RESEARCH ARTICLE

Molecular Markers for Patients with Thymic Malignancies: not Feasible at Present?

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Abstract

Background: Thymomas and thymic carcinomas are rare malignancies and devising clinically effective molecular targeted therapies is a major clinical challenge. The aim of the study was to analyze BLC2 and vascular endothelial growth factor receptor (VEGFR) expression and *KRAS* and *EGFR* mutational status and to correlate them with the clinical characteristics of patients with thymomas and thymic carcinomas. Materials and Methods: A total of 62 patients (mean age: 50.4±13.2 years) with thymomas and thymic carcinomas were enrolled. The expression of BLC2 and VEGFR in tumor cells and normal tissues was evaluated by RT-PCR. The mutational status of the *KRAS* and *EGFR* genes was investigated by PCR with sequence specific primers. Results: The BLC2 and VEGFR expression levels did not differ significantly between tumor and normal tissues. Moreover, there were no clearly pathogenic mutations in *KRAS* or *EGFR* genes in any tumor. None of the molecular markers were significantly related to clinical outcomes. Conclusions: Changes in levels of expression of BLC2 and VEGFR do not appear to be involved in thymic tumorigenesis. Moreover, our data suggest that *KRAS* and *EGFR* mutations do not play a major role in the pathogenesis of thymomas and thymic carcinomas.

Keywords: Thymoma - Thymic carcinoma- BCL2 - VEGFR - *KRAS* - EGFR

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Introduction

Thymomas and thymic carcinomas are rare mediastinal malignancies (< 1% of all adult cancers) arising from the cells located on the outside surface of the thymus (Girard, 2000; Detterbeck and Zeeshan, 2013; Lamarca et al., 2013). The tumor cells in thymomas closely resemble the normal cells of the thymus (regardless of the presence and relative numbers of non-neoplastic lymphocytes), grow slowly, and rarely spread beyond the thymus (Polo et al., 2013). According to the WHO classification, thymomas can be divided into five subgroups (A, AB, B1, B2, B3), depending on cancer cell shape, degree of atypia, and number of intratumoral thymocytes (Strobel et al., 2005). Differently from thymomas, the epithelial component proliferates of thymic carcinomas are not characterized by the typical lymphocytic admixture (Kelly, 2013). The clinical manifestations of thymomas and thymic carcinomas generally reflect tumor invasion, local compression of neighboring organs, and distant metastasis (Mikhail et al., 2012). The paraneoplastic syndromes, such as myasthenia gravis, red cell aplasia, autoimmune diseases, and opportunistic infections, are uncommon in thymic carcinomas. However, myasthenia gravis has been repeatedly described in the well-differentiated form of thymoma (Venuta et al., 2012).

The etiology and molecular pathology of thymomas and thymic carcinomas continue to remain largely unknown (Girard, 2010). Moreover, these tumors are characterized by heterogeneous histological features and clinical behavior (Lamarca et al. 2013). Until recently, most studies trying to identify molecular alterations in thymic epithelial tumors were single-marker studies (Girard, 2010). The pathogenic role of key components of apoptotic, prosurvival, and angiogenic pathways such as of BLC2, VEGFR, *KRAS*, and *EGFR* has been extensively investigated in solid tumors. BCL2 is a prosurvival molecule and its enhanced expression has been associated with malignant transformation as well as resistance to chemotherapy in various solid cancers (Li et al., 2013). Moreover, an aberrant expression of vascular endothelial growth factor receptor (VEGFR) on epithelial tumor cells may allow VEGF to stimulate growth and migration of cancer cells in an autocrine and/or paracrine fashion (Cao et al., 2013). The commonly mutated *KRAS* and *EGFR* oncogenes have been frequently been studied as potential

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biomarkers in different malignancies. The *KRAS* gene is a member of the RAS family, and encodes a small membrane-bound and growth factor-activated GTPase (Wang et al., 2013). The GTPase controls a "switch" to transmit growth factor signals from the receptor on the cell membrane into the cell. The RAS proteins activate different signaling pathways, and mutations in the *KRAS* gene can cause constitutive activation of these signal transduction pathways (Kim et al., 2011). This may ultimately lead to uncontrolled growth, migration, invasion, and apoptosis resistance. Activating mutations in the epidermal growth factor receptor (EGFR)-a transmembrane protein with cytoplasmic kinase activity that transduces important growth factor signaling from the extracellular milieu to the cell-have been similarly linked to malignant transformation (Ono and Kuwano, 2006).

To gain more insight into the molecular pathogenesis of thymomas and thymic carcinomas, herein we studied BLC2 and VEGFR expression and *KRAS* and *EGFR* mutational status and correlated them with prognosis in patients with these malignancies.

