Molecular characterization of well-differentiated human thyroid carcinomas by cDNA arrays

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Abstract. Well-differentiated papillary and follicular thyroid carcinomas are the two most common types of thyroid cancer. Although cancerous cells in both types phenotypically resemble the epithelial follicular cell, the tumors display different histological characteristics and clinical outcomes. Molecular defects contributing to the separate development pathways remain largely unclear. We evaluated gene expression profiles to generate a detailed molecular characterization of the two tumor types, attempting to detect novel diagnostic and clinical markers. Gene expression profiling of 46 thyroid samples (16 papillary carcinomas, 13 follicular carcinomas and 17 normal thyroid specimens) was performed by using high-density human UniGene cDNA arrays. The identification of differentially expressed genes was based on a comparison of signal intensity ratios of tumor versus normal tissues. A cross-validation of individual filter-array hybridizations and real-time PCR analysis of selected genes were carried out to confirm data reproducibility and reliability. The majority of genes with altered expression were found in both papillary and follicular carcinomas, reflecting a close relationship between the two tumor types. However, 123 genes consisting of 45 known and 78 unknown genes were shown to be differentially expressed between papillary and follicular carcinomas. Follicular variants of papillary carcinoma, clustered together with classical papillary carcinoma, could be differentiated from follicular carcinoma. Our study revealed a set of genes differentiating follicular carcinoma from classical papillary carcinoma and follicular variant. The data generated in this study could serve as a useful source for further investigation of pathways of papillary and follicular differentiation of thyroid cancer.

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Abbreviations: PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; ATC, anaplastic thyroid carcinoma

Key words: gene expression profiling, cDNA array, papillary thyroid cancer, follicular thyroid cancer

Introduction

Thyroid cancer that originates from follicle cells of the thyroid can be subdivided into PTC, FTC and ATC (1). PTC and FTC are the two most common types of thyroid malignancies, representing approximately 80% and 15% of all thyroid cancers, respectively (2). ATC is rare, accounting for approximately 1% of thyroid cancers. PTC and FTC are well-differentiated carcinomas and bear a favorable prognosis, whereas ATC is an undifferentiated tumor and aggressive. Despite arising from the same progenitor cell, the three tumor types differ in histological features and clinical behavior. PTC is characterized by papillary growth pattern, common presence of distinctive nuclear changes and metastasis to the regional lymph nodes. In contrast, FTC is characterized by follicular architecture (lack of papillae) and invasion into the capsule and/or blood vessels. The prognosis of FTC is in general less favorable than that of PTC. ATC that is believed to occur from a terminal dedifferentiation of previously undetected long-standing papillary or follicular thyroid carcinoma (3) has a rapid progression behavior and often manifests a distant dissemination at diagnosis. The most common sites of distant spread of ATC include lung, bone and brain. Although the three types of thyroid carcinomas are usually distinguishable on histopathological or cytological examination, the molecular basis of the phenotypes remains unclear. Differential diagnosis of atypical forms (e.g., follicular variant of PTC) is sometimes also difficult. Therefore, there is a need to characterize genetic alterations in these tumors and to search for new markers for amending differential diagnosis.

Molecular genetic studies have identified several common genetic changes associated with PTC, FTC and ATC. Alterations of the *RET* gene, which is mapped to 10q, are considered to play an important role in PTC development (4,5). On the other hand, the *PAX8-PPARγ1* fusion gene, resulting from the translocation t(2;3)(q13;p25), is implicated in the progression of FTC (6). Genetic changes of several other genes, such as the *RAS* oncogene family (7,8), *BRAF* (9,10), *p53* (3,11,12), *MDM2* (13), *AKT* (14) and *MET* (15), have also been found in a fraction of thyroid cancers including all the three tumor types. However, none of the molecular

Table I. Clinical data of patients suffering from thyroid carcinomas.

Type	No.	Age/sex	Stage	Size (cm)	Histological variant	Lymph node metastases[a]
PTC	19	53/M	pT3	6.0	Papillary	No
	22	69/F	pT2	2.5	Follicular variant	No
	23	22/F	pT2	3.4	Follicular variant	No
	25	22/F	pT4b	2.7	Follicular variant	Yes
	26	35/M	pT2	2.5	Papillary	No
	28	36/M	pT4	1.8	Follicular variant	Yes
	29	17/F	pT4b	4.0	Papillary	Yes
	32	32/M	pT3	4.7	Follicular variant	Yes
	40	28/F	pT2	1.1	Papillary	No
	41	56/F	pT2	1.7	Papillary	No
	42	81/M	pT4	6.5	Papillary	Yes
	47	61/F	pT4	2.5	Papillary	Yes
	48	76/M	pT4	4.3	Papillary	Yes
	52	28/M	pT3	5.5	Papillary	No
	54	41/F	pT1b	<1.0	Papillary	Yes
	63	24/F	pT2	1.5	Papillary	No
FTC	12	70/F	pT4	8.3	WI, CI, VI	No
	15	33/F	PT3	4.5	MI, CI, VI	No
	18	55/M	pT3	4.0	MI, CI, VI	No
	21	21/F	pT2	2.2	MI, CI, VI	No
	24	41/M	pT2a	2.9	MI, CI, VI	No
	34	24/F	pT3	4.2	MI, CI	No
	37	30/F	pT2	3.3	MI, VI	No
	43	35/M	pT3	6.5	MI, CI, VI	No
	46	60/F	pT3	2.8	MI, CI, VI	No
	50	83/F	pT4	5.0	WI, VI, anaplastic foci	Yes
	56	40/M	pT3	4.2	MI, VI	No
	84	41/F	pT3a	6.2	MI, CI, VI	No
	86	80/M	pT3	6.5	WI, VI	No

PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; F, female; M, male; CI, capsule invasion; VI, blood vessel invasion; MI, minimally invasive; WI, widely invasive. [a]The data for extra-thyroidal extension are not available for all patients.

aberrations is convincingly linked to defined histological types of thyroid cancer and the precise mechanisms by which thyroid cancer develops through the papillary or follicular pathway remain unclear. Cytogenetic analyses showed that chromosome imbalances and allelic losses involve multiple chromosomes (16-19), indicating the presence of undetected genes contributing to the initiation and development of the thyroid malignancies of follicular cell derivation.

