Genome-wide gene expression profiles of thyroid carcinoma: Identification of molecular targets for treatment of thyroid carcinoma

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Abstract. In order to clarify the molecular mechanism involved in thyroid carcinogenesis and to identify candidate molecular targets for diagnosis and treatment, we analyzed genome-wide gene expression profiles of 18 papillary thyroid carcinomas with a microarray representing 38,500 genes in combination with laser microbeam microdissection. We identified 243 transcripts that were commonly up-regulated and 138 transcripts that were down-regulated in thyroid carcinoma. Among these 243 transcripts identified, only 71 transcripts were reported as up-regulated genes in previous microarray studies, in which bulk cancer tissues and normal thyroid tissues were used for the analysis. We further selected genes that were overexpressed very commonly in thyroid carcinoma, though were not expressed in the normal human tissues examined. Among them, we focused on the regulator of G-protein signaling 4 (*RGS4*) and knocked-down its expression in thyroid cancer cells by small-interfering RNA. The effective down-regulation of its expression levels in thyroid cancer cells significantly attenuated viability of thyroid cancer cells, indicating the significant role of RGS4 in thyroid carcinogenesis. Our data should be helpful for a better understanding of the tumorigenesis of thyroid cancer and could contribute to the development of diagnostic tumor markers and molecular-targeting therapy for patients with thyroid cancer.

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Abbreviations: PTC, papillary thyroid cancer; LMM, laser microbeam microdissection; TG, thyroglobulin; ATC, anaplastic thyroid carcinoma

Key words: gene expression profile, microarray, thyroid carcinoma, molecular target gene

Introduction

Thyroid carcinoma represents 1% of all malignant diseases and accounts for nearly 90% of neuroendocrine malignancies. Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor, representing 80-90% of thyroid malignancies. Papillary, follicular and anaplastic thyroid carcinomas are known to arise from follicular epithelial cells while medullary thyroid carcinomas arise from the parafollicular C cells of the thyroid. PTC is usually well-differentiated; however, the clinical characteristics of PTC vary widely. For example, incidental microcarcinomas are often noninvasive or minimally invasive and grow very slowly. However, invasive PTC often causes metastasis and can be lethal. PTCs often recur many years after their surgical operation. The prognosis for PTC is frequently favorable, though ~20% of patients with PTC experience recurrence and some of them die of the disease (1,2). A postoperative follow-up for monitoring recurrence is critically important for a favorable outcome. Although PTC can be diagnosed by a cytological examination of fine-needle aspiration materials, this method is not applicable for the prediction of a post-operative clinical outcome. Serum thyroglobulin (TG) immunoassay has been used for monitoring the recurrence of differentiated thyroid carcinoma, though measurement of serum TG is sometimes hindered by the presence of circulating factors, particularly anti-TG antibodies and a residual normal thyroid gland tissue that produces TG (3).

The genome-wide microarray analysis enabled us to obtain comprehensive gene expression profiles related to phenotypic and biological information in cancer cells (4-10). We have identified multiple applicable targets for the development of novel anti-cancer drugs and/or diagnostic markers. For thyroid

carcinoma, this approach is useful for identifying unknown molecules involved in the carcinogenic pathway (5,11-14).

Through gene expression profile analysis of 18 papillary thyroid cancers coupled with the purification of the cancer cell population by laser microbeam microdissection (LMM) on a microarray consisting of ~38,500 transcripts, we identified a number of transcripts that were overexpressed in thyroid cancers. We report important information regarding the mechanisms of thyroid carcinogenesis as well as the discovery of potential targets for the development of diagnostic markers and signal-suppressing therapeutic strategies for thyroid cancer treatment.

