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Alteration in gene expression profiles of thymoma: Genetic differences and potential novel targets

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Abstract

Background: This study was conducted to investigate the gene expression profiles associated with thymoma to better understand the molecular mechanism underlying the pathogenesis of thymoma.

Methods: Eight patients with thymomas (type A, AB, B1, and B2) and four controls with thymic cysts were analyzed using microarray profiling to identify changes in gene expression.

Results: Across all of our samples, 2319 messenger RNAs were upregulated and 2776 were downregulated in thymomas relative to thymic cysts. Gene ontology and pathway analyses revealed that a large number of genes participate in cellular functions, among which MHC class II protein complex assembly, assembly with peptide antigen, calcium activated phosphatidylcholine scrambling, and release of cytoplasmic sequestered NF-κB were dysregulated, whereas intestinal immune network for immunoglobulin A production, cytokine–cytokine receptor interaction, the calcium signaling pathway, and pathways related to autoimmune diseases were downregulated.

Conclusions: Our results revealed gene expression differences between thymomas and thymic cysts, and identified key candidate genes/pathways that might be used as diagnostic markers and potential therapeutic targets to treat cancer metastasis.

Introduction

Thymomas are rare mediastinal tumors that originate from the epithelial cells of the thymus and may be benign or malignant. The annual incidence of thymomas in China is 0.17/100 000.[1] According to the World Health Organization (WHO) classification, thymomas are histologically distinguished into types A, AB, B1, B2, B3, and C.[2] Types A and AB are mostly benign with lower mortality and a less aggressive rate of growth, whereas

types B1, B2, and B3 are more aggressive and have a greater tendency for intrathoracic spread.

Surgical resection is the main therapeutic intervention for thymoma at early stages, whereas radiation therapy and chemotherapy are used for advanced or recurrent patients who are unable to undergo surgical resection. The average 10-year survival rate for thymoma types A, AB, and B1 is > 80% when paired with early detection, but is dependent on therapeutic strategies and treatments. Because of the limited development of molecularly targeted drugs for thymoma and the rarity of the disease, a better understanding of the molecular pathogenesis of thymoma is urgently needed to identify novel drug targets and biomarkers.[3,4] The rapid development of "-omic" technologies, including whole genome expression analysis and next-generation sequencing (NGS), have provided new insights into the complexity and profiles of genomic alterations of thymoma by: (i) incorporating clinical characteristics, including residual disease, stage, tumor histology, age, albumin level, family history of thymoma, and physical status; and (ii) identifying biomarkers and actionable therapeutic targets for the development of prognostic models of this rare cancer. Petrini *et al.* reported several somatic mutations in thymic carcinomas, including *TP53*, *CYLD*, *CDKN2A*, *BAP1*, and *PBRM1*.[5] Alberobello *et al.* revealed alterations of PI3K caused by mutations in a subgroup of thymomas, leading to an effective strategy to treat these tumors by targeting PI3K.[6] Enkner *et al.* revealed genetic differences between thymomas and thymic carcinomas, suggesting potential novel therapeutic targets.[3] Okuda *et al.* reported mutations in five tyrosine kinase genes (*KIT*, *DDR2*, *PDGFRA*, *ROS1*, *IGF1R*).[7] However, there is still limited knowledge of the pathogenesis of thymoma.

Cancer treatment is shifting toward molecular targeted agents and individually tailored regimens using global gene expression profiling. The purpose of the present study was to elucidate the molecular mechanisms underlying thymoma by molecular gene expression profiling. Using a panel of seven genes, including oncogenes and tumor suppressor genes frequently altered in a variety of tumors, we compared the gene expression profiles of thymomas with those of thymic cysts to analyze oncogenicity in vitro and by real-time (RT) PCR. The results of the present study strengthen our understanding of the molecular mechanisms underlying thymoma and provide potential biomarkers for prognosis and potential therapeutic targets.

Methods

Ethics

The Research Ethics Committee of the Second Hospital of Tianjin Medical University approved this investigation (No. KY2018K055). All patients provided written informed consent before treatment commencement.

Table 1 Thymoma types in the enrolled patients

Type	Control	Thymoma	Total
Thymic cyst	4	0	4
Thymoma type A	0	2	2
Thymoma type AB	0	3	3
Thymoma type B1	0	2	2
Thymoma type B2	0	1	1
Total	4	8	12

Clinicopathological patient features

All of the patients were pathologically diagnosed with thymoma, and were grouped into different subtypes according to pathological features (Table 1). Patients with thymic cysts were used as controls.