Examining tumors for alterations in genome-wide gene expression is a potentially useful approach to determining the detailed molecular characterization of tumors (20). It could provide significant insight into tumorigenesis as well as facilitate diagnosis and thereby guide treatment. Recently, gene expression profiling based on array techniques has been used to study various tumors including thyroid carcinomas of follicular cell derivation (21-30). One of these studies directly compared two independent previous studies to reveal gene expression differences between PTC and FTC (27). The present study analyzed gene expression profiles by using filter-based high-density cDNA arrays in a series of thyroid tissues including 16 PTCs, 13 FTCs and 17 normal thyroid samples. Despite a similar involvement of the majority of genes with altered expression in these tumors, a small number of genes were identified to be differentially expressed between the two major tumor types. The data may provide important clues to the development and progression of papillary and follicular thyroid tumors through separate pathways.

Materials and methods

Tissue samples. Forty-six deep-frozen thyroid samples, which were taken intraoperatively from 29 patients during primary thyroidectomy, were collected for this study after obtaining informed consent. According to WHO criteria, PTC was diagnosed in 16 patients, and FTC in 13 patients. The 16 PTCs included 11 classical papillary carcinomas and 5 follicular variants. Of the 13 FTCs, 10 had minimal invasions and 3 had wide invasions, one of which had anaplastic foci (Table I).

The tumor with anaplastic foci included in this study showed predominant follicular neoplastic growth, indicating a progression from FTC to ATC. The patients were euthyroid and had a normal value of thyroid-stimulating hormone (TSH). They were not treated with any drugs before surgery. Macroscopically unchanged surrounding thyroid tissue samples were obtained from 17 of these patients. To confirm the diagnoses, all tissue samples used for the array analyses were histologically reviewed by one senior pathologist (P.H.). Histological assessments were carried out according to the WHO classification (1,31). Frozen sections were taken from each tissue block and stained with hematoxylin and eosin (H&E). Representative areas containing >80% of tumor cells or only normal tissue were selected for isolating RNA.

RNA preparation and hybridization. Total RNA was extracted from frozen tissues by using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The reverse transcription of total RNA into cDNA was performed as described in standardized protocols (Research Genetics, Huntsville, AL) with minor modifications. cDNA was synthesized from 2 μg of total RNA by using reverse transcriptase (Qiagen, Basel, Switzerland) in the presence of oligo-dT primer, nucleotide mix (Ambion, Austin) and α-[^{33}P]-dATP (3000 Ci/mmol, Amersham Pharmacia, UK). Labeled cDNA was purified with MicroSpin™ Columns S-300 (Amersham Pharmacia). High-density cDNA filters (RZPD library 956, Human Unigene Set 2) were obtained from the Resource Center of the German Human Genome Project (RZPD; www.rzpd.de). The filters contained a low-redundancy selection of 75,000 human IMAGE (integrated molecular analysis of genomes and their expression) cDNA clone sequences, including 12,000 known genes and 63,000 expressed sequence tags (EST), which were spotted in duplicate. The cDNA arrays were first used to screen for genome-wide gene expression profiles in 6 matched tumor/normal samples that were obtained from 3 PTC and 3 FTC patients, respectively. Based on the initial results (described in detail in Data analysis), approximately 8,600 cDNA clones consisting of 2,900 known genes and 5700 ESTs were subsequently selected for constructing subarrays. Hybridization and washing were performed essentially following the manufacturer's instructions with modifications. Filter arrays were prehybridized in 15 ml MicroHyb (Research Genetics) and 7 μg of each of human Cot-1 DNA and poly-dA at 42˚C for 2 h. ^{33}P-labeled cDNA probes were denatured and then added to the prehybridization mixtures. Hybridization was carried out at 42˚C overnight.

As a control experiment to test the array reproducibility, we repeated hybridization for one sample on four different filter arrays in one experiment.

Data analysis. The cDNA array was visualized at 50-μm resolution and 16 bits on a phosphor-imaging system (Fuji, Japan) and interpreted using ArrayVision™ software (Version 5.1, www.imaging-research.com). The signal intensity of each spot on the array was calculated as the mean pixel value minus a local region background and then normalized to the mean of all spots on the array, which allows generating the mean intensity from multiple arrays and subsequent comparison of different hybridizations. For target selection to construct the subarray, signal intensity ratios of tumor versus corresponding normal tissue were calculated for each target on the array. If the signal intensity value of the average of the two replicates fell below a threshold of background at 0.02, the intensity value for that target was reset to a value of 0.02 in order to avoid negative or spuriously large ratio values. The fold change value was used to define gene expression level. A gene that had a ratio value of ≥2 was considered overexpressed, whereas one with a ratio of ≤0.5 was regarded as underexpressed. Targets were selected for constructing the subarray, if their ratios of tumor versus the corresponding normal tissue were consistently <0.5 or >2 in at least two of the three matched pairs for each tumor type.

