Elucidation of the molecular mechanisms of anaplastic thyroid carcinoma by integrated miRNA and mRNA analysis

GUOPING LIU, KAINAN WU and YUAN SHENG

Department of Breast Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, P.R. China

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Abstract. To elucidate the complex molecular mechanisms of anaplastic thyroid carcinoma (ATC), the mRNA and miRNA expression profiles of ATC were systematically explored. A total of 55 common differentially expressed genes (DEGs) were obtained from two mRNA expression datasets including 23 ATC samples and 24 paired normal samples. Gene expression levels of three randomly selected DEGs, VCAN, COL5A1 and KCNJ16, were examined using RT-PCR in 10 ATC samples. Notably, the ATC and normal samples were clearly classified into two groups based on their common DEGs. Moreover 23 common DEGs, such as TG, NKK2-1, KCNJ16 and CTHRC1, were predicted to be the potential targets of 17 identified miRNAs in ATC. Meanwhile, several miRNA target genes were associated with biological processes related to tumor progression such as angiogenesis, cell migration or growth and potassium channel regulation. In summary, the poor prognosis of ATC is possibly caused via complex biological processes. Firstly, angiogenesis was activated by the high expression of CTHRC1, VCAN and POSTN, providing necessary nutrition for tumor cells. Then tumor distant metastasis was induced via stimulation of cell migration and cell growth or regulation of cell-cell interaction. Moreover, intracellular potassium concentration changes promoted ATC progression indirectly. Hence, identification of these critical DEGs was valuable in understanding the molecular mechanisms of ATC.

Introduction

The incidence of thyroid cancer, one of the most common endocrine malignancies, has increased rapidly in recent years based on worldwide statistics (1). The annual number of newly diagnosed thyroid cancer cases is 129/1,000,000, and the associated deaths are 5/1,000,000 (2). Thyroid cancer can be divided into well-differentiated carcinomas (such as papillary or follicular carcinomas) and anaplastic thyroid cancer (ATC) (3). The percentage of ATC is low, ranging from 1 to 5% of all thyroid cancers, but accounts for ~14-50% of deaths (4). The poor prognosis and the high rate of distant metastases of ATC lead to a 5-year survival rate of 7% (5).

Considering the complexity of the molecular mechanisms, various studies have been carried out such as mRNA (6) and miRNA expression profiling (5) by microarray, gene mutation whole exome sequencing (7), cytogenetic analysis and comparative genomic hybridization (CGH) microarray (8). Several de novo mutations including TP53, β-catenin and PIK3CA have been identified in ATC whereas in pre-existing mutations in PTC (papillary thyroid cancer) mutations such as RAS and BRAF have been found (4). mRNA and miRNA expression levels have also been demonstrated to be critical in tumor progression. A study by von Roemeling et al showed that stearoyl-CoA desaturase 1 (SCD1) associated with fatty acid metabolism is highly expressed in ATC compared with that in normal samples (9). Gene expression analysis of ATC and PTC demonstrated that most of the DEGs were common in both, but ATC contained more genes associated with epithelial to mesenchymal transition (EMT), dedifferentiation and glycolytic phenotypes (6). Furthermore, miRNA expression profile analysis of 11 ATC samples showed that 17 common miRNAs were downregulated and one was upregulated (5).

Although previous research has been carried out for ATC, the combination analysis of mRNA and miRNA expression profiles has not yet been systematically explored. A substantial amount of mRNA expression datasets have been submitted to the GEO (Gene Expression Omnibus) database and bioinformatic methods have been demonstrated to be valuable for molecular mechanism investigation (10). In the present study, mRNA expression datasets from different laboratories were analyzed and several DEGs were identified by RT-PCR. Then common DEGs were subjected to subsequent function analysis. Furthermore, miRNA and mRNA expression levels were integrated for the elucidation of the molecular mechanisms.

