Abstract. The incidence of thyroid cancer has recently experienced a rapid increase in China, and papillary thyroid carcinoma (PTC) accounts for nearly 80% of human thyroid cancers. In the present study, the differential expression of microRNAs (miRNAs) and their target genes were identified in order to analyze the potential roles of miRNAs as biomarkers and in papillary thyroid carcinogenesis. One hundred and twenty-six PTC samples were collected from patients at the China-Japan Union Hospital, China, and the gene/miRNA expression profiles were examined with Illumina BeadChips and verified by real‑time RT‑PCR. Gene Ontology (GO) categories were determined, and pathway analysis was carried out using KEGG. miRNA target genes were predicted by implementing three computational analysis programs: TargetScanS, DIANA-microT and PicTar. Two hundred and forty-eight miRNAs and 3,631 genes were found to be significantly deregulated (gene, P<0.05; miRNA, P<0.01) in PTC tissues when compared with their matching normal thyroid tissues. hsa-miR-206 (target gene, MET), hsa-miR-299-3p (target gene, ITGAV), hsa-miR-101 (target gene, ITGA3), hsa-miR-103 (target gene, ITGA2), hsa-miR-222 (target genes, KIT and AXIN2), hsa-miR-15a (target genes, AXIN2 and FOXO1) and hsa-miR-221 (target gene, KIT) were identified. Together with the functions of the target genes, we further elucidated the role of miRNAs in papillary thyroid carcinogenesis and suggest the use of miRNAs as biomarkers for early diagnosis. Our findings provide the basis for future studies in the field of miRNA-based cancer therapy.

Introduction
Thyroid cancer is the most common endocrine malignancy and recently its worldwide incidence is rapidly rising (1,2). It is considered as a highly prevalent malignant disease. The American National Cancer Institute reported that during the years of 2005-2009, the median age at diagnosis for thyroid cancer was 50 years, and 56,460 new cases and 1,780 deaths from thyroid cancer were estimated in the United States in 2012. There are four main types of thyroid cancer: papillary, follicular, medullary and anaplastic thyroid cancer. Papillary thyroid carcinoma (PTC) accounts for approximately 80% of human thyroid cancers. Thyroid cancer constitutes one of the most curable tumors; however, the differential diagnosis can often be elusive. Although fine-needle aspiration biopsy is currently the most useful diagnostic technique for evaluating a thyroid nodule, preoperative diagnosis of thyroid nodules is frequently imprecise, with up to 30% of fine-needle aspiration biopsy cytology samples reported as ‘suspicious’ or ‘indeterminate’ (3). Therefore, molecular-based diagnostic approaches are needed for the preoperative distinction of these lesions.

It has been demonstrated that numerous miRNAs (miRNAs) are transcriptionally upregulated in PTC compared with unaffected thyroid tissue. miRNAs are small (~21 nucleotides long) non-coding RNAs, which regulate gene expression at the post-transcriptional level and play important roles in a multitude of biological processes, including tumorigenesis (4,5). A specific miRNA may function either as an oncogene or as a tumor suppressor by regulating the expression of target oncogene(s) and tumor suppressors, respectively. Generally, miRNAs bind to the 3'-untranslated regions (3' UTRs) of target mRNAs, leading to mRNA degradation or repression of translation. In the present study, miRNA and gene profiles were analyzed to identify the differential expression of miRNAs and target
genes in PTC vs. normal thyroid tissues in an attempt to elucidate the potential roles of miRNAs as biomarkers and in the carcinogenesis of PTC.

Materials and methods

Tumor specimens. A total of 126 thyroid samples were collected from PTC patients undergoing thyroidectomy at China-Japan Union Hospital, China between 2010 and 2011. After surgical excision, the specimens including PTC and their matching normal thyroid tissues were snap frozen in liquid nitrogen until use. The pathological types of the tumors were verified by pathologists. All tissues were obtained following prior informed consent from each patient.