The software GeneSight (BioDiscovery, Inc., Los Angeles, CA) was used for ratio calculation, clustering and statistical analyses including ANOVA, Student's t-test, and permutation t-test. For data analyses, we first performed ANOVA analysis to identify genes that exhibited significant expression differences between normal, PTC and FTC samples. To smooth the variation in gene expression among normal thyroid samples, signal intensity ratios of each tumor versus the median value of 17 normal samples were calculated for all genes. On the basis of gene expression ratios of tumor versus normal samples, we carried out fold change analysis to detect genes with ≥2-fold change. Student's t-test and permutation 2-sample t-test were used to compare the two main tumor groups (PTC vs. FTC) as well as PTC subtypes (classical vs. follicular variant) for statistical significances of the differentially expressed genes that were identified by ANOVA and fold change analyses. Permutation test is a statistical method, in which the significance level for each gene is determined by comparing its statistic to the permutation distribution of the tumor samples that were randomly divided into two groups for 10,000 times. Hierarchical clustering was used to classify the tumors on the basis of gene expression similarity.

Quantitative real-time PCR. To confirm that the cDNA array data accurately reflect levels of transcripts, four genes (*TG, PAX8, FN1* and *CNN3*) were subjected to the quantitative real-time PCR analysis in 12 random selected samples. Two μg of total RNA from tumors, normal tissues and the pool of normal samples were denatured at 65˚C for 5 min and then reverse-transcribed in a 20-μl reaction mixture containing 250 μM of each dNTP, 50 units of reverse transcriptase enzyme (Omniscript, Life Technologies), 100 ng/μl oligo $(dT)_{16}$ primers (Life Technologies), 1X PCR buffer, 2.0 mM $MgCl_2$ (Applied Biosystems, Inc., Foster City, CA) at 37˚C for 60 min.

The sequences of specific primers and TaqMan probes for CNN3 were as follows: forward, CCTTTTGACCAGACCA CAATTAGTC; reverse, TGTCTCTTCTGGTACCTGGTGC TA; probe, FAM-CATCCCTGCCTGGCTGGCTCCTAMRA. Assays-on-demand™ primers and probes, obtained from Applied Biosystems, Inc., were used for the other three genes (*TG, PAX8* and *FN1*). These primers and probes were designed to work in the same cycling conditions (95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min) generating products with sizes 100-150 bp. *ACTB* (ß-actin) was tested as an endogenous control. Real-time PCR was carried out by means of an Applied Biosystems PRISM 7700

Table II. A list of known genes differentially expressed between PTC and FTC.[a]

Accession no.[b]	Gene symbol	Function and description	Frequency (%)[c] PTC	FTC
Overexpression				
		DNA or RNA structure and configuration		
aa460957	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	0	23
aa402352	SMARCD2	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin d2	0	23
		Transcription regulation		
h11161	TGFB1I4	TGF ß 1 induced transcript 4	63	31
ai393202[d]	ZBTB3	Zinc finger and BTB domain containing 3	31	0
		Cell division and growth regulation		
aa455303[d]	GFER	Growth factor, augmentor of liver regeneration	31	0
r55711[d]	GPC1	Glypican 1	25	8
n63669[d]	TMSB4X	Thymosin, ß 4, X-linked	81	15
		Signal transduction		
aa282352[d]	BMP7	Bone morphogenetic protein 7	38	0
		Biosynthesis and degradation		
r18814[d]	CTSH	Cathepsin H	44	8
w01726[d]	SERPINA1	Serine/cysteine proteinase inhibitor, clade A, member 1	31	8
		Cell adhesion, cell-cell and cell-matrix interaction		
n69991	ADAM19	A disintegrin and metalloproteinase domain 19	0	23
		Immunology		
h11608[d]	HLA-B	Major histocompatibility complex, class I, B	25	8
		Others		
n39009	SELS	Selenoprotein S	31	0
n23608	CNN3	Calponin 3, acidic	56	8
h14383	KCNAB	Potassium channel, shaker-related subfamily, b2	56	0
Underexpression				
		DNA or DNA structure and configuration		
aa149889	NAKAP95	Neighbor of A-kinase anchoring protein 95	13	62
		Transcription regulation		
ai654035	IRX2	Iroquois homeobox protein 2	44	77
ai393202[d]	ZBTB3	Zinc finger and BTB domain containing 3	0	23
aa281718	SP100	Nuclear antigen Sp100	13	62
aa425324	LHX1	LIM homeobox protein 1	13	77
w67713	SREBF1	Sterol regulatory element binding transcription factor 1	6	39
		Cell division and growth regulation		
ai277754	CNNM3	Cyclin M3	25	15
aa455303[d]	GFER	Growth factor, augmentor of liver regeneration	6	39
r55711[d]	GPC1	Glypican 1	6	23
n63669[d]	TMSB4X	Thymosin, ß 4, X-linked	0	39
		Signal transduction		
aa282352[d]	BMP7	Bone morphogenetic protein 7	6	69
w69648	MAP2K5	Mitogen-activated protein kinase kinase 5	6	62
		Immunology		
h50622	HLA-DRB3	Major histocompatibility complex, class II, DR, ß 3	0	23
h11608[d]	HLA-B	Major histocompatibility complex, class I, B	0	23
		Cell adhesion, cell-cell and cell-matrix interaction		
aa453795	PCDH16	Protocadherin 16 dachsous-like	13	31

Table II. Continued.

