Original paper

C-KIT mutation in thymic carcinomas

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Thymic epithelial tumours are rare malignancies of the anterior superior mediastinum. Several studies have analysed the presence of c-KIT mutations in thymic carcinoma. Immunohistochemical c-KIT expression and mutations in exons 8, 9, 11, 13, 14, 17, and 18 of the KIT gene and in the promoter region of the TERT gene (chr5, 1,295,228C>T/A and 1,295,250C>T) were analysed by PCR based direct sequencing using representative formalin-fixed paraffin-embedded tumour samples of 18 thymic carcinomas. Of 18 patients, 4 test samples were excluded from the study due to inadequate DNA quality. Of 14 patients with thymic carcinomas, KIT and TERT mutation was not detected in any samples. C-KIT expression was associated with nearly a worse overall survival (median time 24.160-49.840, log-rank, $p = 0.05$). We showed that squamous cell carcinomas led to worse survival than other subtypes. As expected, TNM stage II was significantly correlated with better OS ($p = 0.015$). Thymic carcinoma is characterised by a KIT-positive and CD5-positive staining pattern. We report a worse overall survival for patients with c-KIT expressing tumours. These data suggest a negative prognostic role for c-KIT expression especially within the first 5 years.

Key words: thymic cancer, KIT, immunohistochemical staining, mutation analysis, survival analysis.

Introduction

Thymic epithelial tumours (TET) are rare malignancies of the anterior superior mediastinum with an overall annual incidence of 0.15 per 100 000 inhabitants. They typically occur in people over 40 years old and peak in the seventh decade of life [1]. Males have a slightly higher risk of developing thymic carcinomas (TCs) than females [2]. The WHO 2015 classification distinguishes type A, AB, B1, B2, and B3 thymomas, micronodular thymoma with lymphoid stroma, metaplastic thymoma, rare thymomas, TCs, thymic neuroendocrine tumours, and combined thymic carcinomas [3]. For TETs, the Masaoka classification was published in 1981, and Koga *et al*. modified this classification in 1994. This Masaoka-Koga classification has been widely used for a long time. Recently a new TNM classification stated that tumours that all totally encapsulated, extend into the adjacent fat tissue, and invade the mediastinal pleura be defined collectively as T1 tumours. Only tumours that invade the pericardium are classified as T2. T3 and T4 tumours invade lung, intrathoracic large vessels, and other tissues. Concerning the N descriptor, perithymic nodes were newly defined as N1 and deep intrathoracic/cervical nodes are defined as N2. Regarding the stage grouping, T1N0M0 was classified as stage I, T2N0M0 as stage II, T3N0M0 as stage IIIA, and T4N0M0 as stage IIIB. Tumours with

positive N1 nodes and pleural dissemination were classified as stage IVA. Stage IVB tumours had lung nodules, N2 node involvement, and distant metastases [3].

They represent a wide variety of histological and molecular malignant entities that may have disturbing or aggressive behaviours [1]. The resulting data yields separate entities in the clinical and biological behaviour of thymoma and TC [3]. Unlike thymoma, TC is a highly aggressive tumour with frequent lymphatic and haematogenous metastasis.

Surgical treatment remains the only curative treatment and represents the most important prognostic factor in terms of complete resection, and overall survival [4].

The development of molecularly targeted drugs has so far been limited by the lack of information on the molecular alterations of TETs [5].

Several studies have analysed the presence of c-KIT mutations in the TC, which is rare, but is present in about 10% of cases of mutational conditions [2]. Immunohistochemical (IHC) c-KIT positivity has been found in more than 80% of thymic malignancies [6].

We performed a molecular profiling study to derive further insight into the pathogenesis of TCs and to identify potential novel targets for therapy. We focused the analysis on TCs, because of their aggressiveness and due to the need to improve therapy.

We explored 18 TCs with a panel of immunohistochemical stains for antigens (CD117/ c-KIT, CD5, p63, chromogranin, synaptophysin, CD56). Furthermore, we carried out DNA sequencing of TCs with a panel of comprising oncogenes and tumour suppressor genes known to be frequently altered in various tumours. Currently, such gene panels are increasingly utilised in diagnostic molecular pathology for the identification of therapeutic targets in various malignancies.