Materials and Methods

The local Ethics Committee approved this study that was conducted in agreement with the Declaration of Helsinki. All patients provided their written informed consent.

Patients

The thymoma registry at the Department of Medical Oncology, Uludag University School of Medicine (Bursa, Turkey), was established in 1990 with the aim of collecting the general characteristics and the follow-up data on all new cases of thymomas and thymic carcinomas cancer diagnosed at our institution. The diagnosis was independently confirmed by two pathologists. Tumors were staged according to both the Masaoka staging system (Masaoka, 2010) and the 2004 WHO classification (Strobel et al., 2005).

Expression analysis of BCL2 and VEGFR

Paraffin sections of primary tumors and their adjacent normal tissue were deparaffinized using the BiOstic FFPE Tissue Kit (MO BIO Laboratories, Carlsbad, CA, USA). After two washing steps with 100% ethanol, samples were air-dried. Thereafter, RNA from microdissected cells was isolated using the RNeasy FFPE Kit (Qiagen, Hilden, Germany), digested with DNase, and eluted in RNase-free water (15 μL). The concentration and quality of RNA samples was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from 5 ng of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). Samples were analyzed for the mRNA expression of BCL2 and VEGFR using custom qRT-PCR assays (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The conditions for quantitative real-time polymerase chain reactions (qRT-PCR) were as follows: preheating at 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec and 60°C for 60 sec. All qRT-PCR reactions were carried out on a using a LightCycler 480II instrument (Roche Diagnostics). To control for variations in RNA quality and quantity, the expression of the gene of interest was normalized to the expression of beta-actin (ACBT) as a housekeeping gene. mRNA expression levels were calculated according to the following formula: 2-ΔCT, where ΔCT (sample) was defined as CT (gene of interest)-CT (ACBT).

KRAS and EGFR mutational analysis

DNA was extracted from paraffin sections of primary tumors using the QIAamp® DNA FFPE tissue kit (Qiagen) according to the manufacturer's instructions. All DNA samples were checked using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Two codons in the *KRAS* gene (codons 12 and 13 in the exon 2) are mainly known to generate alternated proteins that are constitutively activated without the signal of a ligand bound to the receptor. the *KRAS* gene exon 2 was amplified by co-amplification at lower denaturation temperature-PCR (COLD PCR) with the following primer set: forward 5'-AGGTACTGGTGGAGTATTTGA-3' and reverse: 5'-AACTTTCAGCATAA-TTATCTTGTA-3'. The COLD-PCR protocol consisted of 25 initial cycles of conventional PCR amplification for an initial build up of all amplicons, followed by 30 COLD-PCR cycles aimed at selectively enriching the mutant sequences. The cycling conditions were as follows: preheating at 95˚C for 10 min; 25 cycles at 95˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 1 min followed by additional 30 cycles at 82˚C for 20 sec, 58˚C for 30 sec, 72˚C for 1 min, with a final elongation step at 72˚C for 10 min. After checking the amplification products on agarose gels, the PCR reactions were purified with the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and used for sequencing with both the forward and reverse primers. The sequence data were compared with the consensus *KRAS* sequence (GenBank accession no: NM_004449.3). For hot-spot mutational analysis of the *EGFR* gene, specific PCR primers were used to cover exons 18, 19, 20, and 21 (Roma et al., 2013). The PCR protocol was as follows: preheating at 95˚C for 10 min followed by 35 cycles of 94˚C for 20 sec, 55˚C for 30 sec, 72˚C for 1 min, with a final elongation step at 72˚C for 10 min. PCR products were purified and sequenced as described above. The sequence data were compared with the consensus *EGFR* sequence (GenBank accession no: NM_201284.1).

Data analysis

All calculations were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Normally distributed continuous variables were presented as mean±standard deviation. Categorical data were presented as counts and percentages analyzed by using the χ^2 test. Spearman rank correlation was used to examine the relationship between variables. A two-tailed p value <0.05 was considered statistically significant.