Accession no.[b]	Gene symbol	Function and description	Frequency (%)[c] PTC	FTC
		Biosynthesis and degradation		
w01726[d]	SERPINA1	Serine/cysteine proteinase inhibitor, clade A, member 1	13	54
r18814[d]	CTSH	Cathepsin H	0	31
		Others		
ai650518	AARSL	Alanyl-tRNA synthetase like	6	77
r80823	COX15	Cytochrome c oxidase subunit 15	6	31
h84889	CRYAA	Crystallin, alpha A	19	31
aa443998	NUDT1	Nucleoside diphosphate linked moiety X, motif 1	44	23
r09023	WRCH-1	Ras homolog gene family, member U	0	46
w93621	RREB1	Ras responsive element binding protein 1	6	46
w57742	PPGB	Protective protein for ß-galactosidase	6	39
ai125365	ODF4	Outer dense fiber of sperm tails 4	25	0
aa412342	TBL2	Transducin (ß-like) 2	31	15
ai811630	LOC142678	Sleletrophin	38	77

[a]The listed genes were differentially expressed between papillary thyroid carcinomas (PTC) and follicular thyroid carcinomas (FTC), significant at P<0.01 by Student's t-test. [b]All accession numbers are from GenBank at the NCBI. [c]Percentage of tumors with 2-fold or greater alterations. [d]These genes were frequently up-regulated in one tumor group and down-regulated in the other group.

Sequence Detector and TaqMan® reagents, according to the manufacturer's protocol. All samples were run in triplicate. Reactions omitting enzyme or RNA were used as negative controls. Relative transcript amounts of these genes were calculated by comparison with the pooled RNA of the 17 normal samples and normalized to *ACTB* in the reaction.

Results

Genes showing frequent expression change in both FTC and PTC. ANOVA analysis revealed approximately 1,950 cDNA targets displaying significant expression differences between PTC, FTC and normal thyroid samples. Fold change analysis detected 603 genes with at least 2-fold underexpression and 176 genes with at least 2-fold overexpression, which frequently occurred in papillary and/or follicular tumors (>40%). Although these genes were differentially expressed between tumor and normal samples, most of them showed no significant differences between the two types of thyroid carcinomas. This finding is consistent with the hypothesis that most gene expression alterations may be similarly involved in both PTC and FTC due to their close histological relationship.

The frequently deregulated genes identified in both tumor types fell into several functional categories including cell cycle, cell proliferation and differentiation, signal transduction, transcription and translation, cell-cell or cell-matrix interaction, and positive or negative regulation of tumor cells. The most frequently overexpressed genes, detected in ≥60% of PTC and/or FTC, include those belonging to signal transduction (*PTPRF* and *TBL2*), cell cycle control (*CDK5R1* and *RBBP6*), growth factor receptors (*EGFR* and *PDGFRB*) and transcriptional factors (ESR1). Some genes associated with cell-cell and cell-matrix interaction, including *ITGAL*, *TNFAIP6* and *MMP12*, were also frequently overexpressed. The most commonly underexpressed genes observed in both tumors (≥60%) are associated with cell cycle, proliferation and differentiation (*AKAP11*, *CCND3*, *RIP60* and *TGFb1*), signal transduction (*ACK1*, *CARD6*, *GPR48*, *GRIN2D*, *MATK* and *PPP1R9B*), transcription and translation regulation (*MKRN3*, *ETV3* and *SOX17*), transport (*ABCB1*, *BAP29* and *STX3A*), and immune response (*IGHG3, IGKC* and *SIGIRR*).

Strikingly, several members of the RAS oncogene pathway were frequently deregulated in both PTC and FTC. *NRAS*, *RAB11B* and *RAB40C* were overexpressed, while *BRAF*, *RAB1A*, *RAP1A* and *RAGD* were underexpressed. Some genes (e.g., *MET* and *LGALS3*) that have been reported to be involved in the development of thyroid tumors were also overexpressed in a portion of PTC and FTC specimens.

Some thyroid-specific genes, such as *TG* encoding thyroglobulin (the glycoprotein precursor of thyroid hormones) and *PAX8* (a member of the PAX family that is able to activate expression of endogenous thyroid-specific genes including *TG*), were underexpressed. It should be pointed out that a few thyroid-related genes previously described to be deregulated in thyroid tumors, e.g., *TSHR*, *NIS*, *DIO1* and *DIO2*, were not present on the filter array we used.

Genes displaying expression differences between PTC and FTC. One of our main aims was to identify genes expressed differentially in the two types of thyroid carcinomas in order to facilitate the discovery of critical genes for the different development pathways of thyroid carcinomas. On the basis of significant expression variation, 123 genes including 45 known genes and 78 unknown genes were identified to be differentially expressed between PTC and FTC. Strikingly, these genes displayed similar expression patterns between

Table III. A list of genes differentially expressed in widely invasive FTC and/or the follicular carcinoma with anaplastic foci[a].