Material and methods

In total, 18 TC patients were enrolled in this study. Cases were obtained from the archives of the Department of Pathology, Istanbul University, Istanbul, Turkey. This study was approved by the Medical Ethics Committee of the Health Sciences University Istanbul Education and Research Hospital (Approval No: 14.09.2018/1425) and was conducted according to the principles of the Declaration of Helsinki.

Immunohistochemistry

Paraffin-embedded specimens were cut into 5-μm sections and slides were autoclaved for 10 min in the target retrieval solution with 0.01 mol/l Tris buffer, 0.001 mol/l EDTA (pH 9.0) and 0.01 mol/l citrate buffer. The primary antibodies used in all cases were the following: c-KIT /CD117 (Polyclonal Rabbit anti-Human CD117: A4502, DakoCytomation Japan, Tokyo, Japan), CD5 (monoclonal Mouse anti-human CD5: NCL-CD5-4C7; Mitsubishi Kagaku Iatron,Tokyo, Japan), p63 (clone 4A4, NeoMarkers, Fremont, CA, USA; 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). Immunopositivity was scored as 0, undetectable; 1+, heterogeneous positivity less than 50% of tumour cells; 2+, strong positivity in 50-90% of tumour cells; and 3+, diffuse positivity in more than 90% of tumour cells.

Mutation analysis

Mutations in exons 8, 9, 11, 13, 14, 17, and 18 of the KIT gene and in the promoter region of the *TERT* gene (chr5, 1,295,228C>T and 1,295,250C>T) were analyzed by previously described polymerase chain reaction (PCR)-based direct sequencing (analytical sensitivity 25%) with appropriate primers (Table I) [7, 8].

Results

Clinicopathological features are summarised in Table II.

Table I. Primer sequences

Gene	Forward primer sequence (5→3′)	Reverse primer sequence (5→3′)
KIT exon 8	TCAGGAAGGTTGTAGGGATT	AATTGCAGTCCTTCCCCTCT
KIT exon 9	AAGTATGCCACATCCCAAGT	ATGGTCAATGTTGGAATGAA
KIT exon 11	CCAGAGTGCTCTAATGACTGA	GTTTCAGGTGGAACAAAACA
KIT exon 13	CATCAGTTTGCCAGTTGTG	ATCTAGCATTGCCAAAATCA
KIT exon 14	GACCACCCTTGGGTATTTTTATG	AACCCTTATGACCCCATGAACT
KIT exon 17	AAAAAGTTAGTTTTCACTCTTTACAA	TCGAAAGTTGAAACTAAAAATCC
KIT exon 18	GTACTCAAGTTATCACTCCACATTT	TCAAGAAGATGCTCTGAGTCTAAT
TERT	CAGCGCTGCCTGAAACTC	GTCCTGCCCCTTCACCTT

Table II. Clinicopathologic characteristics of thymic carcinomas (n = 18)

Patients' characteristics	Patient No. (%)
Age	Mean, 55.94 years; median 61.5 years
	(range, 11-78 years)
Sex	
Male	13 (72.2%)
Female	5 (27.7%)
Histotype	
Squamous cell	12 (66.6%)
Undifferentiated	1 (5.5%)
Neuroendocrine carcinoma (Large cell)	3 (16.6%)
Lymphoepithelioma like	2 (11.1%)
Stage	
II	5 (27.7%)
III	8 (44.4%)
IV	5 (27.7%)
Survival	
Alive	8 (44.4%)
Dead	10 (55.5%)

Eighteen patients with a histological diagnosis of TC were recruited at the Department of Pathology of the university from March 2008 to June 2018. Thirteen patients were males (72.2%), and 5 were females (27.7%); their mean and median ages at diagnosis were 55.94 and 61.5 years (range 11-78 years).

Histopathologically the tumour samples were 12 squamous cell carcinoma (66%), 1 undifferentiated carcinoma (5.5%), 3 large cell neuroendocrine carcinoma (16.6%) and 2 lymphoepithelioma like carcinoma (11.1%).

At the time of analysis, all but 8 patients were alive. Median follow-up was 30 months (range 15-64 months). Overall, 13 cases (72.2%) of the tumours were in stage III or IV at diagnosis. All patients had received at least one prior line of chemotherapy for metastatic or locally advanced disease and presented metastatic disease at the time of enrolment, with pleura being the most common site of metastasis.

