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High expression level of SOX2 is significantly associated with shorter survival in patients with thymic epithelial tumors

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ABSTRACT

Objectives: Thymic epithelial tumors (TET) are heterogenous tumors which are composed of thymoma (TM) and thymic carcinoma (TC). We attempted to determine differences in gene expression between TM and TC, and to determine the effect of such genes on the prognosis of patients with TET.
Materials and methods: Gene expression profiles of SOX2, OCT-4, IGF-1, IGF-1R and IR mRNA transcripts in tumor tissues of TM and TC were determined using real-time PCR (RT-PCR). We constructed tissue microarray with 140 paraffin-embedded tumor tissues and performed immunohistochemistry (IHC) for IGF-1R-related signaling molecules, including SOX2, IGF-1, IGF-1R and pAKT.
Results: SOX2 mRNA expression was notably higher (216-fold) in TCs than in TMs. However, there was no significant difference in expression of IGF-1, IGF-1R, OCT-4 or IR between the two tumor types. In IHC results, SOX2 (HR: 7.57, P = 0.001) and IGF-1 (HR: 9.43, P = 0.001) expression levels in TC were significantly higher than those in TM. There was a significant correlation in expression of SOX2 with IGF-1 (P = 0.021) and pAKT (P = 0.026). In univariate analysis, clinical TNM stage, WHO classification, serum LDH, expression of SOX2, IGF-1R, IGF-1 and pAKT, were significantly correlated with overall survival (OS). Multivariate analysis using a forward-selection procedure revealed that clinical N stage (HR: 4.08, P < 0.001), M stage (HR: 3.37, P = 0.001) and SOX2 expression (HR: 4.53, P = 0.010) were significantly associated with OS.
Conclusions: SOX2 is expressed significantly higher in TC than in TM. SOX2 expression is also closely related to IGF-1 and pAKT expression. The higher expression of SOX2 is significantly associated with shorter survival in patients with TET.

1. Introduction

The histopathology of thymic epithelial tumors (TET) consists of six subtypes (A, AB, B1, B2, B3 and C) according to the WHO classification system, based on the morphology of epithelial cells and the relative proportion of non-tumoral lymphocytic component. There is a close relationship between pathological subtypes (WHO classification) and clinical behavior (Masaoka-Koga stage) [1,2]. Thymic carcinoma (TC) patients are diagnosed more frequently in advanced stage with poor prognosis and unfavorable response to systemic chemotherapy compared to thymoma (TM) patients [3,4]. TET, defined by the 2015 WHO histological classification, displays different molecular characteristics. Type B3 TM and TC have been distinguished from type A and type B2 TMs by genomic profiling [4]. Studies on oncogenic molecules such as EGFR, HER2, KIT, VEGF and RAS can help us to understand the molecular pathogenesis of TET. However, there have been no established therapeutic molecular targets for TET [4,5].

The insulin-like growth factor 1 receptor (IGF-1R) signaling pathway has been proposed as a potential therapeutic target for TET [6]. IGF-1R is known to be more frequently expressed in TC than in TM

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[7,8]. However, in a phase II clinical trial, the objective tumor response to cixutumumab, an IGF-1R monoclonal antibody, was observed only for TM among advanced and refractory TETs [9]. The functions of IGF-1R and insulin receptor (IR) are complex. They interact with many signaling pathways, making it difficult to find a uniform set of predictive biomarkers for IGF-1R inhibitors [10]. For this reason, additional investigation regarding the dimerization and downstream signaling of IGF-1R is warranted.

Recently, it has been proven that the IGF-1R pathway is closely related to the stem cell-related pathway and the epithelial-mesenchymal transition (EMT) pathway. Many studies on the interaction between IGF-1R and stem cell–related molecules strongly support the theory that the IGF-1R pathway plays a key role in the induction and maintenance of cancer cell "stemness" and "EMT" [11]. However, studies that compare gene expression related to IGF-1R and stem cell between TM and TC are lacking.

Therefore, the objective of this study was to determine difference(s) in gene expression related to IGF-1R and stem cell pathways between TM and TC, and to identify the effect of such molecules on the prognosis of patients with TET.

2. Materials and methods

2.1. Real-Time polymerase chain reaction of fresh tissues

Fresh tissue specimens were collected from Seoul St. Mary's Hospital Biobank (TM5 and TC2) and the Korea Biobank Network (TM1 and TC3). All patients provided informed consent for the use of their materials in this study. Samples were acquired in accordance with the Declaration of Helsinki. As soon as the fresh tissue was removed from the operating room, it was transported to the pathology department in an ice box. After visual confirmation, tissue from the tumor site and adjacent normal tissue were placed in cryovials and rapidly frozen in liquid nitrogen (LN2). Tissues were stored in a LN2 dewar until use.