Materials and methods

Tissue samples and microdissection. Eighteen thyroid cancer tissue samples (17 female and 1 male patients; median age, 46 years; range, 20-71 years; Table I) were obtained with written informed consent along with adjacent normal thyroid tissue samples from patients undergoing surgery at the Clinic of Endocrine Surgery (Sofia, Bulgaria). These cancer tissues were histopathologically diagnosed as papillary thyroid carcinoma. Clinical information was obtained from medical records. Clinical stage was judged according to the UICC TNM classification. Each speciman was embedded in TissueTek OCT medium (Sakura, Tokyo, Japan) immediately after surgical resection and stored at -80˚C until use. These frozen tissues were cut into 8-μm sections using a cryostat (Sakura) and then stained with hematoxylin and eosin (H&E) for histological examination. Thyroid cancer cells and corresponding normal thyroid epithelial cells were selectively collected using the EZ cut system with a pulsed ultraviolet narrow beam-focus laser (SL Microtest GmbH, Jena, Germany) according to the manufacturer's protocols. RNA isolated from the microdissected normal thyroid follicular epithelial cells of 13 individuals was mixed and used as a 'universal control' for each of the 18 cancer samples.

Cell lines. The human thyroid carcinoma cell line, FTC-133, was used in this study. FTC-133 was grown in a monolayer in appropriate media supplemented with 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). FTC-133 was maintained at 37˚C in an atmosphere of humidified air with 5% CO_2.

Affymetrix GeneChip hybridization. The Affymetrix human genome U133 Plus 2.0 GeneChip arrays were used for microarray hybridizations. This GeneChip comprises of >54,000 probe sets and analyzes the expression level of >47,000 transcripts. For microarray hybridization, we followed the protocol described in the Affymetrix GeneChip eukaryotic two cycles target preparation protocol (Affymetrix). For the first-round synthesis of double-stranded cDNA, 100 ng of total RNA were reverse transcribed using the two-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA) and T7-oligo-dT primer according to the manufacturer's instructions followed by IVT amplification with the MEGAscript T7 kit (Ambion, Inc., Austin, TX). After cleanup of the cRNA with a GeneChip sample cleanup module IVT column (Affymetrix), second-round double-stranded cDNA was amplified using the IVT labeling kit (Affymetrix). A 20 μg aliquot of the labeled product was fragmented by heat and ion-mediated hydrolysis at 94˚C for 35 min in H_2O and 8 μl of 5x fragmentation buffer (Affymetrix). The fragmented cRNA was hybridized for 16 h at 45˚C in a hybridization oven 640 to a U133 Plus 2.0 oligonucleotide array (Affymetrix). The washing and staining of the arrays with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR) was completed in a fluidics station 450 (Affymetrix). The arrays were then scanned using a confocal laser GeneChip scanner 3000 (Affymetrix).

Table I. Clinical features of PTC clinical samples used for microarray analysis.

No	Age	Gender	Clinical Stage
TH_T_1	37	Female	III
TH_T_3	69	Female	III
TH_T_4	54	Female	II
TH_T_6	22	Female	III
TH_T_7	60	Female	II
TH_T_8	58	Female	II
TH_T_9	34	Female	III
TH_T_10	71	Male	III
TH_T_11	64	Female	II
TH_T_13	49	Female	I
TH_T_14	28	Female	III
TH_T_15	47	Female	I
TH_T_16	26	Female	III
TH_T_20	44	Female	II
TH_T_21	36	Female	II
TH_T_26	45	Female	II
TH_T_28	20	Female	II
TH_T_31	59	Female	II

Data analysis. Global normalization at a target value of 500 was applied to each of the 19 arrays (18 cancer arrays and one array of the universal control) under consideration using GeneChip operating software (Affymetrix). Normalized data from text files were imported to a Microsoft Excel spread sheet. Since data derived from low signal intensities are less reliable, we excluded transcripts with low intensities from further analysis when the signal intensities of both normal and cancer cells were lower than that of the cut-off. For the other genes, we calculated the signal intensities of cancer/normal ratio using the raw data of each sample. We selected up-regulated or down-regulated genes based on their signal intensities of cancer/normal ratios (r): up-regulated ($r>5.0$) and down-regulated ($r<0.2$).