Materials and methods

Gene expression profiles. Two gene expression profiles GSE33630 and GSE65144 submitted by Dom et al (11) and von Roemeling et al (9), respectively, were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). For the GSE33630 dataset, 11 ATC samples...
and 11 paired normal samples were selected for subsequent analysis. The GSE65144 dataset consisted of 12 ATC samples and 13 normal samples (12 matched and 1 unmatched). Experiments for the two datasets were carried out using GPL570 platform (HG-U133_Plus_2, Affymetrix Human Genome U133 Plus 2.0 array).

Data preprocessing and screening of DEGs. The mRNA expression profiles were subjected to log2 transformation, background correction and normalization using the GeneChip Robust Multi-array Analysis (GCRMA) method within the Bioconductor package (http://www.bioconductor.org) (12). The uninformative probe sets such as control probe sets, and genes with low expression variance were filtered out. For genes with multiple probes, the average expression value was calculated. Finally, the significant DEGs were identified using the Limma (Linear Models for Microarray Analysis) (13) package with criteria of adjustment of p<0.01 and |log2 fold change (FC)| ≥2. DEGs with a fold change >5 were used for downstream analysis. The heat map was constructed using the heat map method in Bioconductor.

Quantitative RT-PCR analysis. The mRNA expression levels of three randomly selected DEGs, *VCAN*, *COL5A1* and *KCNJ16*, were examined using RT-PCR. The total RNA was extracted from the 10 ATC samples and the adjacent normal tissues using TRIzol reagent (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions. cDNA was obtained using M-MLV reverse transcriptase based on the manufacturer's protocol (Promega, Madison, WI, USA). The *VCAN*, *COL5A1* and *KCNJ16* mRNA expression levels were detected using 7500 Real-Time PCR system (Thermo Fisher Scientific). Relative quantification was normalized using *GAPDH* mRNA expression and calculated with the 2^-ΔΔCt method. The primer sequence list is as follows: *VCAN* forward, 5'-CACAACCCGCATTTGAACTTG-3' and reverse, 5'-CGCAGCGCCTGGAGTTCTT-3'; *COL5A1* forward, 5'-ACAAGGCTGGATGAAATAACAA-3' and reverse, 5'-CCGGGCCTTTGGGAAGATC-3'; *KCNJ16* forward, 5'-TCAATGCGGACGAATAACATAC-3' and reverse, 5'-AATCGTCTTCTTGCTCTTCTCTC-3'; and *GAPDH* forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTTGCTGTACAAATAAC-3'.

Functional and pathway enrichment analysis. In order to explore the biological processes involved in ATC, functional and pathway enrichment analyses for the common DEGs were carried out using Database for Annotation, Visualization and
Integrated Discovery (DAVID) online tools (14), which is based on the Gene Ontology (GO) (15) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (16) databases. The criterion for significantly enriched pathways was set as p≤0.05. GO terms, consisting of biological processes (BP), cellular components (CC) and molecular functions (MF), were screened with a p≤0.05.

Gene interaction and miRNA regulation network analysis. The physical interaction and validated pathway interaction network for the common DEGs was constructed using the Gene Multiple Association Network Integration Algorithm (GeneMANIA, http://www.genemania.org/) (17). Moreover, 17 deregulated miRNAs from the study of Hebrant et al (5) were integrated for the exploration of miRNA-mRNA interaction by using the CyTargetLinker plugin of Cytoscape (18).

Results
Common DEGs. After background correction and normalization, the medians of the gene expression values were almost at the same level indicating that the data were suitable for subsequent analysis (Fig. 1). DEGs for the two datasets were identified independently, and a total of 174 and 64 DEGs were screened out for GSE33630 and GSE65144, respectively. Moreover, 55 DEGs (accounting for 32 and 85% for GSE33630 and GSE65144, respectively) were found to be simultaneously differentially expressed in these two datasets (Fig. 2A). Among the common DEGs, 15 genes were upregulated and 40 were downregulated (data not shown). In addition, correlation of expression values for the 55 common DEGs was 0.98 (p<2.2e-16, Fig. 2B).