Stem cell and IGF-1R pathway related-gene microarray analysis was performed on these fresh tissues to determine their expression profiles. Real-time polymerase chain reaction (RT-PCR) amplification and relative quantification of IGF-1, IGF1R, IR, SOX2, OCT-4 and GAPDH were performed using the TaqMan gene expression assay (Applied Biosystems, Foster City, CA, USA). All assays used similar amplification efficiency and a ΔCT experimental design was used for relative quantification. GAPDH served as an endogenous control. A non-template control was included in each quantitative RT-PCR experiment to confirm the absence of DNA contamination in reagents used for amplification. Experiment results were analyzed using light cycler 480 instrument software 1.2 (Roche, Mannheim, Germany).

2.2. Immunohistochemistry

We found the 175 archival tissues of thymic epithelial tumors which were attained from the 4 hospitals of the Catholic University of Korea, Catholic Medical Center between 2002 and 2013. Of these, 140 tissues were available for immunostaining. Tissues were fixed in formalin and embedded in paraffin. Slices were prepared as 3-μm thick paraffin sections. Immunohistochemistry (IHC) was performed using an automated immunohistochemical stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's protocol. These sections were deparaffinized, pretreated with cell conditioning solution (CC1, Ventana) and UV irradiated to abrogate endogenous hydroperoxidase activity. Primary antibodies were diluted in antibody diluent containing background-reducing components (Dako Cytomation, Glostrup, Denmark), including a rabbit monoclonal anti-SOX2 antibody (dilution, 1:100; Cell Signaling Technology, USA), a rabbit polyclonal anti-IGF-1 antibody (dilution, 1:100; Abcam, United Kingdom), a rabbit monoclonal anti-IGF-1R antibody (dilution, 1:100; Cell Signaling Technology, USA), and a rabbit monoclonal anti-pAKT antibody (dilution, 1:100; DAKO, USA). These sections were incubated with primary antibodies at room temperature for 32 min and then hybridized with HRP-conjugated secondary antibody (Ventana) at room temperature for 8 min. The reaction was developed with diaminobenzidine (DAB; Dako) at room temperature for 5 min. The slides were counterstained with hematoxylin II (Ventana) for 4 min and bluing reagent (Ventana) for 4 min at room temperature. Sections were observed and analyzed under a light microscope (BX50, Olympus, Japan).

We observed nuclear staining of SOX2 and cytoplasmic staining of IGF-1R and pAKT. IGF-1 staining was predominantly cytoplasmic, with some mixed nuclear and cytoplasmic staining. A nuclear staining pattern of IGF-1 was very rare. The immunohistochemical score of proportion was divided into scores of 0 (0%), 1 (1–29%), 2 (30–50%) and 3 (> 50%). The intensity was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong).

2.3. Statistical analysis

Differences in mRNA expression of stem cell and IGF-1R-related genes between TM and TC were assessed using Mann-Whitney U test. Correlations between these two TET subtypes (TM vs. TC), and expression levels of SOX2, IGF-1R, IGF-1 and pAKT, were assessed using Fisher's exact test and Pearson's chi-square test. We also checked the relevance between SOX2 (a stem-cell marker) and three IGF-1R related markers by Fisher's exact test and Pearson's Chi-square test. Univariate and multivariate analyses of clinical and molecular pathologic factors affecting overall survival (OS) were performed using Cox's proportional hazards model. Analysis was performed with SPSS 20.0 software package.

3. Results

The mRNA transcripts of IGF-1R-related signaling molecules and stem cell-related signaling molecules (SOX2, OCT4, IGF-1, IGF-1R and pAKT) in TM and TC were measured using RT-PCR. SOX2, IGF-1, IGF-1R and pAKT were immunohistochemically stained with 140 paraffin-embedded tumor tissues. The relationship between the expression of these molecules and their clinical characteristics was also examined.

3.1. mRNA expression levels of cancer stem cell and IGF-1R-related genes in TM and TC

Relative mRNA expression levels (target/reference) of cancer stemness and IGF-1R-related signaling molecules were measured in six TMs and five TCs using quantitative RT-PCR. Among these cancer stemness genes, mRNA expression of SOX2 was significantly higher in TCs than in TMs (Mann-Whitney U = 0, $P = 0.004$). However, no statistically significant difference in OCT-4 expression was found between the two tumors (Mann-Whitney U = 10, $P = 0.429$). Among IGF-1R-related genes, the mRNA expression level of IGF-1 was numerically higher in TCs than in TMs, but the difference was not statistically significant (Mann-Whitney U = 5, $P = 0.082$). mRNA expression levels of IGF-1R and IR were similar between the two tumors (Mann-Whitney U = 9, $P = 0.329$; Mann-Whitney U = 15, $P = 1.000$, respectively) (Fig. 1).