Calculation of contamination percentage. Calcitonin (CALCA), a hormone that is expressed exclusively in parafollicular C cells, was used to evaluate the proportion of C cells present in the population of microdissected normal follicular epithelial cells. Each intensity was normalized to the

Table II. Primer sequences for semi-quantitative RT-PCR experiments.

Gene	Forward primer	Reverse primer
CHI3L1	5'-tgaggcatcgcaatgtaaga-3'	5'-taggatgtttggctccttgg-3'
FAM20A	5'-cagctaggaaggaagcagga-3'	5'-tgccaagcctgacctatacc-3'
KLHDC8A	5'-cctcctgcattctcaggcta-3'	5'-gctttggctgaatgaactga-3'
LRP4	5'-tctgagcctccccacattac-3'	5'-attctgaacagcccaagtgc-3'
NMU	5'-gtgtcgtcagttgtgcatcc-3'	5'-tggaaaattagctggcatcc-3'
TM7SF4	5'-cctggttatgcctcctttca-3'	5'-tgcttgtgaggctgtggtag-3'
RGS4	5'-ctgcattcgttgctccagta-3'	5'-ggcaagattgccagagagac-3'

Table III. Sequences of specific double-stranded oligonucleotides inserted into the siRNA expression vector.

psi-U6BX-RGS4 #1	5'-CACCGCTTCTTGCTTGAGGAGTGCACGAATGCACTCCTCAAGCAAGAAGC-3' 5'-AAAAGCTTCTTGCTTGAGGAGTGCATTCGTGCACTCCTCAAGCAAGAAGC-3'
psi-U6BX-RGS4 #2	5'-CACCGCTTGCGAATTCCAAGCTGTTTTCAAGAGAAACAGCTTGGAATTCGCAAGC-3' 5'-AAAAGCTTGCGAATTCCAAGCTGTTTCTCTTGAAAACAGCTTGGAATTCGCAAGC-3'
psi-U6BX-Mock	5'-CACCGTGTCTTCAAGCTTGAAGACTA-3' 5'-AAAATAGTCTTCAAGCTTGAAGACAC-3'
psi-U6BX-Scramble	5'-CACCGCGCGCTTTGTAGGATTCGTTCAAGAGACGAATCCTACAAAGCGCGC-3' 5'-AAAAGCGCGCTTTGTAGGATTCGTCTCTTGAACGAATCCTACAAAGCGCGC-3'

intensity of the ß actin gene (ACTB) as follows: (Ratio A) the CALCA/ACTB intensity ratio in whole normal thyroid tissue (where some of the cells correspond to C cells); the signal intensity of poly $(A)^+$ RNA isolated from whole normal thyroid tissue was 0.18.

(Ratio B) the CALCA/ACTB intensity ratio in microdissected normal follicular epithelial cells = 0.0046 (a mixture of normal follicular epithelial cells from 13 individuals as a universal control). On the basis of these measurements, the proportion of contaminated C cells in microdissected normal follicular epithelial cells was calculated to be 2.6%.

Semi-quantitative RT-PCR. RNAs from the purified populations of thyroid cancer cells and from normal follicular epithelial cells were extracted using an RNeasy micro kit (Qiagen, Valencia, CA) and were treated with DNase I according to the recommendation of the manufacturer. Extracted RNAs were subjected to two rounds of RNA amplification using T7-based *in vitro* transcription (Invitrogen) and amplified RNAs were reverse transcribed to single-stranded cDNAs using a random primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification and monitored their reactions using GAPDH as a quantitative control since this showed the smallest fluctuations of cancer/normal ratio in our thyroid cancer microarray data. PCR amplification was performed using single-strand cDNAs as templates and gene-specific primers (Table II). PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Northern blot analysis. Human multiple tissue Northern blots (MTN) (BD Biosciences, Palo Alto, CA) were hybridized for 16 h with ^{32}P-labeled PCR product of the regulator of G-protein signaling 4 (*RGS4*) cDNA. The cDNA probe of *RGS4* was prepared by RT-PCR using the primers 5'-GACAAAG TGGTTATTTGCCAGAG-3' and 5'-AGGTGAGAATTAG GCACACTGAG-3'. Prehybridization, hybridization and washing were performed according to manufacturer's recommendations. The blots were autoradiographed with intensifying screens at -80˚C for 14 days.