Immunohistochemistry

Fourteen TCs (77.7%) revealed heterogeneous or diffuse membranous immunoreactivity for KIT (Fig. 1). The percentage of positive staining was 100% in lymphoepithelioma like carcinoma and large cell neuroendocrine carcinoma, whereas squamous cell carcinoma showed less (72%) and undifferentiated carcinoma showed none (0%).

Table III. Immunohistochemical and molecular features of 18 thymic carcinomas

Feature	Cases (n = 18)
	n (%)
CD117	
Positive	14 (77.7%)
Negative	4 (22.2%)
CD5	
Positive	16 (88.8%)
Negative	2 (11.1%)
p63	
Positive	13 (72.2%)
Negative	5 (27.7%)
Synaptophysin	
Positive	3 (16.6%)
Negative	15 (83.3%)
Chromogranin	
Positive	3 (16.6%)
Negative	15 (83.3%)
c-KIT	
Wild type	14 (100%)
Mutated	0 (0%)
TERT	
Wild type	14 (100%)
Mutated	0 (0%)

CD5 expression was observed in 16 tumour samples (88.8%) (100%/100%/91.6%/50% undifferentiated carcinomas, large cell neuroendocrine carcinomas, squamous cell carcinomas and lymphoepithelioma like carcinomas respectively) (Fig. 1). Thirteen cases (72.2%) immunoreacted with p63 (all squamous cell carcinomas and 1 lymphoepithelioma like carcinoma) (Table III).

Immunoexpression was score 3+ or score 0 in all cases. The scoring system was not related to other parameters.

Sanger sequencing PCR

Mutation analysis of the *c-KIT* gene and *TERT* gene was performed in 12 squamous cell carcinomas, 1 undifferentiated carcinomas, 2 lymphoepithelioma like carcinomas and 3 large cell neuroendocrine carcinomas. In the remaining four samples, DNA was not successfully extracted. Mutation analysis was completed in 14 of 18 samples, but none of the tested samples showed mutations in any of the four exons. Also none of the tested samples showed mutations in the *TERT* gene.