RNA extraction. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quantity and the integrity of extracted RNA were determined by UV spectrophotometer. Total RNA was quantified by formaldehyde denaturing gel electrophoresis.

Real-time reverse transcription-PCR. Real-time reverse transcription-PCR was used to verify the gene expression using Stratagene Mx3000P (Japan). Reverse transcription was performed with 500 ng RNA for the 10-µl reverse transcription reaction, and 1 µl cDNA was then used for each 25-µl reaction mixture that contained an optimal concentration of primers and SYBR-Green Supermix (SYBR Premix Ex Taq II; Takara Co., Osaka, Japan). A total of 45 cycles was performed, consisting of 20 sec at 95˚C and 20 sec at 60˚C for each cycle. GAPDH was used as an internal control.

Illumina BeadChip and data analysis. Total RNAs were hybridized to the Illumina Human Genome BeadChip Array and miRNA BeadChip (Illumina). The arrays were scanned on an Illumina BeadStation 500 System, and the hybridization data were analyzed using Illumina BeadStudio software. The following filtering criteria were used for selection of differentially expressed genes: for a positive gene or miRNA in the tumor (T) or control (N) (matching normal thyroid tissues): detection, P<0.05; ratio (groupT/groupN) >2 or ratio (groupT/groupN) <0.5. All data were MIAME compliant, and the raw data were deposited in the ArrayExpress database along with the normalized data.

Gene Ontology (GO) category and pathway analysis. The differentially expressed genes were analyzed for inclusion in GO categories and pathways. Categorization in significant biological processes was performed using tools of the Gene Ontology project (http://www.geneontology.org). The test of statistical significance considers the number of differentially expressed genes found in each category compared with the total number of genes in the category represented on the chip. The pathway analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Two-sided Fisher's exact test and the \( \chi^2 \) test were used to classify the GO category and pathway, and the false discovery rate (FDR) was calculated to correct the P-value. P-value <0.05 and FDR <0.05 were used as a threshold to select significant GO categories and KEGG pathways.

Table I. Result of the gene and miRNA chips.

<table>
<thead>
<tr>
<th>Genes</th>
<th>miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated</td>
<td>2,013</td>
</tr>
<tr>
<td>Downregulated</td>
<td>1,618</td>
</tr>
<tr>
<td>Total</td>
<td>3,631</td>
</tr>
</tbody>
</table>

The gene network analysis of the differentially expressed genes involved in significant pathways was carried out using the KEGG database. Interactions of genes in the database were analyzed and gene networks were established.

Prediction of miRNA target genes. In the present study, three websites including TargetScanS (http://www.targetscan.org/), DIANA-microf (http://diana.cslab.ece.ntua.gr/microT/) and PicTar (http://pictar.mdc-berlin.de/) were used to predict miRNA targets. A gene that was predicted in two or three of these target prediction programs was selected as a potent candidate.

Results

miRNA and gene expression in PTC tissues. Two hundred and forty-eight miRNAs and 3,631 genes were significantly differentially expressed (for genes, P<0.05; miRNA, P<0.01) between PTC tissues and the matching normal thyroid tissues. Of these 248 miRNAs, 180 were overexpressed and 68 were underexpressed in PTC tissues. Of the 3,631 genes 2,013 were overexpressed and 1,618 were underexpressed in PTC tissues (Table I). To verify the results of the chips, real-time RT-PCR was carried out for verification.

Deregulated genes involved in carcinogenesis. After the differentially expressed genes were analyzed for inclusion in GO categories and KEGG pathways, 68 of the deregulated genes were found to be involved in tumor signaling pathways or to play important roles in cancer (data not shown) including the Wnt signaling pathway, mTOR signaling pathway, JAK-STAT signaling pathway, ErbB signaling pathway, MAPK signaling pathway, VEGF signaling pathway, PPAR signaling pathway, adherens junction, ECM-receptor interaction, focal adhesion, cytokine-cytokine receptor interaction, apoptosis and the cell cycle (Fig. 1).