Accession no.[b]	Gene symbol	Function and description	WiFTC /MiFTC[c]	FTCa /MiFTC[d]
		Chromosome conformation and function		
w92272	CHD3	Chromodomain helicase DNA binding protein 3	3.11	
r13812	MAPT	Microtubule-associated protein tau	0.19	2.26
r14959	NIN	Ninein (GSK3B interacting protein)	0.12	6.13
r18964	NR2E1	Nuclear receptor subfamily 2, group E, member 1	0.23	3.06
r35385	POLS	Polymerase σ	3.24	
		Cell cycle and Cell proliferation		
r31950	CSF3R	Colony stimulating factor 3 receptor	0.31	
h73225	CDK6	Cyclin-dependent kinase 6	0.25	5.06
r23410	DGSI	Digeorge syndrome critical region gene	0.22	
ai191900	GRLF1	Glucocorticoid receptor DNA binding factor 1	0.25	
		Apoptosis		
h01053	NOL3	Nucleolar protein 3	0.29	3.39
ai762184	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	0.31	
		Transcription and translation		
aa910862	HIC1	Hypermethylated in cancer 1	0.31	
ai553882	MKRN3	Makorin, ring finger protein, 3	3.61	2.61
		Transport		
r91904	AQP3	Aquaporin 3	0.27	3.59
ai694073	GJB6	Gap junction protein, ß 6	5.20	
w90118	SLC1A5	Solute carrier family 1, member 5	0.14	2.07
r73003	SLC16A4	Solute carrier family 16, member 4	0.16	
ai690393	SCARA3	Scavenger receptor class A, member 3	0.26	3.85
aa633631	SLC17A7	Solute carrier family 17, member 7	4.59	2.66
		Cell adhesion and cell-cell interaction		
r48162	ITGB4	Integrin, ß 4	0.24	3.36
w57892	FN1	Fibronectin 1	3.78	6.19
		Signal transduction		
r54450	BCR	Breakpoint cluster region	0.24	
t98794	ELK3	SRF accessory protein 2	0.28	2.18
h61757	ELK4	SRF accessory protein 1	0.33	4.16
r18284	FLOT2	Flotillin 2	0.22	
aa516129	FLT1	Fms-related tyrosine kinase 1	0.17	2.91
r68663	ITPK1	Inositol 1,3,4-triphosphate 5/6 kinase	0.29	2.32
aa447894	IFNAR1	Interferon receptor 1	0.31	
r39221	MAPK10	Mitogen-activated protein kinase 10	0.31	4.12
aa402274	NF1	Neurofibromin 1	0.20	
ai288166	SHB	SHB adaptor protein B	0.30	6.78
aa397726	STK11	Serine/threonine kinase 11	0.25	0.45
h12312	TXK	TXK tyrosine kinase	0.29	0.36
		Metabolism		
h03154	BCLP	ß-casein-like protein	0.25	
ai216166	B3GALT4	ß 3-galactosyltransferase 4	0.32	
h01521	CTSD	Cathepsin D	0.31	2.20
h67886	FACL4	Fatty-acid-Coenzyme A ligase, long-chain 4	0.24	2.43
ai683074	FDXR	Ferredoxin reductase	3.53	
r52626	HASJ4442	Putative methyltransferase	0.30	3.53
r06458	LCAT	Lecithin-cholesterol acyltransferase	0.33	

Table III. Continued.

Accession no.[b]	Gene symbol	Function and description	WiFTC /MiFTC[c]	FTCa /MiFTC[d]
		Metabolism		
r25544	PCCB	Propionyl Coenzyme A carboxylase, ß polypeptide	0.27	
aa032245	PPIC	Peptidylprolyl isomerase C	0.21	3.37
h11442	SDS	Serine dehydratase	0.32	
aa868515	SIAT4B	Sialyltransferase 4B	0.22	0.43
aa458899	SPUVE	Protease, serine, 23	0.23	4.05
aa393664	UBC6P	Ubiquitin conjugating enzyme 6	0.31	
		Others		
n51499	AKAP2	A kinase anchor protein 2	0.33	3.94
aa456315	BZRP	Benzodiazapine receptor	0.27	2.48
h94731	DISC1	Disrupted in schizophrenia 1	0.32	
r54061	HR	Hairless homolog (mouse)	0.25	2.73
r10121	IGKC	Immunoglobulin κ constant	0.32	
aa427578	NTF5	Neurotrophin 5	0.33	
aa458981	PTMS	Parathymosin	0.27	2.82
h06521	SECP43	tRNA selenocysteine associated protein	0.23	0.45
r36289	SYNPO	Synaptopodin	0.18	
r59170	WBSCR1	Williams-Beuren syndrome chromosome region 1	0.27	

[a]Considering mean value, these genes were not differentially expressed in minimally invasive follicular thyroid carcinomas. [b]All accession numbers are from GenBank at the NCBI. [c]Ratio of mean value of the two widely invasive follicular thyroid carcinomas (WiFTC) versus mean value of the ten minimally invasive follicular thyroid carcinomas (MiFTC). [d]Ratio of the single follicular carcinoma with anaplastic foci (FTCa) versus mean value of the ten minimally invasive follicular thyroid carcinomas (MiFTC).

follicular variants of papillary carcinomas and classical papillary ones, but significant differences between the follicular variants and follicular carcinomas. Of these genes, 51 showed an at least 2-fold expression change frequently occurring in either PTC or FTC. A list of selected known genes is given in Table II. Using permutation 2-sample t-test to determine genes that were most differently expressed between two tumor groups, we identified 19 'most significant' genes, among which there were 7 known genes *(BMP7*, *CEACAM7*, *CNN3*, *LHX1*, *MAP2K5*, *WRCH-1* and *ZFP36L1*). Other genes displaying major differential expression between the two types of thyroid tumors included *CTSH*, *GFER*, *GPC1*, *HLA-B*, *SERPINA1*, *TMSB4X* and *ZBTB3*. These genes were frequently overexpressed in PTC but underexpressed in FTC.

In addition, several genes that were previously reported to be involved in cancer development, e.g., *BSG* and *FN1* (22,23), showed slightly differential expression between *PTC* and *FTC* ($0.05>P<0.01$). *FN1* that is related with cell adhesion and extracellular matrix was highly increased in 81% of PTCs and 33% of FTCs, respectively. *BSG*, a cell surface antigen, was overexpressed in FTC but not in PTC.