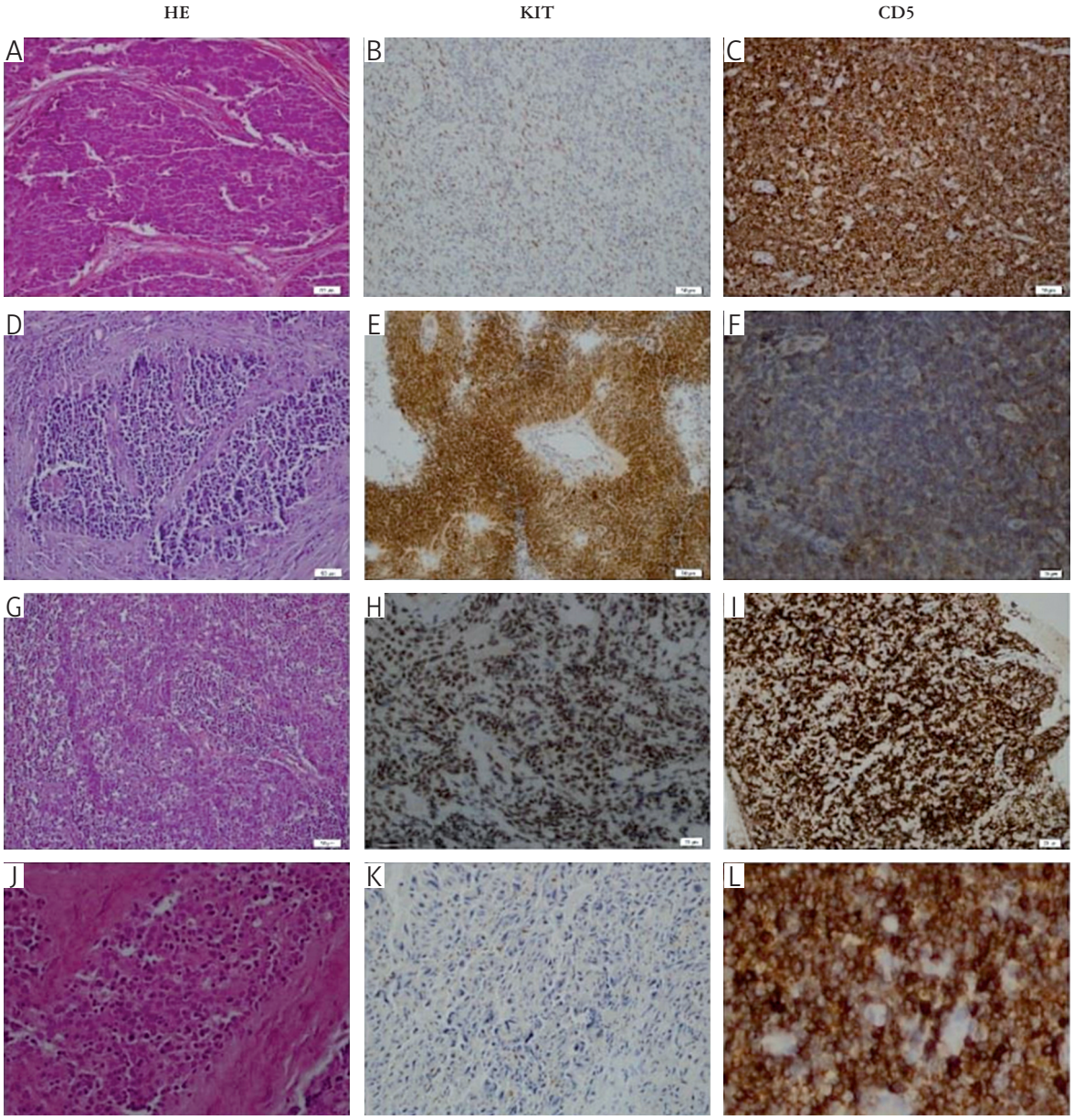


Fig. 1. Light microscopic and immunohistochemical expressions of KIT and CD5 were shown: A-C) squamous cell carcinoma of the thymus in 50-year-old female patient; D-F) large cell neuroendocrine carcinoma of the thymus in 63-year-old male patient; G-I) lymphoepithelioma like carcinoma of the thymus in 17-year-old female patient; J-L) undifferentiated carcinoma of the thymus in 36-year-old male patient. KIT and CD5 expressions were positive in squamous cell carcinoma, large cell neuroendocrine carcinoma and lymphoepithelioma like carcinoma of the thymus (B, C, E, F, H, I), but KIT expression was not positive in undifferentiated carcinoma of the thymus (K)

