Negative	15 (31)
p63	
Positive	41 (85)
Negative	7 (15)
Synaptophysin	
Positive	10 (21)
Negative	38 (79)
Chromogranin	
Positive	7 (15)
Negative	41 (85)
c-KIT	
Wild type	42 (87.5)
Mutated	6 (12.5)
Exon 11 (V559A, L576P, Y553N, W557R)	4 (66)
Exon 17 (D820E)	1 (17)
Exon 9 (E490K)	1 (17)

**Figure 1.** Example of poorly-differentiated thymic carcinoma (A, haematoxylin–eosin staining $\times 200$) expressing CD5 (B, immunohistochemistry $\times 200$), p63 (C, immunohistochemistry $\times 200$) and CD117 (D, immunohistochemistry $\times 200$).

Overall, activating c-KIT mutations were observed in six cases (12.5%). When considering only CD117-positive neoplasms, the rate of c-KIT mutations raised to 21% (6 of 29 cases). Most important, despite positivity for CD117, no mutation was detected in atypical carcinoids. Excluding atypical carcinoids, the observed frequency of c-KIT mutations in CD117-positive TCs was 26% (6 of 23 cases). All gene alterations consisted of missense mutations in heterozygosis

involving exon 11 (four cases), exon 9 (one case) and exon 17 (one case). More in detail, mutations in exon 11 were V559A, L576P, Y553N and W557R (supplemental Figure S1, available at *Annals of Oncology* online): three of these mutations are unprecedented in TCs, while L576P was previously found in a poorly-differentiated TC [9]. Also the type of c-KIT mutation detected in exon 9 (E490K) was not previously reported in TC (supplemental Figure S1, available at *Annals of Oncology* online).

At statistical analysis, only CD117 expression was significantly correlated with c-KIT mutations ($P = 0.034$). All the 6 c-KIT-mutated cases were robustly positive (score 3+ in $>50\%$ of tumor cells) for CD117. Among wild-type cases ($n = 42$), 23 tumors stained with CD117, while 19 cases were completely negative.

Finally, a striking relationship between morphology, immunoprofile and c-KIT mutations was observed. All c-KIT-mutated TCs consisted of poorly-differentiated squamous cell carcinomas showing a solid growth of monomorphic cells with moderate cytoplasm and nuclei with a single prominent nucleolus dissected by bands of dense collagen. Tumor cells strongly expressed CD117, CD5 and p63 but did not stain for neuroendocrine markers. Taking in consideration only TCs displaying these latter characteristics (12 cases overall), c-KIT mutations were detected in half of the cases (6 cases).

discussion

TCs are aggressive neoplasms presenting as unresectable mediastinal masses in the majority of the cases, with multimodal chemoradiotherapy resulting often ineffective in advanced stage [1, 2]. Despite TCs may show several genetic alterations, no reliable molecular targets and relevant targeted therapies have been so far identified [3, 15–17]. Among different molecular pathways, a consistent body of evidence suggests a critical role of the proto-oncogene c-KIT in TC [16, 17]. In fact, many TCs are characterized by high levels of c-KIT protein transcripts and overexpression of CD117, the product of c-KIT [5, 6]. This biomarker is very useful in the differential diagnosis with thymomas and other mimicking neoplasms (i.e. squamous cell carcinoma of the lung), which are typically negative [5, 6]. Most importantly, in a subgroup of TCs, CD117 expression is related to a constitutive somatic activating c-KIT mutation [7, 9, 10, 13, 14]. It is well known that, among CD117-positive tumors [namely gastrointestinal stromal tumors (GIST), small-cell lung cancer, seminoma, melanoma, adenoid cystic carcinoma], only those showing c-KIT mutations have a clinical benefit from selective c-KIT inhibitors (i.e. imatinib, sunitinib, sorafenib) [18].

Several works have analyzed c-KIT mutations in thymomas and TCs: results are summarized in Table 3 and supplemental Table S2 (available at *Annals of Oncology* online). Pan et al. [5] evidenced CD117 expression in 86% of TCs, but direct sequencing of the c-KIT juxtamembrane (exons 9 and 11) and tyrosine kinase (exons 13 and 17) domains failed to evidence mutational alterations in 22 TCs. Similarly, Tsuchida et al. [9] demonstrated CD117 immunostaining in 65% of 17 TCs, but no mutations were detected in the 13 analyzed cases. Petrini et al. [12] recently tested c-KIT mutations in eight TCs, five thymomas and one TC cell line (T1889) by direct sequencing

Table 3. Summary of clinicopathological features of published thymic carcinomas with c-KIT mutations and/or treated with c-KIT inhibitors