Microarray analysis

Total RNA was isolated using Trizol Reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. RNA was assessed for quality control utilizing the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and then purified using the RNeasy mini kit and RNase-Free DNase Set (Qiagen, Hilden, Germany), according to the manufacturer's protocols. Messenger RNA (mRNA) was amplified and labeled using the Agilent Low Input Quick Amp Labeling Kit, one-color, and full genome chip (4 × 44K, design ID: 014850, Agilent Technologies) according to the manufacturer's protocol. The RNeasy Mini Kit was used to purify and conjugate the complementary RNA. Gene chips were hybridized for 17 hours in a hybridization oven at < 65°C and 10 rpm using the Agilent Gene Expression Hybridization Kit with a sample quantity of 1.65 μg complementary RNA according to manufacturer's instructions and then screened using an Agilent Microarray Screener. Slides were rinsed in staining dishes (Thermo Shandon, Pittsburg, PA, USA) with a Gene Expression Wash Buffer Kit. The software was set to the green dye channel at 5 μm scan resolution and 100%, 10%, and 16-bit of photoelectric multiplication tube. Data were captured by Feature Extraction software version 10.7 and Gene Spring software version 11.0, uniformly treated with a Quantile algorithm, and analyzed using an online analysis system (SAS version 1.0). Fold changes ≥ 2 (upregulated) or ≤ 0.5 (downregulated) were used as a cutoff to screen differentially expressed genes.[8]

Real-time (RT) PCR

Total RNA was extracted from thymoma and thymic cyst tissues using TRIzol Reagent (Goldenbridge Biotech,

Beijing, China) according to the manufacturer's instructions. Complementary DNA was digested with DNase 1 and then synthesized via reverse transcription from RNA samples using oligo (dT) primers and TransScript RT/RI/Enzyme Mix (Takara, Beijing, China). Quantitative RT (qRT) PCR was used to determine the relative mRNA transcription levels of PLK5, HMGA2, REG4, SFRP1, CXCL14, and CAV1 to the control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with SYBR green PCR master mix buffer (Takara) and specific primers. As shown in Table 2, the qRT-PCR primers were designed and synthesized by Sangon Biotech Company (Shanghai, China). PCR amplification was conducted as follows: predenaturation at 94°C for three minutes, followed by 35 cycles of 94°C for 30 seconds, 56–58°C for 30 seconds, 72°C for one minute, and finally, extension at 72°C for five minutes. The relative expression levels of targeted genes were normalized to the GAPDH and analyzed by $2^{-\Delta\Delta Ct}$.[8]

Gene ontology (GO) and pathway analysis

Genes and gene products were characterized using gene ontology (GO) analysis with regard to cellular components (CP), molecular functions (MF), and biological processes (BP). Pathway analysis is a potent approach to predict the underlying biological functions of differentially expressed genes,[5] and is widely used to identify major pathways in which differentially expressed mRNAs are distributed. The P value was calibrated with a false discovery rate ($P < 0.05$) to determine the significance of GO term enrichment.

Statistical analysis

SPSS version 19.0 was used for data analysis and t-tests of independent samples were used for continuous variables. The significance level and misjudgment rate of each GO term were estimated by Fisher's exact and chi-squared (χ^2) tests. All data are expressed as the mean ± standard deviation of each group of patients, and an alpha of $P < 0.05$ was used to determine statistical significance.

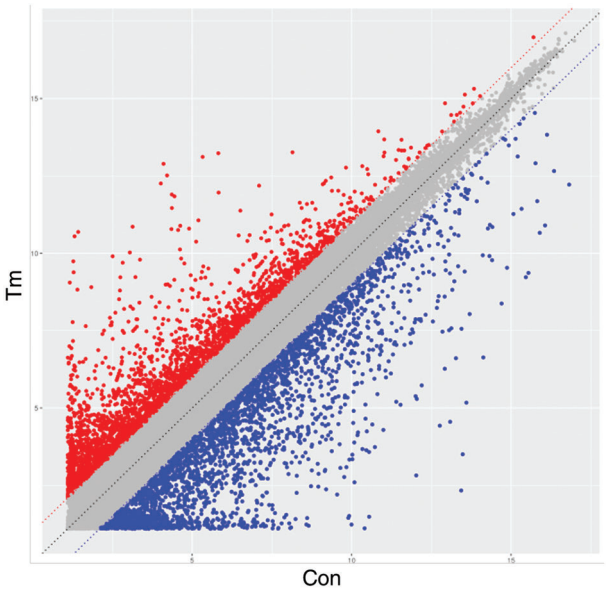

Figure 1 Messenger RNA (mRNA) profile comparisons between thymoma patients (Tm) and thymic cyst controls (Con). The vertical lines correspond to twofold upregulation or downregulation, and the horizontal lines represent $P = 0.05$. The red and blue points highlight the upregulated and downregulated genes, respectively.