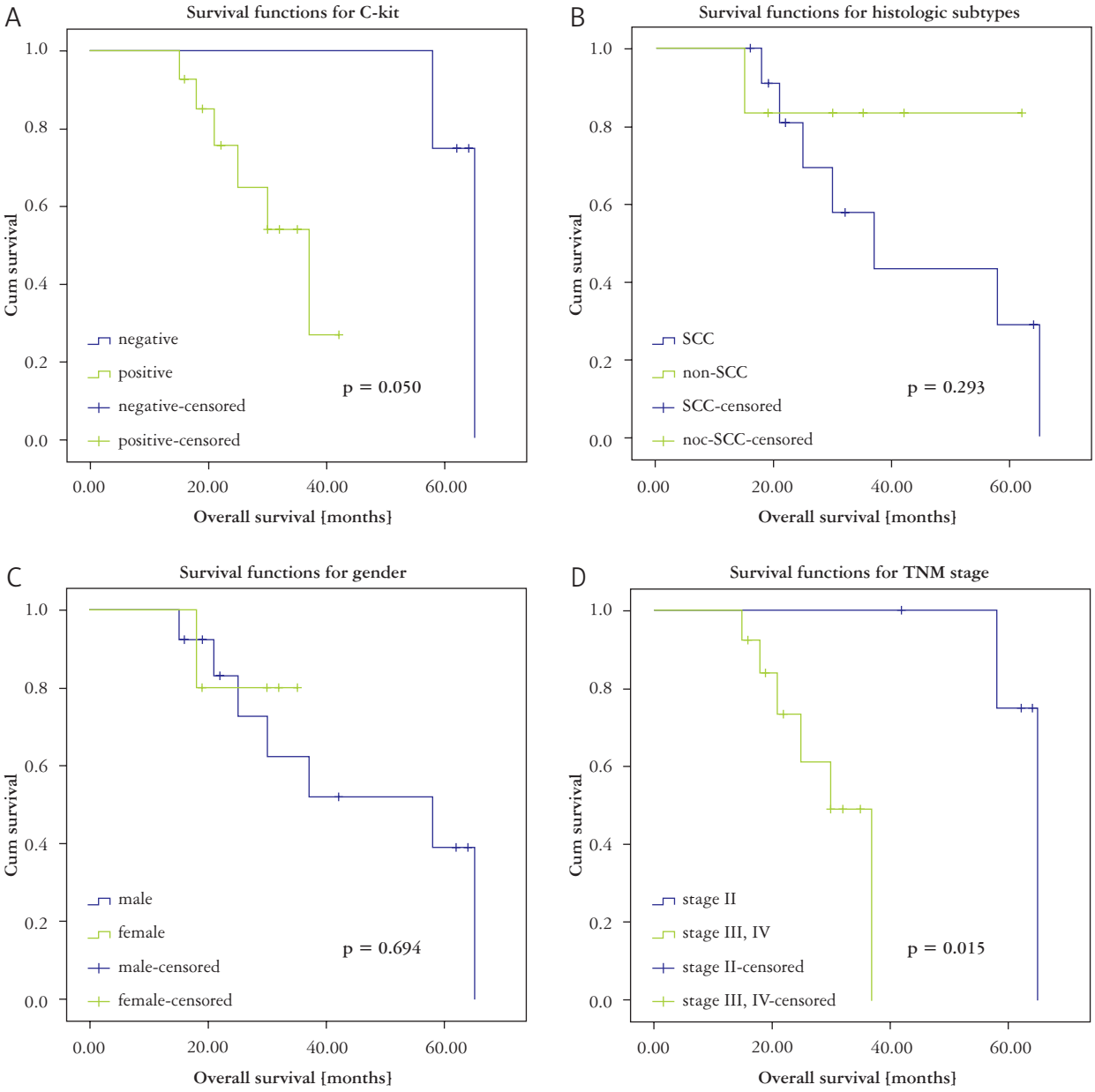
Survival analysis

C-KIT expression was associated nearly with a worse OS (overall survival) (median time 24.160-49.840, log-rank, p = 0.05) (Fig. 2A). While 5-year OS was 75% for c-KIT-negative patients, 5-year OS was not observed in any of the c-KIT-positive patients.

We plotted the Kaplan-Meier curves according to the histological subtype, gender and TNM staging (Fig. 2B-D). There was no statistical difference for OS analysis between histological types and gender (median time 20.359-53.641, log-rank, p = 0.293 and median time 19.643-96.357, log-rank, p = 0.694, respectively). However, we showed that squamous cell carcinomas led to worse survival than other subtypes. As expected, TNM stage II was significantly correlated with better OS (median time 23.572-36.428, log-rank, p = 0.015).

Discussion

KIT is a transmembrane receptor with tyrosine kinase activity encoded by the proto-oncogene c-KIT



scc – squamous cell carcinoma; non-scc – non-squamous cell carcinoma

Fig. 2. OS was worse in cases showing c-kit expression than cases without c-kit expression (p = 0.050) (A). Squamous cell carcinomas showed worse OS than other histological subtypes (p = 0.293) (B). There was no relationship between OS and gender (p = 0.694) (C). There was no relationship between OS and TNM (stage II and stage III, IV) (p = 0.015) (D)

and plays a major role in the development and maintenance of Gastrointestinal stromal tumours (GISTs). KIT expression does not predict the presence and/or type of the c-KIT mutation, however its expression in GISTs has been associated with all c-KIT activating mutations that mainly occur in exons 9 (extracellular domain), 11 (juxtamembrane domain), 13 (first kinase domain), and 17 (activation loop). These mutations lead to the activation of the KIT kinase [9].

C-KIT expression in TCs has been reported to range between 50% and 88% [9]. Thymic epithelial cells are considered the cells of origin for thymic carcinoma, and normal thymic epithelium does not express c-KIT [10, 12].