3.2. Clinical characteristics of patients

The clinical characteristics and pathologic findings were assessed for 140 patients with TETs who were classified into two groups depending on histological pattern: 110 T Ms and 30 TCs. Elderly patients aged > 60 years (51.7% vs. 32.4%, $P = 0.06$) and male gender (65.5% vs. 45.9%, $P = 0.06$) were predominant in the TC group. Advanced disease was noticeably more frequent in TC than in TM: T3-4 (89.7% vs. 28.0%, $P < 0.001$), N 1–3 (48.1% vs. 7.5%, $P < 0.001$), and M1 (58.6% vs. 8.1%, $P < 0.001$). TM patients were categorized according to WHO

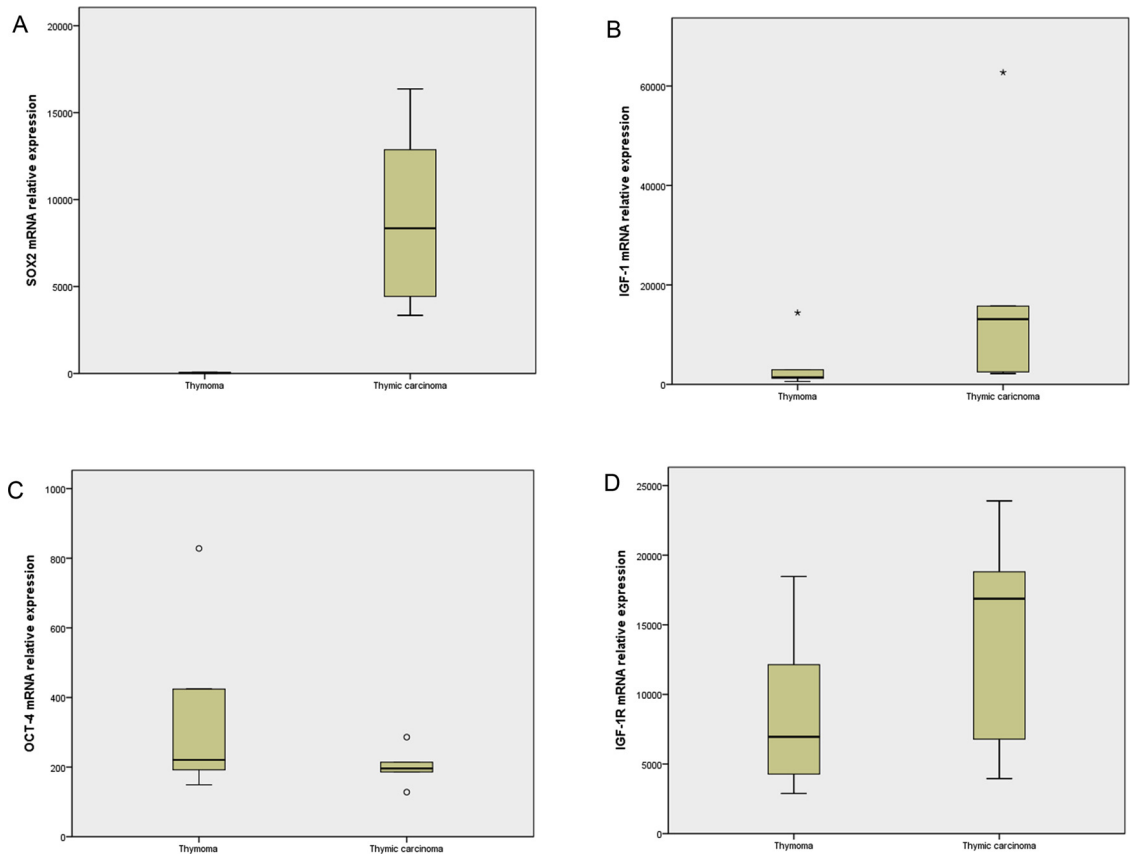

Fig. 1. mRNA expression of stem cell and IGF-1R related genes in TM and TC. Different expression levels of SOX2, IGF-1, OCT-4, IGF-1R and IR were observed between TM and TC. (A) Relative mRNA expression level of SOX2. (B) Relative mRNA expression level of IGF-1. (C) Relative mRNA expression level of OCT-4. (D) Relative mRNA expression level of IGF-1R. (E) Relative mRNA expression level of IR.

classification in the following order: AB (27.9%), B1 (26.1%), B3 (15.3%), B2 (14.4%) and A (9.9%). The remaining cases consisted of 4 mixed types and 1 metaplastic TM (very rare subtypes). A higher serum LDH level (≥ 480 IU) occurred significantly more frequently in TCs than in TMs (41.4% vs. 15.3%, $P = 0.001$). The clinical characteristics of all TET patients are shown in Table 1.