Furthermore, the 55 common DEGs were used for the classification of ATC and normal samples. As indicated in Fig. 3,
the ATC and normal samples were clearly classified into two groups except for sample ATC_2, which was possibly due to the smaller expression value variation, ranging from 8-12.

**mRNA expression validation by RT-PCR.** To verify mRNA expression levels of the identified DEGs, RT-PCR experiments were carried out for three randomly selected genes including VCAN, COL5A1 and KCNJ16. The mRNA expression levels in the 10 ATC samples and adjacent normal tissues were analyzed. Results showed that the expression levels of VCAN and COL5A1 were higher in the tumor tissues than the levels in the adjacent normal tissues; whereas, the mRNA expression level of KCNJ16 was lower in the tumor tissues (Table I and Fig. 4). These results were nearly consistent with those in the microarray analysis.

**Functional and pathway enrichment analysis.** In order to explore the functions of these common DEGs, functional and pathway enrichment were carried out. The results indicated that the two pathways were significantly enriched for the common DEGs (Table II) and that TG, TPO and TSHR participated in the autoimmune thyroid disease pathway (p=0.0083), and CLDN8, CDH1 and VCAN were significantly involved in the cell adhesion molecule (CAM) pathway (p=0.049).

Furthermore, the common DEGs were mainly related to the cellular components of the extracellular region (p=2.5E-05) and the proteinaceous extracellular matrix (p=7.3E-05). Some common DEGs, such as TNFAIP6, VCAN, POSTN and COL5A1, were significantly enriched in the carbohydrate binding activity (p=0.002) and glycosaminoglycan binding function (p=0.006). In addition, hormone regulation-related processes such as regulation of hormone levels (p=4.6E-05), hormone biosynthetic processes (p=7.3E-05) and thyroid hormone generation (p=1.0E-04) biological processes were also enriched.

**Gene interaction and miRNA regulation analysis.** Furthermore, the interaction between these common DEGs was explored to elucidate the potential regulatory mechanism. The results indicated that several important subnetworks were formed based on different pathways. Fig. 5A shows that LRP2 can interact with SERPINE and TG physically and TSHR can interact with TSHB physically. Also these common DEGs participated in the autoimmune thyroid disease pathway similar to our results from the pathway enrichment analysis. In addition, CDH1,
CLDN8 and VCAN were found to independently participate in cell adhesion molecules. The SLC26A gene family was significantly involved in the multifunctional anion exchanger pathway (Fig. 5B).

For the discovery of miRNAs that can regulate the expression of these common DEGs, the potential targets of 17 reported miRNAs (5) in ATC were predicted. The results showed that 870 and 13,841 target genes were found in the miRTarBase (version 4.4) (19) and TargetScan (version 6.2) (http://www.targetscan.org/) databases, respectively. Among these target genes, 23 genes were identified among the 55 common DEGs, and the miRNA and mRNA interaction network between them is shown in Fig. 6. The network indicated that hsa-let-7f-5p miRNA regulates TG and NKX2-1, and hsa-miR-148b-3p miRNA regulates KCNJ16 and CTHRC1 which was validated by our experiments. Other interactions were predicted based on the TargetScan database.

Discussion

ATC is a type of thyroid cancer with poor prognosis and has been widely studied in mRNA expression, miRNA expression or genome mutational landscape levels. However, efforts trying to elucidate the molecular mechanisms of ATC with the combination of different omics data are rare. In this study, mRNA and miRNA expression levels were combined to explore the potential molecular mechanisms. The mRNA expression analysis showed that 55 common DEGs were simultaneously differentially expressed in the tumor samples of the GSE33630 and GSE65144 datasets. Based on the 15 upregulated and 40 downregulated genes, the ATC and normal samples were clearly classified into two groups. The error assignment of ATC_2 was possibly due to sample quality or tumor heterogeneity. RT-PCR analysis established that VCAN and COL5A1 were significantly expressed in 10 tumor tissues, compared with that in adjacent normal tissues and that the mRNA expression level of KCNJ16 was lower in the 10 tumor tissues.