Matching target genes were determined for the deregulated miRNAs in PTC. The most popular three target prediction programs were used to identify putative target genes for the 248 deregulated miRNAs in PTC. Matching target genes for 88 differentially expressed miRNAs in PTC were successfully found (data not shown). Of the 88 miRNAs, 64 were upregulated and 24 were downregulated. Using the results of the target genes, we compared the data with the deregulated genes from the gene BeadChip data to find those genes similarly expressed. After the data analysis, 578 target genes for the differentially expressed miRNAs in PTC were finally identified. Of these 578 genes, 298 were upregulated and 280 were downregulated.
Association between the deregulated miRNAs and their putative target genes. To find a correlation between miRNAs and their target genes in cancer pathways of PTC, the 578 target genes of the deregulated miRNAs were compared with 68 genes which participate in cancer pathways. Twenty-eight genes and the related signaling pathways were consistent. We found that the target genes of the deregulated miRNAs were involved in the Wnt signaling pathway, mTOR signaling pathway, JAK-STAT signaling pathway, ErbB signaling pathway, MAPK signaling pathway, VEGF signaling pathway, ECM-receptor interaction, focal adhesion, cytokine-cytokine receptor interaction, apoptosis and the cell cycle (Fig. 1). Of these 28 genes, 16 were upregulated and 12 were downregulated. We further investigated the relationship between the miRNAs and their target genes in the cancer pathways of PTC. There were 29 miRNAs and 28 genes. Of these 29 miRNAs, 17 were upregulated (12 weakly expressed related target genes) and 12 were downregulated (16 highly expressed related target genes) (Tables II and III). We next sought to identify mRNAs and their putative target genes which were
significantly differentially expressed and which had both a high ratio (upregulated ratio >2 or downregulated ratio <0.5) and a high signal (either signal value >200). Finally, hsa-miR-206 (target gene, MET), hsa-miR-299-3p (target gene, ITGA5), hsa-miR-101 (target gene, ITGA3), hsa-miR-103 (target gene, ITGA2), hsa-miR-222 (target genes, KIT and AXIN2), hsa-miR-15a (target genes, AXIN2 and FOXO1) and hsa-miR-221 (target gene, KIT) were identified (Table IV).

To verify the results, hsa-miR-222, hsa-miR-15a and their consistent putative target gene AXIN2 were validated by real-time PCR. hsa-miR-222 was 94.11% upregulated, consistent with previous research, hsa-miR-15a was 64.7% upregulated and AXIN2 was 80% downregulated between PTC and their matching normal thyroid tissues.

In our study, hsa-miR-222, hsa-miR-15a and their consistent putative target gene AXIN2 were verified by real-time PCR. hsa-miR-222 was 94.11% upregulated, consistent with previous research, hsa-miR-15a was 64.7% upregulated
and AXIN2 was 80% downregulated, and we found that 94.12% of the samples had regulatory relationships between hsa-miR-222 and AXIN2, and 70.59% between hsa-miR-15a and AXIN2. The consistent trend suggested that the microarray assays were reliable. It was previously reported that hsa-miR-221 and hsa-miR-222 were upregulated in PTC tissues (14-18), consistent with our results, while hsa-miR-15a and hsa-miR-299 were downregulated (19). At the mRNA levels, MET was demonstrated to be upregulated (20) and KIT was downregulated (21,22). In thyroid oncocytic tumors, ITGA5 was found to be upregulated (23). The putative target gene of hsa-miR-221 and hsa-miR-222 has been identified as KIT, a tyrosine kinase receptor that plays an important role in cell growth and differentiation, acting as an oncogene in many types of cancers (24,25). The above-mentioned results are consistent with our studies. Several deregulated miRNAs and genes have been identified by other researchers, such as hsa-miR-221, hsa-miR-222, hsa-miR-15a, hsa-miR-299, MET and KIT using the same gene array and real-time PCR. However, other miRNAs, such as hsa-miR-206, hsa-miR-101, hsa-miR-103, and the putative target genes ITGA5, ITGA2, ITGA3, FOXO1 and AXIN2 analyzed in our study were not reported previously. Their functions and significance still need further examination.