Comparison of gene expression among subtypes. Follicular variant of PTC and classical PTC showed very similar gene expression patterns. Only a small set of genes displayed statistically significant expression differences between the two subtypes (data not shown). However, a clear segregation of the two subtypes was not established either by hierarchical clustering analysis or by immunohistochemical analysis. Genes that were found to be highly expressed in follicular variant PTC but not in classical PTC included *CCNA2*, *CLPP*, *CENTB5*, *CTSH*, *DLG5*, *GFER*, *F2RL3*, *GPC1*, *IGHM*, *MAP3K10*, *MGC3036*, *MGC5178*, *PPARBP*, *SURF4* and *ZYG*. Several genes such as *DSIPI*, *SRRM2*, *SDS*, *KIAA0947* and *KIAA1464* were underexpressed in these follicular variants of papillary carcinoma compared with classical PTC.

In contrast to the PTC subtypes, gene expression alterations of FTC subgroups appeared to be well correlated to the tumor progression of FTC. Although the limited number of tumors is not suitable for statistical analysis, direct comparison revealed expression differences among the subtypes of FTC. A total of 80 genes were detected, for which expression ratios of widely invasive FTC vs. minimally invasive FTC reached >3-fold. In comparison with normal thyroid tissues, most of these genes were not differentially expressed in minimally invasive FTC but were underexpressed in widely invasive FTC (Table III). Several genes were overexpressed in widely invasive FTC. Furthermore, 212 genes were identified to show >3-fold variations between the single follicular carcinoma with anaplastic foci and minimally invasive FTC. Many of these genes showed overexpression in this tumor.

Hierarchical cluster analysis of thyroid cancer samples. Two-dimensional hierarchical cluster analysis of the selected 123

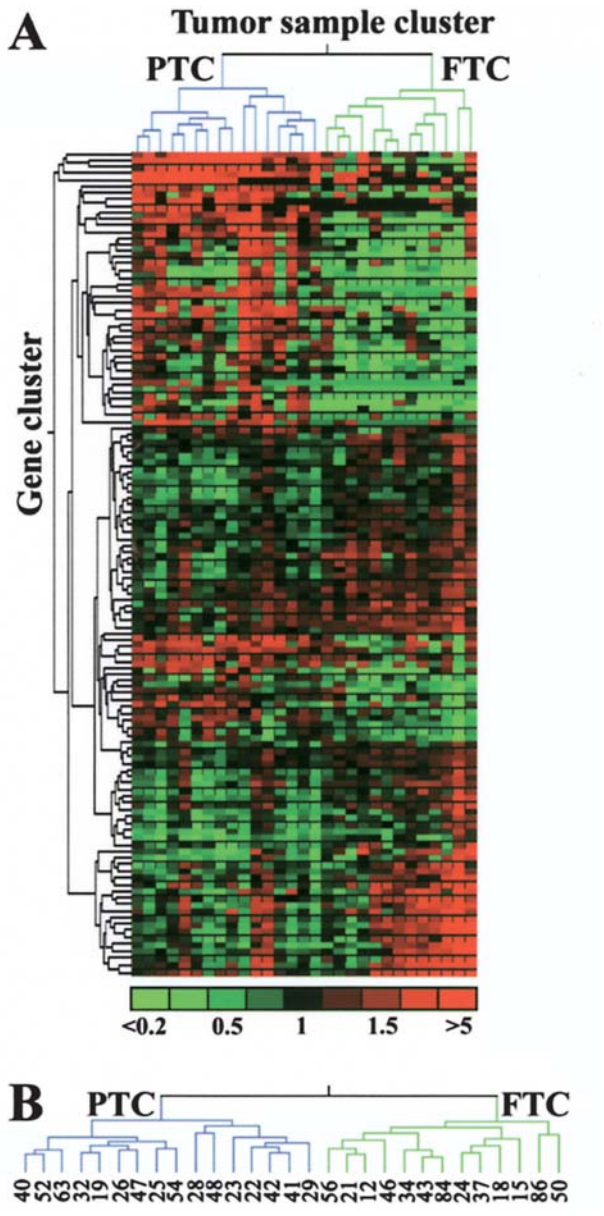

Figure 1. Hierarchical cluster analysis of 123 genes whose variations in expression were significantly different between papillary (PTC) and follicular thyroid carcinomas (FTC). (A) Gene clusters are shown in rows and tumor sample clusters in columns. The height of nodes of hierarchical trees reflects the degree of similarity of gene expression. The 29 tumors examined fell into two major groups, as shown by the two branches of the dendrogram at the top (A and B). (B) The number is the tumor-identification (ID). The group at the left includes all PTC samples, whereas the group at the right consists exclusively of FTC samples. The colored bar at the bottom of A indicates signal ratios of each tumor sample versus the median of 17 normal thyroid tissues. Red, overexpression; green, underexpression; black, unchanged expression.

genes was performed to determine the relationship of the examined thyroid cancer samples on the basis of their gene expression profiles (Fig. 1). The 29 tumors examined fell into two major groups, as shown by the two branches of the dendrogram in Fig. 1, top. The group at the left includes all PTC samples, whereas the group at the right consists of FTC samples. Using permutation 2-sample t-test, we demonstrated that all of the 123 genes were at a significant level for segregating PTC from FTC samples. These genes together gave an overall significance value of P=0.000058, supporting the potential use of the identified gene set in the histology-associated classification of the two tumor types. However, the hierarchical clustering cannot distinguish between subtypes, suggesting that these genes might only differentiate the papillary from the follicular feature of thyroid cancer. The follicular carcinoma with anaplastic foci was clustered together with all FTCs, representing the genetic feature of a follicular growth of this tumor.

Validation of data. Repeated analyses of a single tumor RNA probe on 4 different filter arrays showed that approximately 95% of data points fell within 2-fold limits. The variations in our repeated analyses were lower than these previously reported by others (32). This cross-validation procedure confirmed the high reproducibility of the filter-array approach that we used (Fig. 2). In addition, we selected four genes (*FN1, PAX8, TG* and *CNN3*) to verify the array results by using relatively quantitative real-time PCR (Fig. 3). The results obtained from both methods were in general consistent for all four genes.