Notably, the pathway enrichment analysis revealed that three downregulated DEGs including TG, TPO and TSHR were significantly involved in the autoimmune thyroid disease pathway. Although the relationship between thyroid cancer and autoimmune thyroid disease is unclear, the co-existence of these two clinical afflictions has been demonstrated by retro-
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Figure 6. miRNA and mRNA interaction network from the miRTarBase (version 4.4) and the TargetScan database. The pink hexagons indicate the target genes predicted using the miRTarBase and the green circles indicate the miRNAs.

Figure 7. Possible molecular mechanism diagram of anaplastic thyroid carcinoma (ATC) based on the analysis results. The inverted triangles associated with the rectangles represent downregulated miRNA; ellipses represent target genes. Combined vertical and horizontal symbols represent inhibition. Rectangles represent biological processes.
spective cohort analysis. One study showed that thyroid cancer is significantly associated with an elevated concentration of TgAb (thyroglobulin antibodies, OR=1.57; CI=1.11-2.23) based on the study of 253 patients with thyroid cancer (20). Moreover, one review systematically summarized the reports concerning the link between thyroid autoimmunity and differentiated thyroid cancer (DTC) (2). The low expression of TG, TPO and TSHR in this study was possibly caused by the lower expression of NKX2-1 which can bind to thyroglobulin promoter and regulate thyroid functional gene expression (21). Research has shown that promoter hypermethylation of TSHR is significantly related to TSHR gene silencing (22). With relatively rich CpG dinucleotides, TSHR can be methylated (23) and suppress thyroid iodide-metabolizing molecules. Hence thyroid tumor cells are unable to concentrate iodine and are insensitive to radiiodine (24). These downregulated genes are possibly the results of thyroid cancer (25,26).

Considering the complexity of cancer, the molecular mechanisms of ATC are far from clear. Based on the integration analysis of mRNA and miRNA expression (5), the unknown mechanisms of ATC were further revealed. Integrative analysis results showed that the progression of ATC is considered to be a multipath process including the angiogenesis process, the Wnt/β-catenin pathway, cell migration or cell-cell interaction and potassium channel function (Fig. 7). Research has shown that angiogenesis is critical for cancer cell proliferation and metastatic spread due to the requirement of tumors for adequate oxygen and nutrient supply (27). In the present study, the angiogenesis process was activated by the upregulated expression of CTHRC1, VCAN and POSTN and based on the miRNA regulation analysis results. The upregulated expression of CTHRC1, VCAN and POSTN was the consequence of the downregulated expression of miR-148b-3p, miR-141-3p, miR-135a-5p and miR-19a-3p, respectively (Fig. 7). Additionally, somatic mutation of VCAN (D748G, missense) is possibly related to its functional upregulation (7). CTHRC1, as a novel oncogene, has been proved to be abnormally overexpressed in malignant tumors such as melanoma, breast, pancreas, human non-small cell lung and thyroid cancers (28-32). Overexpression of CTHRC1 may contribute to vascular remodeling and cell migration by suppressing collage matrix deposition (33). Moreover, reports have shown that CTHRC1 anchoring on the cell membrane may stabilize the physical interaction between frizzled receptors and Wnt ligands, and activate the non-canonical Wnt pathway regulating cell motility (34). Moreover, VCAN, as a member of the versican proteoglycan family, is also valuable for angiogenesis. Yang and Yee reported that VCAN-transfected tumor cells, exhibited enriched vascularization and accumulation of red blood cells by H&E staining compared with vector-transfected cells (35). In addition, the endothelial marker of blood vessel formation CD34 was significantly overexpressed in tumor sections (35). Immunocytochemistry analysis also showed that VCAN-transfected cells contained more and larger blood vessels than the control cells (36). These angiogenesis-related processes participated in the enhancement of endothelial cell activities and fibronectin and vascular endothelial growth factor (VEGF) expression (36). Furthermore, POSTN, an adhesion molecule in osteoblasts, was identified to be overexpressed and be related to the angiogenesis process. Research has been carried out to explore the relationship between POSTN and angiogenesis. In keloids, overexpression of POSTN was found to promote angiogenesis by inducing ERK1/2 and focal adhesion kinase pathways and by upregulating expression of VEGF and angiopoietin-1 (37). Additionally, the upregulation of VEGF receptor 2 was identified in human breast cancer with acquired POSTN expression (38), and POSTN promoted angiogenesis via the paracrine pathway by interacting in a α,β- and α,β-dependent process in ovarian cancer (39). All in all, these three overexpressed genes possibly enhance ATC metastasis and progression via the angiogenesis process.