3.3. Surgical staging and pathologic findings

Ninety-nine (70.7%) patients were diagnosed by surgical excision, while the remaining patients (n = 41) were diagnosed by CT-guided core needle biopsy. There were wide differences in T3-T4 (87.5% vs. 23.2%), N1-N3 (46.2% vs. 4.0%) and M1 (63.4% vs 0%) between the two tumor groups, indicating that needle biopsy was prioritized for patients with advanced disease. Masaoka staging of surgical specimens showed 41 (41.4%) in stage I, 43 (43.4%) in stage II, 13 (13.1%) in stage III, and 2 (2.0%) in stage IV. In accordance with TNM staging (AJCC 8th edition), patients were classified as: 41 (41.4%) in T1, 39 (39.4%) in T2, 8 (8.1%) in T3, and 11 (11.1%) in T4. With regard to N staging, there were 90 (84.1%) patients in N0, 2 (1.9%) in N1, and 1 (0.9%) in N2. All patients who underwent surgery had no distant metastasis (M0). When surgical specimens were examined, capsular invasion of lymph node was detected in 30 (30.3%) cases, lymphatic invasion in 7 (7.1%), vascular invasion in 6 (6.1%), and perineural invasion in 1 (1.0%) case. In addition, mitosis greater than 3 per 10 high-power fields was observed in 16 (16.2%) patients. Surgical biopsy showed locoregional invasion of lung (10.1%), pericardium (8.1%) and great vessel (7.1%). Specimens obtained from needle biopsy revealed pleura (41.5%), lung (29.3%) and pericardium (4.8%) invasion.

3.4. Different expression levels of IGF-1R-related molecules between TM and TC

There were significant variations in SOX2 and IGF-1 expression levels between TM and TC (Fig. 2). The positivity of SOX2 and IGF-1 expression (proportion multiplied by intensity ≥1) in TCs was significantly greater than that in TMs (HR: 7.57, $P = 0.001$; and HR = 9.43, $P = 0.001$, respectively). However, there was no significant difference in IGF-1R expression between the two groups ($P = 0.163$). The expression of pAKT tended to be higher in TC than in TM, but the difference was not significantly different ($P = 0.08$) (Table 2).

3.5. Interrelationships among four IGF-1R related molecules

SOX2 expression was significantly correlated with IGF-1 expression and pAKT expression ($P = 0.021$ and $P = 0.026$, respectively). IGF-1 expression was also significantly correlated with pAKT expression ($P < 0.001$). However, IGF-1R expression was not correlated with SOX2, IGF-1, or pAKT expression ($P = 0.193$, $P = 0.624$ and $P = 0.164$, respectively) (Table 3).

3.6. Clinical and molecular factors affecting overall survival

We analyzed clinical and molecular variables affecting OS in 140 TET patients. In univariate analysis, serum LDH, clinical TNM stage, WHO pathologic classification and expressions of SOX2, IGF-1R, IGF-1 and pAKT were significantly associated with OS (Table 4). Multivariate analysis using forward-selection procedure revealed that clinical N stage (HR: 4.08, $P = 0.001$), M stage (HR: 4.53, $P = 0.001$) and SOX2 expression (HR: 4.53, $P = 0.010$) were significantly associated with OS

Table 1
Clinical characteristics of patients with thymoma and thymic carcinomas.

	Thymoma (N = 110)	Thymic carcinoma (N = 30)	*P* value
Age (Median, range)	53 (20-85)	60 (40-78)	0.06
Age < 60	74 (67.3%)	15 (50.0%)	
Age ≥60	36 (32.7%)	15 (50.0%)	
Sex			0.06
Male/ Female	51 (46.4%)/59 (53.6%)	19 (63.3%)/ 11 (36.7%)	
Clinical staging			
cT1-T2/ T3-T4	78 (70.9%) / 32 (29.1%)	3 (10.0%)/ 27 (90.0%)	**< 0.001**
cN0/ N1-N3	102 (92.7%)/ 8 (7.3%)	14 (46.7%)/ 16 (53.3%)	**< 0.001**
cM0/ M1	101 (91.8%)/ 9 (8.2%)	13 (43.3%)/ 17 (56.7%)	**< 0.001**
Metastasis: Lung/ bone/ liver/ other sites	10 (9.1%)/ 1 (0.9%)/ 0 (0%)/ 11 (10.0%)	11 (36.7%)/ 10 (33.3%)/ 5 (16.7%)/ 10 (33.3%)	
Tissue type (WHO classification)			
A	11 (10.0%)		
AB	31 (28.2%)		
B1	28 (25.5%)		
B2	16 (14.5%)		
B3	19 (17.3%)		
C		29 (96.7%)	
Mixed type			
predominantly B2 combined with B3	3 (2.7%)		
predominantly B1 combined with B3	1 (0.9%)		
predominantly C combined with B1		1 (3.3%)	
Metaplastic thymoma	1 (0.9%)		
Thymic carcinoma subtypes			
Squamous cell carcinoma		18 (62.1%)	
Undifferentiated carcinoma		5 (18.2%)	
Lymphoepithelioma-like carcinoma		2 (6.9%)	
Neuroendocrine carcinoma		2 (6.9%)	
Adenocarcinoma		1 (3.4%)	
Adenosquamous carcinoma		1 (3.4%)	
Hemoglobin (g/dL)			0.68
Hb ≥10	102 (92.7%)	29 (96.7%)	
Hb < 10	8 (7.3%)	1 (3.3%)	
LDH (IU/L)			**0.001**
LDH < 480	85 (77.3%)	15 (48.3%)	
LDH ≥ 480	17 (15.5%)	12 (41.4%)	

(Table 4). Additionally, we performed correlation analysis of clinical and molecular variables with OS for 110 T M patients. SOX2 expression, pAKT expression and clinical TNM staging were significantly associated with OS by univariate analysis (Table 4). N stage (HR: 4.79, $P = 0.016$) and M stage (HR: 5.64, $P < 0.001$) were significantly associated with OS by multivariate analysis. In 30 TC cases, only clinical N stage was a significant prognostic factor for OS by both univariate and multivariate analysis (HR: 3.08, $P = 0.038$).