Results

Messenger RNA profiles differ between patients with thymoma and controls

Agilent Whole Human Genome Microarray was used to detect mRNA from eight thymoma patients and four thymic cyst controls. A scatter plot of all of the genes measured revealed notable differences in mRNA levels in many genes between the groups. As shown in Figure 1, using a 2/0.5-fold change cutoff, we observed changes in 5095 mRNAs, including 2319 upregulated and 2776 downregulated mRNAs, in thymoma patients compared to controls.

Quantitative RT-PCR validation

To independently validate gene expression changes in thymomas, six mRNAs were randomly selected from 2319

Table 2 Primers for qRT-PCR

Gene	Forward	Reverse
PLK5	GTCAGAATTCAACCTG	GTCAGTCGACGAGGT
	CGCAGATGGAGCCC	CTGCTCAGACTTCAG
HMGA2	TGGTGCAAGACTCAGGAG	CAGTCGGAAAGCAAAGG
REG4	GAACTGGTCTGATGCCGAGC	ATCCACTGCCACTGCTGCCT
SFRP1	CGAGTTTGCACTGAGGATGA	CAGCAAGCTTCTTCAGGTC
CXCL14	AGCCAAAGTACCCGCACTG	AGACCCTGCGCTTCTCGTTC
CAV1	CGGGAACAGGGCAACATGTACA	TCCCTTCTGGTTCTGCAATCACAT
GAPDH	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT, quantitative real-time.

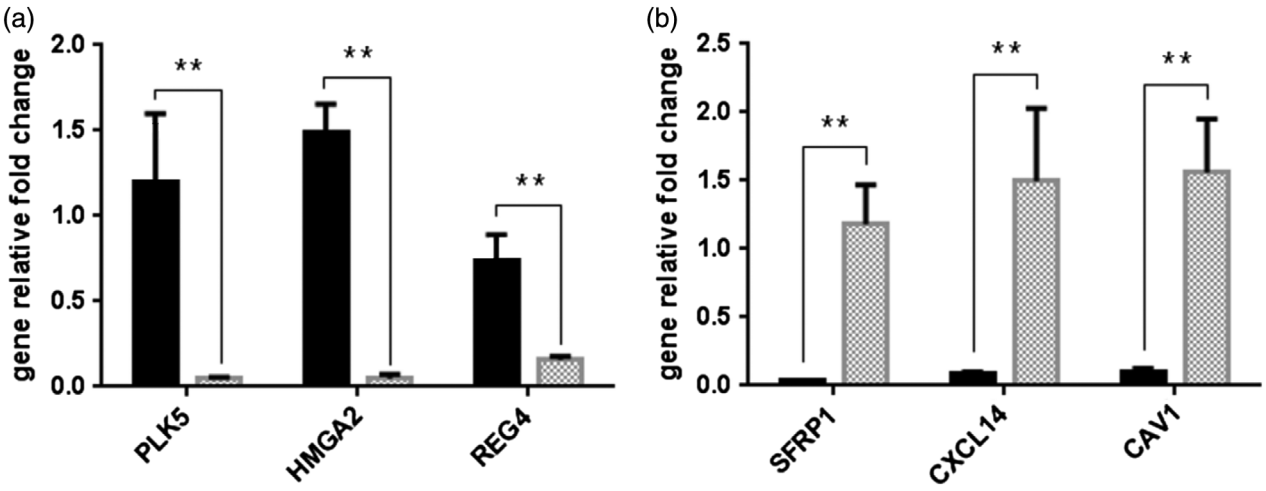

Figure 2 Validation of messenger RNA (mRNA) microarray data by quantitative real-time (qRT) PCR. The relative expression level of (**a**) upregulated and (**b**) downregulated mRNAs was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as means ± standard deviation. ** indicates $P < 0.01$. (■) Tm Group, (▨) Con Group.

upregulated and 2776 downregulated mRNAs in thymoma patients for comparison to controls (Fig 2). Consistent with our microarray analyses, the expression levels of these six mRNAs in thymoma patients were significantly different from the controls, among which PLK5 was the most elevated (55.96-fold higher expression), followed by HMGA2 (46.06-fold higher expression) and REG4 (15.59-fold higher expression). SFRP1, CXCL14, and CAV1 displayed 34.41, 17.55, and 16.26-fold lower expression, respectively.