Table 1. General Characteristics of the Study Participants (n=62)

		Number of patients (%)	
Sex	Male	27	(43.5%)
	Female	35	(56.5%)
Masaoka stage	I	19	(30.6%)
	II	20	(32.4%)
	III	10	(16.1%)
	IV	9	(14.5%)
	V	4	(6.4%)
WHO stage	A	3	(4.8%)
	AB	6	(9.7%)
	C	7	(11.3%)
	B1	15	(24.2%)
	B2	12	(19.3%)
	B3	7	(11.3%)
	B2-3	2	(3.2%)
	Unknown	10	(16.2%)
Clinical presentation			
Cough		11	(17.5%)
None (chance finding)		15	(24.2%)
Chest pain		7	(11.3%)
Myasthenia gravis		15	(24.2%)
Dyspnea		11	(17.7%)
Superior vena cava syndrome		2	(3.2%)
Hoarseness		1	(1.6%)

Data are given as counts (percentage)

Results

A total of 54 cases of thymomas and 8 cases of thymic carcinomas (mean age: 50.4±13.2 years, range: 19-74 years) were included in the present study. The general characteristics of the study participants are depicted in Table 1. In total, 57 patients underwent surgery, whereas 5 were not deemed to be eligible for surgical resection. Two patients underwent thymectomy plus pneumoctomy, one thymectomy plus lung wedge resection, whereas the remaining 54 patients underwent thymectomy alone. Eight patients received chemotherapy with a combination of cisplatin, adriamycin, and cyclophosphamide (CAP), six with doxorubicin plus cisplatin, vincristine, and cyclophosphamide (ADOC), one with cisplatin plus etoposide, one with carboplatin plus etoposide, with with doxorubicin alone, whereas the remaining patients did not receive chemotherapy. One patient showed partial response, two patients had disease progression, whereas 59 patients showed a complete response. Forty-one subjects received radiotherapy, and six cancer-related deaths were observed at follow-up. The median overall survival was 24 months (range: 1-168 months).

The BLC2 and VEGFR expression levels did not differ significantly between tumor and normal tissues. Moreover, there were no clearly pathogenic mutations in *KRAS* or *EGFR* genes in any tumor. None of the molecular markers were significantly related to clinical outcomes or patient characteristics (data not shown).

Discussion

Targeted therapies based on predictive biomarkers are eagerly awaited for rare tumors like thymomas and thymic carcinomas (Kelly, 2013). However, the results of our study have shown that changes in BCL2 and VEGFR expression levels are not useful molecular markers of thymic tumorigenesis. Moreover, our data suggest that *KRAS* and *EGFR* mutations do not play a major role in the pathogenesis of thymomas and thymic carcinomas.

Sentman et al. (1991) have previous demonstrated that BCL2 is regionally localized to the mature T cells of the thymic medulla. However, multiple death pathways independent of BCL2 were shown to operate within the thymus to regulate apoptosis of cortical thymocytes. Our data show that thymic malignancies are not characterized by alterations in BCL2 expression. Furthermore, the clinical relevance of BCL2 expression is limited in thymic tumors because it does not correlate with prognosis and there was no strong association between BCL2 and any of the clinical characteristics of the participants in the present study.

VEGF has been involved in the cross-talk between the hematopoietic and epithelial compartments of the neonatal thymus (Cuddihy et al., 2009), but its role in the regulating the growth and proliferation of adult thymocytes remains unclear. Although VEGF and VEGFR were found to be hyperexpressed by immunohistochemistry in thymoma and thymic carcinomas (Cimpean et al., 2008), VEGF levels were previously found to be similar in the sera of patients with thymic tumors and healthy controls (Sasaki et al., 2001). The minor involvement of VEGF and its receptor in thymic malignancies may explain why an angiogenesis inhibitor like bevacizumab did not elicit a significant tumor response when tested in combination with erlotinib in patients with thymomas and thymic carcinomas (Rajan and Giaccone, 2010).

Activating *KRAS* mutations are among the most common oncogenic lesions detected in human malignancies (Schubbert et al., 2007). In the Memorial Sloan-Kettering Cancer Center series, *KRAS* mutations were identified only in 3 of 45 thymic epithelial tumors analyzed (Strobel et al., 2004). The mutations included a G12A, a G12V and a G13V mutation (Strobel et al., 2004). *KRAS* mutations were also assessed in other series including 17 thymic tumors, and no mutations were found (Kurup et al., 2005; Yamaguchi et al., 2006; Christodoulou et al., 2008). *EGFR* mutations are similarly uncommon in thymic tumors, and only three *EGFR* mutations have been described today from a total of 158 tumors tumor samples (Yamaguchi et al., 2006; Yoh et al., 2008). Our data confirm the low frequency of *EGFR* activating mutations in thymic tumors and might explain why these tumors do not generally respond well to *EGFR* inhibitors (Meister et al., 2007).

The strengths of our study include a large sample size of rare thymic tumors and the fact that our results further strengthen and confirm previous reports in the field. In conclusion, by studying a series of thymomes and thymic carcinomas, we demonstrate that molecular alterations in *KRAS* and *EGFR* are not involved in thymic tumorigenesis. Moreover, our results indicate that changes in expression levels of genes other than BCL2 and VEGFR might be involved in the development of thymic malignancies.

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