3.7. Clinical and molecular factors affecting relapse-free survival

Using the Kaplan-Meier method, we analyzed clinical and molecular variables affecting relapse-free survival (RFS) in 99 TETs receiving surgical resection. Lower Hb level (< 10 g/dL) ($P = 0.012$), advanced clinical T stage (cT3/T4) ($P = 0.008$), advanced clinical N stage (cN1/ N2/N3) ($P = 0.001$), advanced Masaoka staging (stage 3–4) ($P = 0.005$), advanced pathologic T stage (pT4) ($P = 0.002$), pathologic WHO classification (B3/C) ($P = 0.002$), presence of lymphatic invasion ($P < 0.001$), presence of venous invasion ($P = 0.001$), and IGF-1 expression ($P = 0.020$), were correlated with shorter RFS. By multivariate analysis using the forward-selection procedure, lower Hb level (HR: 8.27, $P = 0.028$), advanced Masaoka staging (HR: 3.96, $P = 0.044$) and presence of lymphatic invasion (HR: 22.53, $P < 0.001$) were strongly associated with shorter RFS.

4. Discussion

Based on previous studies and clinical experience, we hypothesized that there might be disparate molecular profiles between TM and TC. Such differences at the molecular level might play a key role in producing wide differences in clinical characteristics, treatment response and prognosis for the two malignant diseases.

Many studies have provided evidence that genetic aberrations of IGF-1R-related signaling molecules are indicative of clinical aggressiveness of TETs [4]. In a previous study, increased IGF-1R expression in TETs had poor prognostic value for OS and time to progression. Zucali et al. [7] evaluated IGF-1R expression by IHC in 132 specimens from patients with TETs receiving surgery. Twenty-two (20%) of 111 cases were positive for IGF-1R. IGF-1R was less common in A, AB and B1 TM (3.4%) compared to B2, B3 and C subtypes (37.2%). Girard et al. [8] also reported a higher degree of IGF-1R expression in TC (83%) than in TM (43%) in a total of 63 thymic tumors. Thus, IGF-1R has been studied as one of the promising therapeutic targets for thymic tumors to date. In an early phase clinical study, figitumumab, an anti–IGF-1R antibody, exhibited clinical activity lasting for more than 1 year in some refractory TM patients [9]. However, in a phase 2 trial of another anti–IGF-1R antibody, cixutumumab, for recurrent or refractory advanced TETs, partial response was observed only for 14% of TMs, while no objective responses were recorded in TCs [10]. The clinical response for the minority of TM patients and lack of response in TC patients to an IGF-1R monoclonal antibody might suggest a lack of specific binding to the receptor. Because IGF-1R interacts with IR via hetero-dimerization, only blocking IGF-1R could be insufficient to completely interfere with IGF-1R signaling. The downstream signaling pathway could also be aberrant in the IGF-1R-related pathway. Several studies have evaluated the resistance mechanism of anti-IGF-1R Ab treatment, with disappointing results from clinical studies of various carcinomas [12]. in vitro study of cell lines established from head and neck squamous cell carcinoma and non-small cell lung cancer patients has revealed that Src activation via integrin β3 plays an important role in resistance to anti-IGF-1R mAb [13]. Blocking both the IGF-1R and integrin β3-Src pathways has been proposed as a way to overcome such resistance. In addition, studies on the tumor microenvironment have revealed that when the anti-IGF-1R Ab is administered, STAT3-mediated IGF-2 secretion is increased in cancer cells, resulting in the recruitment of macrophages and fibroblasts through IGF-2/ IGF-2R activation [14]. Eventually, an angiogenic and metastatic environment was established through the production of stroma-derived CXCL8, leading to cancer progression. This observation suggests that blocking the STAT3/IGF-2/ IGF-2R intercellular signaling loop could overcome resistance to IGF-1R Ab therapy.

In our study, neither IGF-1R mRNA expression in fresh tissues nor IGF-1R protein expression in archived tissues were significantly different between TC and TM. Moreover, IGF-1R expression was not a significant prognostic factor for RFS or OS. Remon et al. have examined cytoplasmic IGF-1R expression along with CD52, CD22, CD26, and EG5, by IHC staining in 103 TETs [15]. There was no significant difference in IGF-1R expression between TM (63–100%) and TC (92%). Meanwhile, Mimae et al. have checked the protein expression and gene copy number of IGF-1R, EGFR, HER2 and c-MET by IHC staining and bright-field in situ hybridization (BISH) in 140 TETs [16]. There was a significant difference in the frequency of IGF-1R expression between TM (10.7%) and TC (83.8%). However, BISH analysis of IGF-1R showed conflicting results compared with IHC staining for TM (27%) and TC (27.3%). These results support our data that mRNA expression of IGF-1R shows no statistical difference between TM and TC, even with small