GO and pathway analysis

We probed significantly altered mRNAs from our microarray studies for gene ontology functions to measure BP, CP, and MF enrichment. We observed that upregulated mRNAs were involved in MHC class II protein complex, assembly with peptide antigen, calcium activated phosphatidylcholine scrambling, and the release of cytoplasmic sequestered NF-κB (Fig 3).

To better understand the potential underlying mechanisms, we performed pathway analyses of upregulated mRNAs with an absolute fold-change ≥ 2 and identified the pathways known to be important in thymoma. As shown in Figure 4, the aberrantly upregulated mRNAs were processes including the intestinal immune network for immunoglobulin A production, cytokine–cytokine receptor interaction, the calcium signaling pathway, and pathways related to autoimmune diseases, such as rheumatoid arthritis, Type I diabetes mellitus, autoimmune thyroid disease, and asthma.

Discussion

We conducted microarray analyses to investigate differential gene expression profiles of human mRNAs in patients with thymoma, presenting a comprehensive gene expression profile and crafting a pathway signature of thymoma using GO enrichment analysis. Compared to thymic cyst controls, thymoma patients differentially expressed 5095 mRNAs. A majority of the identified genes were classified into the intestinal immune network for immunoglobulin A production, cytokine–cytokine receptor interaction, the calcium signaling pathway, and pathways related to autoimmune diseases, such as rheumatoid arthritis, Type I diabetes mellitus, autoimmune thyroid disease, and asthma. Significantly different expression levels of these mRNAs may be related to the development of thymoma-related autoimmune diseases.[9]

Thymomas are rare mediastinal tumors that are distinguished by WHO into type A, AB, B1, B2, and B3 thymomas and other rare subtypes of thymic carcinomas. Type A and AB thymomas are mostly benign, while type B1, B2, and B3 thymomas are more aggressive, with B3 thymomas having the greatest tendency for primarily intrathoracic spread.[10] Malignant thymomas are highly aggressive tumors with frequent lymphatic and hematogenous metastasis. Our results provide novel insights into the nature of this disease by investigating the differentially expressed mRNAs in thymoma patients compared to thymic cyst controls. We identified a total of 5095 differentially expressed mRNAs (2319 upregulated and 2776 downregulated mRNAs) between eight thymoma patients and four thymic cyst controls.

The roles of tumor suppressors SFRP1, CXCL14, and CAV1 have been proposed in many cancers, but have rarely been examined in thymomas. In the present study, we observed the putative tumor suppressors SFRP1, CXCL14 and CAV1 among the downregulated genes in

Figure 3 Gene ontology (GO) enrichment from differentially expressed genes. The top 30 GOs that were dysregulated in thymoma patients compared to controls are shown. Differentially expressed messenger RNAs (mRNAs) were selected for GO analysis. The enrichment factor represents the enrichment of these mRNAs, and the *P* value shows a positive correlation with GO.

thymomas. In non-small cell lung cancer cells, SFRP1 is believed to mediate tumor proliferation, invasion, and migration.[11] Downregulation of CAV1 in gastric cancers is reported to correlate with poor prognosis.[12] CXCL14 prevents cell proliferation and promotes apoptosis by targeting chemokines, resulting in the reduction of many solid tumors.[13] Our results suggest that downregulation of these tumor suppression genes might be a critical molecular component of thymoma, the assay of these genes might serve as a useful biomarker, and overexpression of these genes might be a targeted treatment for thymoma. Further investigation in a well-designed clinical study with appropriate molecular diagnostic gating, including the selection and validation of individual genes as biomarkers, is