Discussion

Using cDNA array analyses, we revealed both similarities and differences in the gene expression profiles of PTC and FTC. The majority of genes with altered expression were detected in PTC as well as in FTC. The shared molecular characteristics parallel the close histological and biological relationship of the two types of tumor. Genes displaying an aberrant expression in most of these cancers may play a causal role in thyroid cell transformation and might represent potential targets for developing novel strategies for prevention and treatment of thyroid cancer.

One of the striking findings is the involvement of the RAS pathway in the development of both PTC and FTC. The RAS gene family includes at least 21 members. They code for small GTPases that act as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. They are activated in response to a wide variety of extracellular stimuli and regulate signaling pathways that control multiple biological processes. Mutations of RAS genes have been found in a variety of human tumors including thyroid cancer (33-35). In our study, several RAS family genes (e.g., *NRAS*, *RAB11B* and *RAB40C*) were highly expressed, while others (*BRAF*, *RAB1A* and *RAP1A*) were underexpressed in both PTC and FTC. The findings support that disturbance of the RAS signaling pathway may play a role in the early transformation of thyroid malignancies. In a study on gene expression profiles of papillary thyroid carcinomas, which harbored *BRAF*, *RAS* or *RET/PTC* mutations, Giordano *et al* revealed a strong relationship between mutational status and gene expression, suggesting mutations of genes in the RAS signaling pathway could confer tumors with discrete mutation-specific phenotypical and biological features (36).

Additional oncogenes including *MET* were shown to be overexpressed in the two common types of thyroid cancer, consistent with previous studies (22,23,29,37,38). *MET* is a proto-oncogene encoding the hepatocyte growth factor (HGF) receptor. Various mutations in the *MET* gene have been reported to be associated with human tumors, such as papillary renal carcinoma and hepatocellular cancer (39,40).

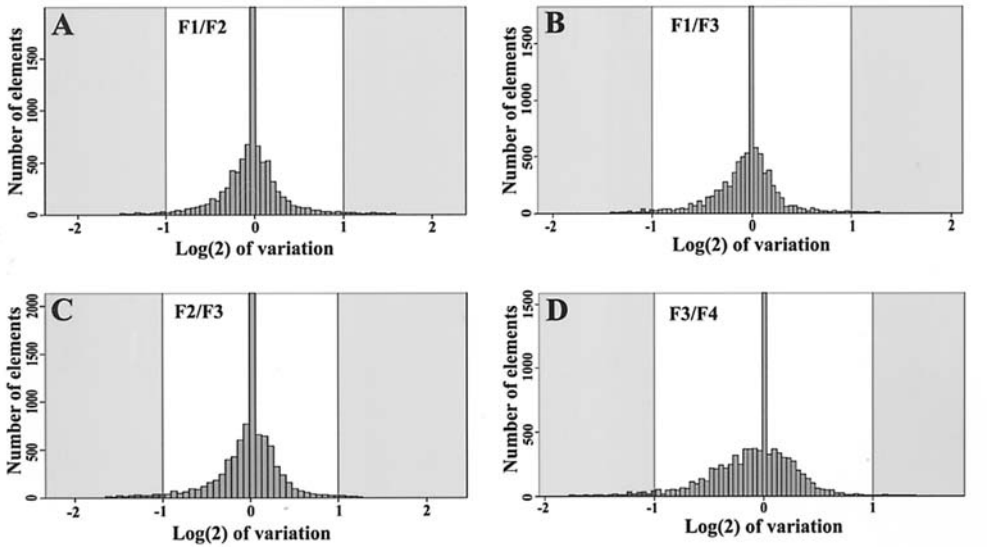

Figure 2. Cross-validation of filter arrays. A single tumor RNA probe was hybridized on 4 different filter arrays in one experiment. Signal intensity ratios of all elements on the filters were calculated from any two of the 4 filters: (A) Filter 1 versus Filter 2; (B) Filter 1 versus Filter 3; (C) Filter 2 versus Filter 3 and (D) Filter 3 versus Filter 4. Shaded areas indicate out of 2-fold limits. In F1/F2 (A), 96.4% of data points fell within 2-fold limits; in F1/F3 (B), 96.8%; in F2/F3 (C), 96.3% and in F3/F4 (D), 96.4%. The data show a high reproducibility of the array approach used in this study.

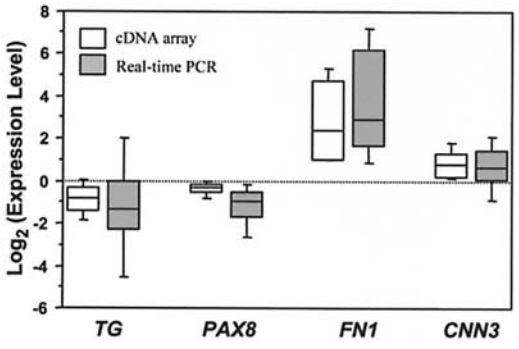

Figure 3. Validation of cDNA array data by relatively quantitative real-time PCR. The box-plots represent the distributions of the $\log_2$ transformed gene expression levels of *TG*, *PAX8*, *FN1* and *CNN3* in 12 samples that were based on the ratio of tumor versus pool of 17 normal samples. Boxed regions enclose the 25th through 75th percentiles. Horizontal lines indicate medians. Whiskers indicate the 5th to 95th percentiles.