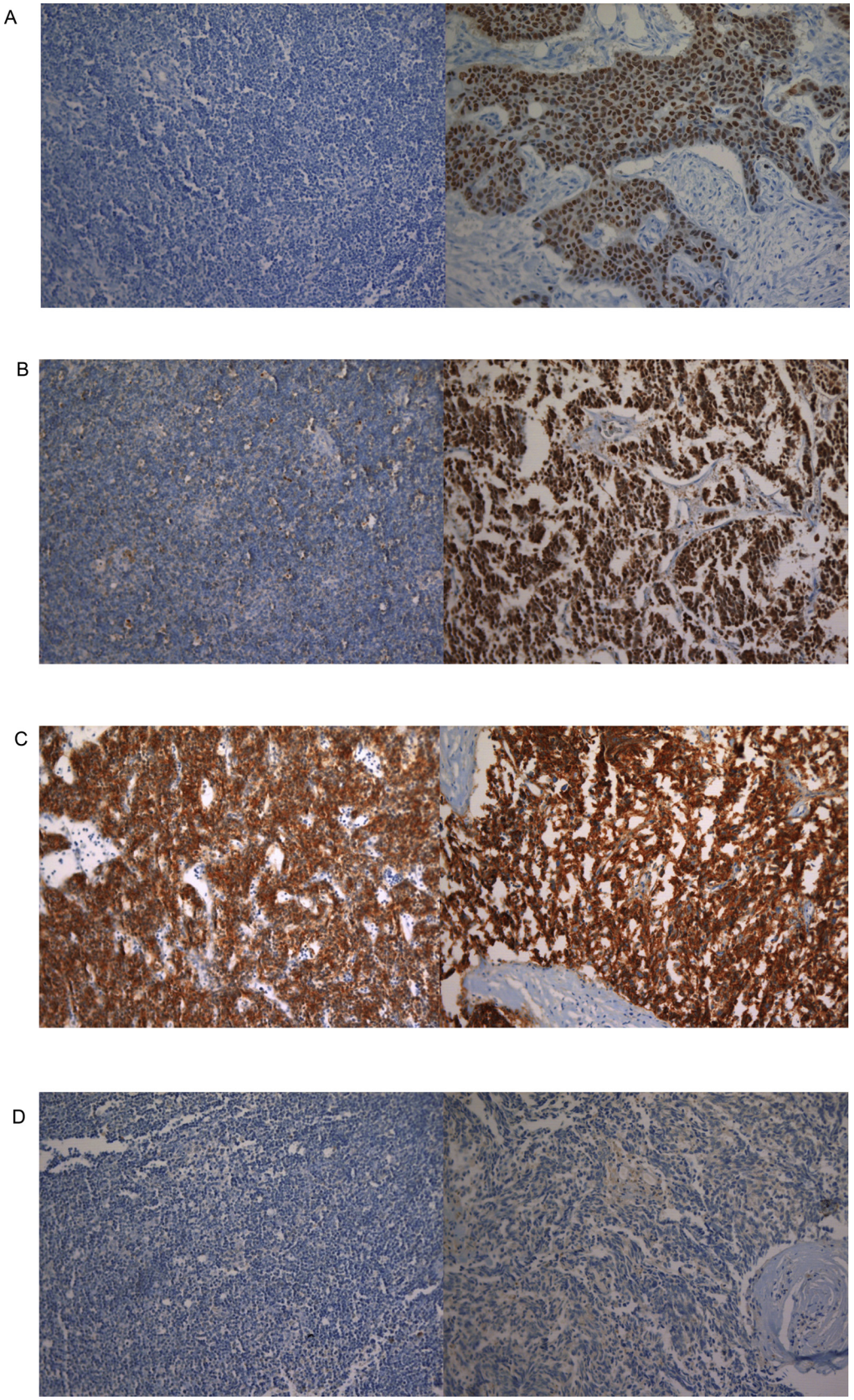

Fig. 2. Immunohistochemical staining for SOX2 (A), IGF-1 (B), IGF-1R (C) and pAKT (D) in thymic epithelial tumors (Lt- TM, Rt-TC). (A) SOX2: TM (negative), TC (positive). (B) IGF-1: TM (negative), TC (positive). (C) IGF-1R: TM (positive), TC (positive). (D) pAKT: TM (negative), TC (negative).

Table 2
Comparison of stem cell marker and IGF-1R-related markers (SOX2, IGF-1R, IGF-1, pAKT) between the thymoma and thymic carcinoma groups.