Reference	Age/sex	Histologic type	c-KIT mutation	Stage	Therapy	Drug	Clinical response
Strobel et al. [7]	54/M	TC, squamous cell, G3	V560del ex11	Metastatic	None	Imatinib	SD (6 months)
Bisagni et al. [10]	46/M	TC, squamous cell, G3	D820E ex17	pT3, N2, M1	S + CT + RT	Sorafenib	PR (>15 months)
Disel et al. [13]	47/F	TC, squamous cell, G3	del577-578-579 ex11	IVA	CT + RT	Sorafenib	SD
Buti et al. [14]	48/M	TC, squamous cell, G3	Y553N	IV	CT	Imatinib	PR (>8 months)
Li et al. [19]	46/M	TC, squamous cell, G3	ND	IV	CT	Sorafenib	SD (>9 months)
Chuah et al. [20]	NA	Type-B2	ND	I	Imatinib + CT	Dasatinib	LR
Hamada et al. [21]	Case 1: 62/M	Atypical carcinoid	None	Invasive	CT	Imatinib	Good clinical response
	Case 2: 58/M	Atypical carcinoid	ND	Invasive	S + RT	Nessuno	Recurrence and metastasis
Giaccone et al. [22]	Case 1: 36/M	TC	ND	IVB	CT	Imatinib	PD
	Case 2: 67/M	Type-B3	None	IVA	S + RT + CT	Imatinib	SD
	Case 3: 47/M	Type-B2/3	ND	IVA	CT	Imatinib	SD
	Case 4: 76/M	TC	ND	IVB	None	Imatinib	PD
	Case 5: 36/M	TC	ND	IVB	CT	Imatinib	PD
	Case 6: 71/M	TC	None	IVB	None	Imatinib	PD
	Case 7: 69/F	TC, squamous cell type	None	IVB	None	Imatinib	PD
	Case 8: 35/M	TC, squamous cell type	None	IVB	CT + imatinib	Sunitinib	PR
Strobel et al. [23]	Case 2: 69/M	TC, squamous cell type	None	IVA	S + RT + CT	Sunitinib	PR
	Case 3: 77/M	TC, squamous cell type	None	II	S	Sunitinib	PR
	Case 4: 28/F	TC, undifferentiated	None	IVB	CT + RT	Sunitinib	PR (2 months)
	15 cases	4 type B2	None	NA	NA	Imatinib	PD
Palmieri et al. [24]		2 type B2/B3	None	NA	NA	Imatinib	PD
		6 type B3	None	NA	NA	Imatinib	1 SD
		3 TC	None	NA	NA	Imatinib	PD

CT, chemotherapy; F, female; G3, grade 3/poorly differentiated; M, male; NA, not available; ND, not done; PD, progression disease; PR, partial response; RT, radiotherapy; S, surgery; SD, stable disease; TC, thymic carcinoma.

analysis from exon 1 to exon 20. Despite a significant difference of CD117 expression between TCs (46%) and thymomas (4%), the authors did not find c-KIT mutations [12]. Of note, CD117 expression was observed only in primary and not in relapsed tumors, but it was significantly associated with a worse overall and progression-free survival [12]. Yoh et al. [8] collected 24 thymomas and 17 TCs, detecting epidermal growth factor receptor mutations in 2 thymomas and a c-KIT missense mutation on exon 11 in 1 TC (L576P).

Finally, Girard et al. [11] evidenced 2 c-KIT mutated of 7 TCs (1 in exon 14, H697Y and 1 in exon 11, V560del). In particular, the novel exon 14 missense mutation H697Y is highly sensitive to sunitinib rather than imatinib. It is noteworthy that all c-KIT-mutated TCs consisted of squamous cell carcinoma histotype with poorly-differentiated morphology (grade 3) without keratinization.

Although prospective trials using imatinib in different thymic malignancies have failed to demonstrate clinical responses [17, 22, 24], a handful of case reports have yielded promising results in selected patients [7, 10, 13, 14, 21].

In 2004, Strobel et al. [7] first reported a case of a chemoresistant undifferentiated TC with exon 11 (V560del) c-KIT mutation experiencing a stable disease for 6 months using imatinib mesylate.

Subsequently, Bisagni et al. [10] reported a long-lasting partial response in a TC showing a missense mutation on exon 17 (D820E). Based on the previous clinical experience dealing with this kind of mutation in GIST, the authors decided to treat the patient with sorafenib, a small molecule inhibiting several

targets, as c-KIT, PDGFRs, vascular endothelial growth factor receptors (VEGFRs), FLT-3, c-RAF, B-RAF.