Moreover, cell migration, cell growth and cell-cell interactions are also critical in the development or metastasis of tumors. In the present study, ADAM12, COL5A1 and ANLN were identified to be overexpressed and their upregulation was possibly caused by the low expression of corresponding miRNAs such as miR-19a-3p, miR-29a-3p, miR-135a-5p, miR-30e-5p, miR-30a-5p, miR-144-3p, miR-135a-5p, miR-29a-3p and miR-200b-3p. Additionally somatic mutations of ANLN (R1095W, missense) and COL5A1 (G1348A, missense) were found to contribute to its upregulation (40). ADAM12, as one of the disintegrins and metalloproteases, has been demonstrated to be involved in several pathological processes. Microarray experiments indicate that ADAM12 is upregulated in aggressive fibromatoses. The mechanism of ADAM12 in breast tumor has been systematically investigated and the results showed that ADAM12 reduced tumor cell apoptosis and simultaneously increased stromal cell apoptosis (41). In serous ovarian carcinoma, high levels of ADAM12 mRNA were detected possibly caused by TGFβ signaling (42). Moreover, COL5A1 encoding an α-chain of fibrillar collagens also regulated cell migration and motility (43,44). COL5A1 binds to α2β1-integrin or β1-integrin receptor and activates the corresponding signaling pathways. In pancreatic ductal adenocarcinoma, high expression of COL5A1 significantly affected cell adhesion, migration and viability based on results from β1-integrin inhibition, siRNA ablation of COL5A1 expression and COL5A1 knockdown experiments (45). In regards to colorectal carcinogenesis, RT-PCR results showed that COL5A1 was co-expressed with COL11A1 in tumor samples rather than in normal samples (46). In addition, the actin-binding protein ANLN is also critical for cell growth, migration and cytokinesis (47). Zhou et al showed that knockdown of ANLN markedly inhibited breast cancer cell line proliferation and colony formation, and more cells were blocked at the G2/M phase (47). Although a detailed mechanism of ANLN in carcinoma progression is still unclear, in silico pathway prediction indicated that the Wnt/β-catenin signaling pathway is associated with ANLN downstream regulation (48).

Moreover, ion channels especially potassium channels have been demonstrated to play a crucial role in tumors (49). In the present study, KCNJ16 was downregulated which led to an ion concentration unbalance between the extracellular and intracellular compartments. Rather than a single mechanism, potassium channel regulation may influence tumor progression via multiple paths such as cell adhesion or migration, angiogenesis and apoptosis (49). The complex regulation mechanism of KCNJ16 in ATC progression still remains to be explored.

In summary, the poor prognosis of ATC is possibly induced by various processes. Firstly, upregulation of CTHRC1, VCAN, POSTN, ADAM12, COL5A1 and ANLN contributes to the development of ATC.
References


16. Liu et al: EXPLORATION OF THE MOLECULAR MECHANISMS OF ANAPLASTIC THYROID CARCINOMA and POSTN promotes angiogenesis and provides necessary nutrition for tumor cells. Then ADAM12, COL5A1 and ANLN induce cell migration, cell growth or cell-cell interaction leading to tumor distant metastasis. Finally, KCNJ16 regulates intracellular and extracellular ion concentrations and promotes ATP progression.

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