According to the different mechanisms of target genes in cancer development, ectopic manipulation of miRNA expression has been recently suggested as a novel therapeutic modality. In our study, the target genes are known to widely participate in the cancer process.

Met, the receptor for hepatocyte growth factor (HGF), is an oncogene that encodes the c-Met protein. c-Met protein is a hepatocellular growth factor receptor and is known to be responsible for the motility and mitogenesis of epithelial cells, including cancer cells (26,27). Met is frequently activated in these carcinomas and may favor tumor growth, and the abundance of Met expression may differentially regulate cell growth, morphogenesis and migration in response to HGF.

ITGA2, ITGA3 and ITGA5, members of the integrins, are invasion-related genes (28,29). Integrins are transmembrane adhesion molecules that mediate cell-cell and cell-extracellular matrix attachment. Integrins regulate cell growth, proliferation, migration and apoptosis and as a consequence, have a potential role in tumor progression and metastasis (30). It has been demonstrated that upregulation of the integrin subclasses could in fact enhance neoplastic cell motility and vascular endothelial growth factor expression (31,32). ITGA5 encodes the integrin α-chain V protein involved in cell adhesion (cell to cell junctions) and signal transduction. Overexpression of ITGA5 was significantly linked to the invasion and metastasis of laryngeal and hypopharyngeal squamous cell carcinoma (33).

Table V. Results of the real-time PCR.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Upregulated</th>
<th>Target gene</th>
<th>Downregulated</th>
<th>miRNA upregulated and target gene downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-222</td>
<td>94.11%</td>
<td>AXIN2</td>
<td>80%</td>
<td>94.12%</td>
</tr>
<tr>
<td>hsa-miR-15a</td>
<td>64.7%</td>
<td>AXIN2</td>
<td>80%</td>
<td>70.59%</td>
</tr>
</tbody>
</table>

FOXO1, acting as a regulator of cell responses to oxidative stress, is required for cell transition from proliferative growth to quiescence (by similarity). It triggers the Akt-dependent phosphorylation and inactivation of FKHR1 functioning, suppresses the transcription of death genes, such as the Fas ligand gene, and thereby promotes cell survival (34).

Axis inhibition protein 2 (AXIN2) is a negative regulator of the Wnt/β-catenin pathway and functions by participating in a negative feedback loop to limit the duration and intensity of a Wnt-initiated signal. This effect was demonstrated in several solid tumors such as colorectal carcinoma, liver tumors, craniopharyngiomas, hepatoblastoma and rare pancreatic tumors (35). Although it has been accepted that altered Wnt signaling is a late event in thyroid cell transformation that affects anaplastic thyroid tumors, recent data suggest that it is also altered in PTC with RET/PTC mutations (36).

The genes described in the present report are associated with the carcinogenesis, unlimited growth and invasiveness. The low or high expression of specific genes leads to malignant transformation of normal thyroid cells, and tumor metastasis may consequently occur in the disease process. Regulation of gene expression by miRNAs may become an important artifice for the treatment of cancer. These genes can also serve as diagnostic biomarkers.

In summary, several thyroid cancer-related miRNAs and their target genes were identified. These include hsa-miR-206 (target gene, MET), hsa-miR-299-3p (target gene, ITGA5), hsa-miR-101 (target gene, ITGA3), hsa-miR-103 (target gene, ITGA2), hsa-miR-222 (target genes, KIT and AXIN2), hsa-miR-15a (target genes, AXIN2 and FOXO1) and hsa-miR-221 (target gene, KIT). According to the functions of the target genes, we can further elucidate the role of miRNAs in papillary thyroid carcinogenesis and determine the potential use of miRNAs as biomarkers for early diagnosis. Due to the limited number of samples in our study, further research is recommended to further validate the accuracy and specificity of our data. Moreover, our findings provide the basis for future research in the field of miRNA-based cancer therapy.

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