	Thymoma	Thymic carcinoma	Odds ratio	*P* -value
SOX2 expression*				
negative	104 (94.5%)	21 (70.0%)		
positive (≥1)	6 (5.6%)	9 (30.0%)	7.57 (95% CI 2.42-23.66)	0.001
IGF-1R expression*				
negative	29 (26.4%)	4 (13.3%)		
positive (≥1)	81 (73.6%)	26 (86.7%)	2.21 (95% CI 0.71-6.93)	0.163
IGF-1 expression*				
negative	44 (40.0%)	2 (6.7%)		
positive (≥1)	66 (60.0%)	28 (93.3%)	9.43 (95% CI 2.13-41.71)	0.001
pAKT expression*				
negative	77 (70.0%)	15 (50.0%)		
positive (≥1)	33 (30.0%)	15 (50.0%)	2.09 (95% CI 0.91-4.83)	0.08

* Proportion multiplied by Intensity: proportion was divided into 0 (0%), 1 (< 30%), 2 (30–50%) and 3 (> 50%). The intensity was rated 0 (negative), 1 (weak), 2 (moderate) and 3 (strong).

Table 3
Correlations among stem-cell marker, SOX2 and three IGF-1R related markers in thymic epithelial tumors (contingency table).

		SOX2 (-) (+)	IGF-1R (-) (+)	IGF-1 (-) (+)	pAKT (-) (+)
	(-)			45 80	86 39
SOX2	(+)			1 14	6 9
	P		NS	0.021	0.026
	(-)				
IGF-1R	(+)				
	P	NS		NS	NS
	(-)	45 1			40 6
IGF-1	(+)	80 14			52 42
	P	0.021	NS		< 0.001
	(-)	86 6		40 52	
pAKT	(+)	39 9		6 42	
	P	0.026	NS	< 0.001	

sample sizes.

With regard to IGF-1R expression, some studies have shown differences between the two tumor types whereas others have not. There might be many possible explanations, including different detection platforms, subjective interpretation of pathologists, and the positive cut-off value of IHC staining. In addition, the higher expression of IGF-1R in B3 TM, rather than A, AB, B1, or B2 subtypes, might affect study results in accordance with the proportion of B3 among TM patients.

It is known that the IGF-1R pathway is closely connected to cancer stem cell and EMT pathways [11]. A large body of evidence supports the theory that IGF-1R functions as a driver of self-renewal, stem cell surface markers, migration and invasion in liver, lung, prostate and breast cancers. Wnt/B-catenin, Notch and Shh act upstream to increase IGF-1R expression with cross-talk and regulate at the promoter level of IGF-1R as stem cell controllers [11,17]. An increase in IGF-1R promotes positive downstream feedback through regulation and interaction with EMT and stemness-linked transcription factors such as Zeb1, NFκB, Snail, Twist, p53, SOX2, Oct4 and Nanog.

Sex determining region-Y-related high mobility group box 2 (SOX2) is a transcription factor that is a major regulator for maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells. However, its overexpression gives rise to extensive epithelial hyperplasia and, ultimately, carcinoma. SOX2 is expressed in squamous cell carcinoma of the lung, and colorectal, castration-resistant prostate and tamoxifen-resistant breast cancers [18,19]. Nakatsugawa et al. analyzed the functions of SOX2 in cancer stem-like cells/cancer-initiating cells derived from human lung adenocarcinoma and demonstrated that SOX2 overexpressing lung adenocarcinoma cell lines showed higher rates of side population cells and higher tumorigenicity [20]. SOX2 is also a candidate for T cell immunotherapy targeting cancer stem-like cell/tumor initiating cells [21].

In the present study, we investigated IGF-1R-related molecules and stem cell markers. SOX2 mRNA expression in fresh tumor tissues from TC was remarkably higher than in those from TM. In IHC with paraffin-embedded tissue, both SOX2 and IGF-1 expressions were significantly higher in TC than in TM. These results suggest that SOX2 and IGF-1 expression are closely related to clinical aggressiveness and poor prognosis in TETs.

Several studies have reported that SOX2 and IGF-1R signaling molecules such as IGF-1 and pAKT have close relationships in a variety of tumors [22–24]. In a previous experiment, murine melanoma cell lines with IGF-1 downregulation were obtained from wild-type B16-F10 cells after transfection with an episomal IGF-1 antisense vector [22]. IGF-1 inhibition greatly reduced stemness features, including the expression of key stem cell markers such as SOX2, Oct-3/4, CD24 and CD133, indicating that IGF-1 is an essential molecule for cancer stemness. Other studies have shown that SOX2 and pAKT are co-expressed and regulated by each other in cancer stem cells and that depletion of either SOX2 or pAKT can reduce clonogenicity of cancer cells, suggesting that the SOX2/AKT axis is a regulator of cancer clonogenicity in gastric and laryngeal cancers [23,24].

We also observed significant relationships between expression levels of SOX2, IGF-1 and pAKT. This result strongly supports that there is crosstalking between stem cell and IGF-1R signal pathways as well as between IGF-1 and pAKT signal pathways.