It is known that mutation of an oncogene may induce a conformation change of the protein that it encodes, thereby leading to oncogenic activation (41). This kind of activating mutation may not significantly influence the transcript level. This could explain why some frequently mutated genes and fusion genes (e.g., *RET/PTC* and *PAX8-PPARγ1*), involved in thyroid tumorigenesis, were not detected by gene expression profiling (24,27,28,37). However, the mutated gene may affect downstream targets in the signaling pathway, generating a distinct gene expression profile (42).

Previous studies have suggested that several genes such as *LGALS3* and *FN1* can be used as markers for malignant transformation of thyroid tumors. The *LGALS3* gene encodes galectin-3 (GAL3), a ß-galactosil-binding lectin involved in regulating cell-cell and cell-matrix interactions, whereas *FN1* codes for fibronectin, an extracellular matrix protein. The overexpression of both genes is believed to be associated with malignant transformation of thyroid follicular cells (43). In the present study, we showed an increase in the expression of both genes in the two common thyroid malignancies, supporting the potential diagnostic use of these genes as markers.

The underexpression of thyroid-specific genes including *TG* has been also suggested as a marker for thyroid malignancy. *TG* gene encoding thyroglobulin (the glycoprotein precursor of thyroid hormones) was found to be underexpressed in both PTC and FTC in our study. Interestingly, the underexpression of *TG* concurred with a decreased transcript level of *PAX8*, a member of the PAX family of transcriptional factors. PAX8 was shown to be able to activate expression of endogenous thyroid-specific genes including *TG* (44,45). PAX8 can also activate the promoter of *TG* by cooperating with thyroid transcription factor-1. The ability of PAX8 to activate transcription of thyroid-specific genes strongly suggests an important role of this transcription factor in the maintenance of functional differentiation in thyroid cells. A decreased expression of the *PAX8* gene in thyroid cancer was also observed by others (46). Efforts to clarify whether the underexpression of *PAX8* is induced through mutation of this gene or other mechanisms could provide important information about the role of *PAX8* in thyroid tumorigenesis.

The present study further detected genes that had not been previously reported in thyroid tumors. Some of these genes, e.g., *PTPRF*, *RBBP6*, *TNFAIP6* and *MMP12*, are known to be related to human tumor development and metastasis. A few genes, including *CCND3*, *TNK2* and *CARD6,* are associated with cell cycle, proliferation and signaling transduction. Thus, our results provide a useful source for further investigation of the biology of papillary and follicular thyroid cancers. Further studies to clarify whether the expression alterations of these genes identified by cDNA arrays are relevant for thyroid tumorigenesis could provide valuable data.

An important goal of this study is the identification of genes differently expressed between PTC and FTC, which

may lead to the discovery of crucial genes associated with the development of papillary and follicular phenotypes. We found a molecular distinction between PTC and FTC on the basis of 123 differentially expressed genes, including 45 known genes and 78 ESTs, as shown by hierarchical clustering analysis. These differently expressed genes fall into different function categories including cell adhesion and extracellular matrix, membrane components, transcription regulation, signal transduction, cell cycle and growth regulation, nuclear acid structure and configuration, etc. In spite of displaying significant expression differences between PTC and FTC, none of these genes was consistently deregulated in all samples of one tumor type but not in the other. This implicates that many genes may somehow interact and thereby contribute to the complex molecular events responsible for the papillary and follicular phenotypes, respectively. It is striking that all tumors with papillary feature can be differentiated from those showing follicular phenotype by gene expression profiling. Although the histological features of PTC and FTC are usually quite distinct, the ability of gene expression profiling to distinguish the follicular variant of PTC from FTC is especially of potential clinical value. Detailed investigation of the differentially expressed genes could further elucidate the molecular events contributing to PTC and FTC development. Aldred *et al* recently compared two data sets, which were separately generated from two previous studies on PTC and FTC, and generated distinctive expression profiles of PTC and FTC (27). They even showed that a minimum of five genes were able to distinguish PTC from FTC. Many genes including the 5 genes (*CITED1*, *CLDN10*, *CAV1*, *CAV2* and *IGFBP6*) they detected, however, were not present in the cDNA arrays that we used. This made it difficult to compare their data with ours. It was reported that different microarray platforms could deliver conflicting data (47). Therefore, there is a need for establishing standards for the microarray technology.

Our data demonstrate that follicular variants and classical PTCs share a very similar expression profile. Only a small number of genes were statistically differentiated between the follicular variant and classical PTC. Immunohistochemistry and hierarchical clustering analysis of these genes, however, failed to separate the two subtypes. In fact, most of these genes showed only slight, albeit statistically significant, alterations at the transcript level. The slight transcript changes might result in very small differences in protein expression between follicular variants and classical PTC, which are usually undetectable on immunohistochemistry or immunoblotting. In contrast to the PTC subtypes, widely invasive and minimally invasive FTC showed significant differences in gene expression. Especially, the follicular carcinoma with anaplastic foci displayed more expression alterations than both widely invasive and minimally invasive FTC. The findings seem to be in agreement with the clinical and biological behavior of these tumors. However, additional studies on large series of samples of each group are required to generate reliable data.

In conclusion, we have identified gene expression alterations either shared by PTC and FTC or occurring differently between the two tumor types. The shared expression alterations reflect a close histological and biological relationship between PTC and FTC. The genes displaying different expression between the two common tumor types may contribute to the progression of thyroid cancer in separate pathways. Differentiation of follicular variant PTC from FTC by gene expression is of potential clinical value. Gene expression profiling also revealed genes that were differentially expressed between widely and minimally invasive FTC. Since the segregation of thyroid tumor samples based on expression profiles was consistent with histological features, gene expression profiling may prove to be a potential molecular tool to distinguish the distinct types of thyroid cancer.

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