Effect of small interfering RNAs on the growth of thyroid cancer cells. In order to evaluate the biological significance of RGS4 in thyroid cancer cells, we used the psi-U6BX3.0 vector for the expression of short hairpin RNA against the target gene, as described previously (15). The U6 promoter was cloned upstream of the gene-specific sequence (19-nucleotide sequence from the target transcript, separated from the reverse complement of the same sequence by a short spacer, (TTCAAGAGA), with five thymidines as a termination signal and a neo-cassette for selection by Geneticin (Sigma). Plasmids designed to express small interfering RNA (siRNA) were prepared by the cloning of double-stranded oligonucleotides into the psi-U6BX vector (Table III).

Human thyroid cancer cells, FTC-133, which express RGS4 at a high level, were plated onto 10-cm dishes ($2.5x10^6$ cells per dish) and transfected with the psiU6BX vector, which included the target sequences for *RGS4* or their scrambled sequences using Fugene6 (Invitrogen). The transfected FTC-133 cells were selected in a medium containing 0.6 mg/ml of neomycin (Geneticin; Gibco BRL,

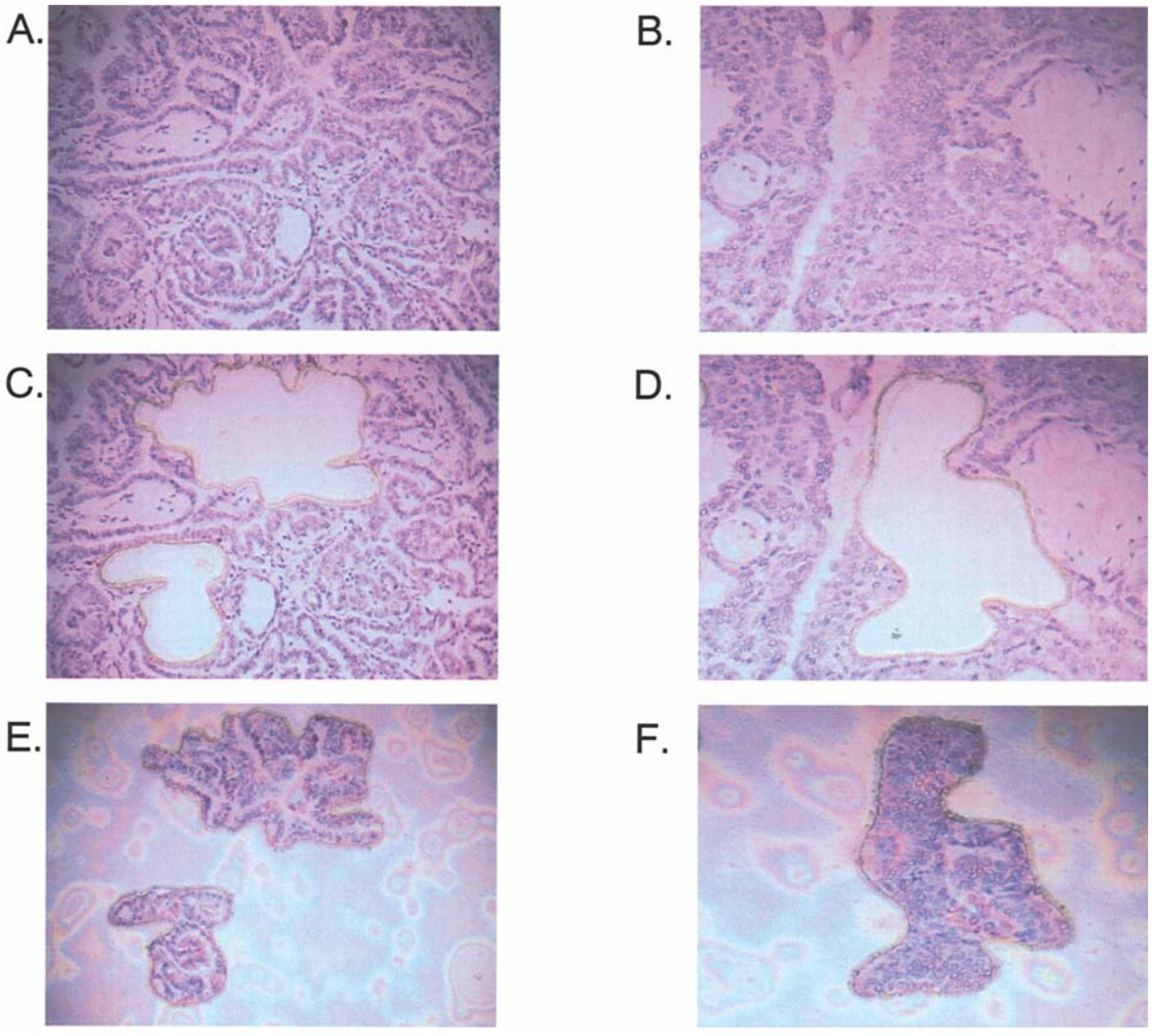

Figure 1. Laser-microbeam microdissection (LMM) of two representative papillary thyroid carcinomas (each case corresponds to either left or right panel). The upper two panels (A and B) show the samples before dissection; the middle (C and D), the same sections after microdissection (H&E staining). The microdissected cancer cells captured on the collecting cap are shown in the bottom panels (E and F).

Carlsbad, CA). Subsequently, total RNA was extracted from the cells at 7 days after Geneticin selection and then the knock-down effect of the siRNAs was examined by a semi-quantitative RT-PCR using specific primer sets: forward, 5'-CATCCACGAAACTACCTTCAACT-3' and reverse, 5'-TCTCCTTAGAGAGAAGTGGGGTG-3' as an internal control