More recently, Disel et al. [13] reported a deletion mutation on exon 11 (577–579del) in an advanced TC with squamous features. The patient had a stable disease using sorafenib. Of note, we are experiencing a consistent partial response (>8 months) in a patient with a poorly-differentiated chemoresistant TC in which a novel missense mutation on exon 11 (Y553N) was detected [14]. The patient was treated with imatinib similarly to what happens in GIST harboring the same mutation.

Other c-KIT mutations have been reported in TCs, involving exon 11 (L576P) and exon 14 (H697Y). Supporting the critical role of c-KIT mutations in TCs, no clinical responses have been registered in 'wild-type' TCs treated with imatinib in recent trials [17, 22, 24], although Hamada et al. [21] described a clinical benefit in a wild-type thymic atypical carcinoid treated with imatinib.

Other case reports have highlighted some effectiveness of sorafenib [19], dasatinib [20] and somatostatin receptor-2 [25] in TC or metastatic thymoma.

Strobel et al. [23] recently reported a partial response in four patients with 'wild-type' TCs (three TCs with squamous cell differentiation and one with undifferentiated TC), treated with sunitinib. However, differently to imatinib, sunitinib is a multi-targeted RTK inhibitor interfering with several targets, as c-KIT, PDGFRs, VEGFRs, FLT-3, then also having an antiangiogenic role. In advanced GIST, sunitinib is adopted as second-line therapy when the tumor develops resistance to imatinib and the drug is particularly effective in GISTs harboring c-KIT mutations on exon 13 and 17 or with PDGFR-alpha mutations [26].

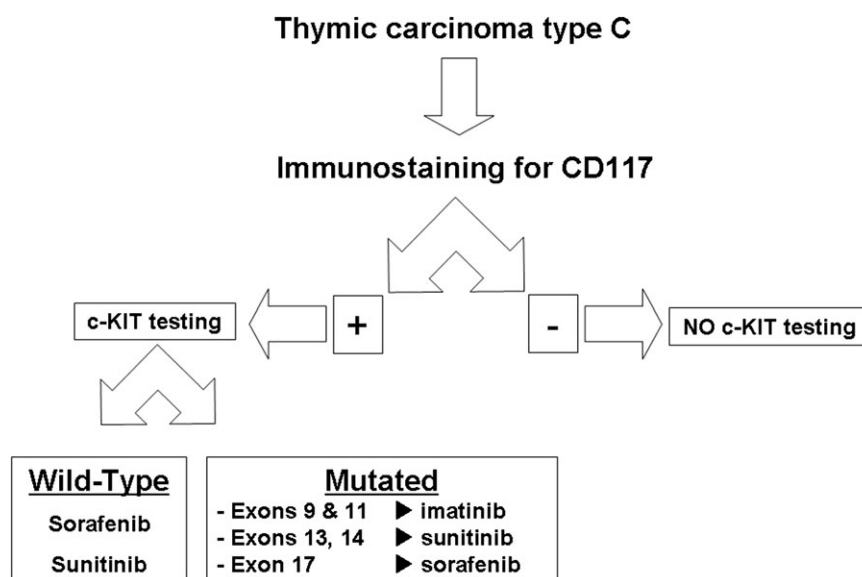


Figure 2. An helpful therapeutic decision tree using targeted therapies in thymic carcinoma based on the expression and mutations of c-KIT.

Far from being a standard of care and in light of our data and previous experiences with imatinib, sorafenib and sunitinib in TC [7, 9, 10, 13, 14], a practical therapeutic algorithm based on c-KIT mutation type was illustrated in Figure 2. Briefly, it seems that the effective use of imatinib mesylate in TCs significantly depends on the presence and type of c-KIT mutation detected in tumor cells, this pharmacological agent selectively inhibiting type III RTKs. On the other hand, sorafenib and sunitinib are less selective than imatinib and can be effectively adopted in TCs harboring imatinib-resistant c-KIT mutations (i.e. involving exons 13, 14 and 17) or in wild-type TCs due to their antiangiogenic role, as suggested in the recent preliminary experience of Strobel et al. [23].

conclusion

In our opinion, an immunohistochemical screening including a small panel of antibodies (CD117, CD5, p63 and neuroendocrine markers) is mandatory in all cases of TC. All CD117-positive TCs should be tested for c-KIT mutations expanding molecular test to exons 9, 11, 13, 14 and 17. The probability to find mutations is higher in CD117-positive thymic squamous cell carcinoma with poorly-differentiated morphology on hematoxylin–eosin and with coexpression of CD5 and p63 in absence of neuroendocrine markers. Mutations involving c-KIT in TCs seem to possess the same biological significance of those observed in GIST, and based on the presence/absence and type of c-KIT mutation, TCs may benefit of a targeted therapy with different RTK inhibitors.

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disclosure

The authors declare no conflict of interest.

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