The overexpression of KIT in TC provides diagnostic utility in discerning this tumour from other carcinomas, especially squamous cell carcinomas arising from the lung and oesophagus, which are the main differential diagnoses in this anatomical site. It is generally thought that thymic carcinomas include a heterogeneous group of carcinomas and that they lack distinctive histological features. In conclusion, some authors claim that the diagnosis of TC can only be made by excluding other primaries [13]. However,

there is evidence that thymic carcinomas may have a special immunohistochemical profile not shared by other morphological mimics. The immunoreactivity for CD5 [11] except some pancreatic carcinomas and cholangiocarcinomas, in TCs, is such an example. Although KIT is not a specific marker for TC, the positive rate of KIT in TCs was significantly higher than that in pulmonary and oesophageal squamous cell carcinomas. Nakagawa *et al*. studied KIT and CD5 expression in thymic epithelial tumours and demonstrated that 16 of 20 cases were positive for KIT and 14 of 20 cases were positive for CD5 [14]. These data suggest that thymic carcinoma is characterised by a KIT-positive and CD5-positive staining pattern. In our series, 14 of 18 tumours expressed KIT and 16 of 18 tumours expressed CD5 immunohistochemically.

C-KIT expression seems to be frequent in squamous cell thymic carcinoma in all published reports. However, its expression has been described in other subtypes as well, including lymphoepithelioma like carcinomas [10] and undifferentiated carcinomas [10]. In our study, neuroendocrine carcinomas among other histologic types, stained strongly positive.

In order to validate the immunohistochemical findings, we further performed Sanger sequence-PCR to identify c-KIT transcripts.

The common KIT genomic mutations that lead to its constitutive activation in GISTs [13, 15], myeloproliferative disorders [13] or mast cell diseases [13] are not present in thymic carcinomas. Only a few KIT mutations in thymic carcinomas have been reported in literature. In 2004, Strobel *et al.* [16] reported a V560del KIT mutation in a case, that was a liver metastasis from poorly differentiated epidermoid carcinoma of the thymus. This patient responded to treatment with imatinib that lasted 6 months. In 2008, Yoh *et al.* [17] identified the L576P KIT mutation in exon 17 of a thymic carcinoma. In 2009, Bisagni *et al.* [18] reported a case of an undifferentiated thymic carcinoma carrying mutation D820E, which is encoded by KIT's exon 17. The patient was treated with sorafenib and received a partial response that lasted more than 15 months. Girard *et al.* [19] recently reported 2 mutations in 7 thymic carcinomas. The authors sequenced exons 10 and 14 in addition to 9, 11, 13, and 17. Interestingly, one mutation, H697Y, was in exon 14. H697Y showed higher sensitivity to sunitinib than to imatinib *in vitro* when transfected in Ba/F3 cells. In 2011, Hamada *et al.* [20] reported one thymic carcinoid case with a good clinical response to imatinib, which has been identified as an overexpression of KIT despite the lack of KIT mutations in exons 9, 11, 13, and 17. These results underline the importance of extending the analysis of thymic carcinomas to KIT regions beyond the most frequent sites of mutations in exons 9, 11, 13, and 17.

In the present study, 18 cases of squamous cell carcinomas and non-squamous cell carcinomas of the thymus were screened for KIT mutations, but no mutations were found despite frequent KIT expression in the same tumours when studied immunohistochemically.

We report a worse OS for patients with c-KIT expressing tumours. These data suggest a negative prognostic role for c-KIT expression especially within the first 5 years (Fig. 2A). We used OS as prognostic endpoint rather because of the long expected survival after radical surgery. However, by multivariate analysis, c-KIT expression was not an independent prognostic factor for TET histotype in our study.

Our series confirms the importance of histopathological diagnosis and immunohistochemistry. The TNM stage has also been established as a prognostic factor [21]. In our series, we were unable to confirm better statistical significance for classification by molecular analysis [3], but we recently validated it for the proposed TNM staging system [5, 22]. Because of this we know our study displays several limitations. Formalin-embedded samples were used for both immunohistochemical and mutation analyses, not fresh frozen samples. DNA was not extracted successfully in four of these eighteen patients. To overcome these limitations, further studies in a larger series are required.

In conclusion, c-KIT status is very important in the treatment and prognosis of thymic carcinomas. It is also used in targeted therapy. Therefore, other criteria affecting c-KIT status and survival should be considered in determining the prognosis of disease and treatment.

The authors declare no conflict of interest.

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