and 5'-CAAAGGGCTTGCAGGTCT-3' and 5'-TAG GCACACTGAGGGACCA-3' for RGS4. In 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, cell viability was evaluated using the Cell-counting kit-8 (Dojindo, Kumamoto, Japan) at 7 or 14 days after the transfection according to the protocol of the supplier. After 21 days of the incubation, these cells were fixed with 4% paraformaldehyde and stained with Giemsa solution for colony formation assay.

Results

Identification of commonly up-regulated or down-regulated genes in thyroid carcinoma. To obtain precise gene expression profiles of thyroid cancer cells, we employed LMM to purify both the cancerous cells and normal follicular epithelial cells (Fig. 1A-F). We estimated the proportion of parafollicular C cells in the microdissected population of normal follicular epithelial cells by measuring the signal intensities of *CALCA* (Calcitonin), which is expressed highly and specifically in parafollicular C cells (16-18). When we compared the signal intensity of this gene in whole normal thyroid tissue with that of the microdissected normal follicular epithelial cells, the average ratio of signal intensity was calculated to be ~97.4%, indicating that the proportion of C cells in the microdissected normal follicular epithelial cells was ~2.6% (section Calculation of contamination percentage in Materials and methods) (19).

We searched genes up- and down-regulated commonly in thyroid cancers according to the following criteria: i) genes for which we obtained expression data defined as described in Materials and methods in ≥50% (at least 9 of the 18 cases) of the cases examined; and ii) genes whose expression ratio was >5.0 or <0.2 in at least 50% of the informative cases. Under the criteria, a total of 243 transcripts were selected as genes that were commonly up-regulated (Table IV) and 138 as

Table IV. Genes commonly up-regulated in thyroid cancer.