Cimpean et al. [25] showed that SOX2 is expressed in a few cells of normal human thymus whereas immunoreactivity to SOX2 is increased in parallel with pathologic stage in TM. A peculiar distribution was found in type B3 TM, with a positive reaction in both tumor and endothelial cells. Another study, in 352 patients with non-small cell lung cancer, has also shown that, among IGF-1, IGF-2, IGF-1R and pIGF-1R, only the expression of IGF-1 was an independent prognostic marker for OS [26]. Meanwhile, in the current study, SOX2 expression was a significant prognostic factor for shortened OS of TETs. However, in TM, only univariate analysis showed a significant correlation with OS ($P = 0.011$) whereas multivariate analysis showed no significant correlation. In TC, univariate analysis showed no significant correlation with OS ($P = 0.49$). The expression of SOX2 was related to capsule invasion and advanced TNM staging. This may explain why SOX2 expression was an independent factor for survival by univariate analysis but not by multivariate analysis in TM. Moreover, TC is considered to be statistically insignificant due to the insufficient number of tissues (n = 30). Our results, together with the aforementioned previous studies, indicate that SOX2, rather than IGF-1R, could more accurately predict poor prognosis in TETs.

In this study, SOX2 expression was not associated with RFS. We believe there are a couple of reasons for this observation. First, this result might be influenced by the inconsistency of adjuvant treatment after surgery because of the retrospective analysis. Second, in other carcinomas such as urothelial carcinomas, SOX2 expression was not significantly associated with postoperative recurrence [19].. These findings suggest that cancer stem-like cells are linked to the more aggressive behavior of TETs, supporting the current cancer stem cell hypothesis.

This study has some limitations. First, because TET is an uncommon disease, the number of cases studied was insufficient to empower statistical significance. Second, this study was performed retrospectively with paraffin-embedded tumor tissues obtained from patients who were diagnosed with TET. Antigen extraction may be a problem in stored tissues, especially those which have been stored for a long period of time. In addition, there is no clear guideline for IHC staining of TETs,

Table 4
Univariate and multivariate analysis of clinical and molecular pathologic factors affecting the overall survival of TET.

	Thymic epithelial tumor (N = 140)				Thymoma (N = 110)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age		0.14				0.15		
< 60	1.00				1.00			
≥60	1.59 (0.86-2.95)				1.87 (0.81-4.33)			
Sex		0.35				0.90		
Male	1.00				1.00			
Female	0.75 (0.40-1.38)				1.05 (0.45-2.47)			
Hemoglobin (g/dL)		0.39				0.23		
Hb≥ 10	1.00				1.00			
Hb < 10	1.58 (0.56-4.44				2.10 (0.62-7.12)			
LDH (IU/L)		**0.003**				0.36		
LDH < 480	1.00				1.00			
LDH ≥ 480	2.66 (1.38-5.10)				1.61 (0.59-4.40)			
Clinical T stage		**< 0.001**				**0.02**		
T 1,2	1.00				1.00			
T 3,4	4.60 (2.18-9.69)				2.77 (1.19-6.43)			
Clinical N stage		**< 0.001**		**< 0.001**		**0.001**		**0.016**
N 0	1.00		1.00		1.00		1.00	
N 1,2,3	7.43 (3.75-14.68)		4.08 (1.83-9.09)		6.17 (2.02-18.84)		4.79 (1.34-17.13)	
Clinical M stage		**< 0.001**		**0.001**		**0.001**		**< 0.001**
M 0	1.00		1.00		1.00		1.00	
M 1	5.02 (2.67-9.46)		3.37 (1.59-7.13)		4.90 (1.90-12.66)		5.64 (2.14-14.84)	
Pathology (WHO classification)		**< 0.001**						
Thymoma (A,AB,B1,B2,B3)	1.00							
Thymic carcinoma (C)	4.34 (2.32-8.10)							
SOX-2 P x I		**0.003**		**0.010**		**0.011**		
0-2	1.00		1.00		1.00			
≥3	5.01 (1.76-14.30)		4.53 (1.44-14.23)		17.26 (1.90-157.16)			
IGF-1R P x I		**0.005**				0.21		
0-2	1.00				1.00			
≥3	2.69 (1.34-5.38)				1.75 (0.74-4.18)			
IGF-1 P x I		**0.032**				0.29		
0-1	1.00				1.00			
≥2	2.19 (1.07-4.47)				1.61 (0.67-3.89)			
pAKT P x I		**0.026**				**0.023**		
0	1.00				1.00			
≥1	2.02 (1.08-3.77)				2.82 (1.15-6.89)			

which may lead to different outcomes depending on the institution or pathologists. However, most previous studies have been performed with fewer than 100 samples. Our results confirmed protein expression of key molecules related to IGF-1R and the stem cell signal pathway in paraffin-embedded tissues on the basis of relative quantitation of mRNA expression in fresh tumor tissues.

5. Conclusion

The current study evaluated mRNA expression of cancer stemness and IGF-1R-related signaling molecules in fresh tumor tissues and validated the expression of SOX2 and IGF-1 in paraffin tumor tissues of TET. The expression of SOX2 and IGF-1 was clearly distinct between TM and TC. SOX2 expression was found to be a significant prognostic marker for OS. These data indicate that SOX2 might be a potential molecular target for the future development of anticancer drugs for treating TETs.

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Conflict of interest statement

All authors have nothing to declare.

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