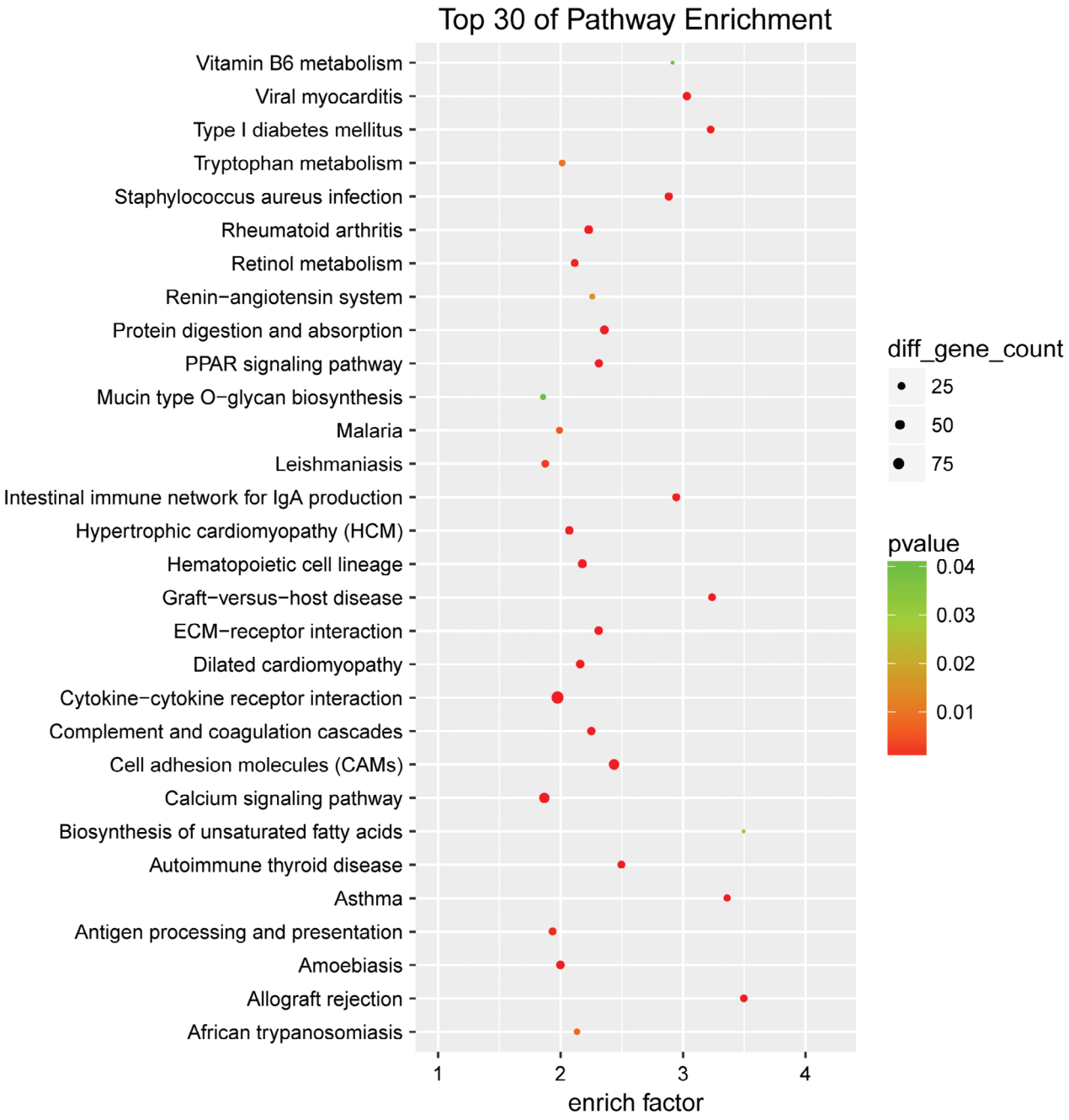

Figure 4 Pathway analysis enrichment of differentially expressed genes. The top 30 pathways that were dysregulated in thymoma patients compared to controls are shown. Differentially expressed messenger RNAs (mRNAs) were selected for pathway analyses. The enrich factor represents the enrichment of these mRNAs, and the *P* value has a positive correlation with pathway.

required to promote precision therapies that specifically target molecules of tumor signaling pathways and predict the response of individual patients to such targeted therapy.

Compared to the control, the mRNAs of PLK5, HMGA2, and REG4 were most significantly overexpressed in thymoma. PLK5 is believed to be oncogenic, and is upregulated in various solid tumors.[14,15] In the present study, however, we found that PLK5 was upregulated in thymoma patients compared to healthy controls, indicating that PLK5 might play an important role in cell death in thymoma. REG4 is reported to promote peritoneal metastasis of gastric cancer,[16] which is supported by our results of the abundant upregulation of mRNA involvement in Reg4 function.

HMGA2 was first identified as an architectural transcription factor regulating the proliferation and differentiation of leukemias.[17] Inconsistent with our findings, other groups have shown that HMGA2 acts as a tumor promoter in malignant tumors and is upregulated and repressed by epigenetic mechanisms.[18] HMGA2, depending on the cellular context, may influence oncogenesis in thymomas. Further study is needed to identify the functional roles of these genes in thymoma carcinogenesis in order to develop new prognostic biomarkers and therapeutic targets.

In conclusion, we successfully identified novel genetic alterations and provide novel molecular profiles for thymoma. These gene expression changes between thymomas and thymic cysts will be helpful to identify key genes/pathways responsible for thymoma and might be used as diagnostic markers and therapeutic targets for thymoma.

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Disclosure

No authors report any conflict of interest.

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