No.	Accession no.	Gene symbol	Gene name
1	AJ276395	FN1	Fibronectin 1
2	M80927	CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)
3	NM_003843	SCEL	Sciellin
4	BC036592	GABRB2	γ-aminobutyric acid (GABA) A receptor, ß 2
5	W73431	FN1	Fibronectin 1
6	AI632223	FAM20A	Family with sequence similarity 20, member A
7	BF791631	KLHDC8A	Kelch domain containing 8A
8	X02761	FN1	Fibronectin 1
9	AA725246		CDNA FLJ11417 fis, clone HEMBA1000960
10	BC005008	CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
11	BC005858	FN1	Fibronectin 1
12	AI884858	TUSC3	Tumor suppressor candidate 3
13	AA088857	LIPH	Lipase, member H
14	AF130095	GAP43	Growth associated protein 43
15	NM_002045	FN1	Fibronectin 1
16	AI656481		CDNA FLJ37386 fis, clone BRAMY2026538
17	AI949827	NFE2L3	Nuclear factor (erythroid-derived 2)-like 3
18	AA772920	GABRB2	γ-aminobutyric acid (GABA) A receptor, ß 2
19	AK026737	FN1	Fibronectin 1
20	NM_006681	NMU	Neuromedin U
21	AW015920		Transcribed locus
22	BE222344		Homo sapiens, mRNA
23	NM_021127	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
24	AI246687	CTSC	Cathepsin C
25	AL514445	RGS4	Regulator of G-protein signalling 4
26	NM_016425	TMPRSS4	Transmembrane protease, serine 4
27	BF672975	LPL	Lipoprotein lipase
28	NM_001935	DPP4	Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)
29	AU156760		CDNA FLJ11044 fis
30	AA991267		Transcribed locus
31	J02761	SFTPB	Surfactant, pulmonary-associated protein B
32	AW294986		Transcribed locus
33	AL133706		CDNA FLJ45325 fis
34	NM_030788	TM7SF4	Transmembrane 7 superfamily member 4
35	T62088	SERPINA1	Serpin peptidase inhibitor
36	NM_007231	SLC6A14	Solute carrier family 6 (amino acid transporter), member 14
37	NM_024650	C11orf80	Chromosome 11 open reading frame 80
38	NM_002848	PTPRO	Protein tyrosine phosphatase, receptor type, O
39	AK095719	FLJ42709	Hypothetical gene supported by AK124699
40	J02761	SFTPB	Surfactant, pulmonary-associated protein B
41	AI333596		Transcribed locus
42	NM_005564	LCN2	Lipocalin 2 (oncogene 24p3)
43	AU146886		CDNA FLJ12023 fis
44	AF146796	SLC34A2	Solute carrier family 34 (sodium phosphate), member 2
45	NM_002607	PDGFA	Platelet-derived growth factor α polypeptide
46	NM_001793	CDH3	Cadherin 3, type 1, P-cadherin (placental)
47	BC011595	GPNMB	Glycoprotein (transmembrane) nmb
48	NM_014031	SLC27A6	Solute carrier family 27 (fatty acid transporter), member 6
49	BF431214		Transcribed locus
50	AI798118		Transcribed locus

Table IV. Continued.

No.	Accession no.	Gene symbol	Gene name
51	AW299924	GOLT1A	Golgi transport 1 homolog A
52	AK024280	SYT12	Synaptotagmin XII
53	AA584297	LRP4	Low density lipoprotein receptor-related protein 4
54	AI422986	ST8SIA4	ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 4
55	AB002365	PRUNE2	Prune homolog 2
56	AF012536	TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c
57	NM_016657	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3
58	AV725825	ZCCHC12	Zinc finger, CCHC domain containing 12
59	M80536	DPP4	Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)
60	NM_000693	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3
61	AI806346	SNAP25	Synaptosomal-associated protein, 25 kDa
62	AK026751		CDNA: FLJ23098 fis, clone LNG07440
63	BE043700	PDLIM4	PDZ and LIM domain 4
64	NM_000698	ALOX5	Arachidonate 5-lipoxygenase
65	N36762		Transcribed locus
66	NM_018700	TRIM36	Tripartite motif-containing 36
67	X57348	SFN	Stratifin
68	BF344237	CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney
69	NM_002203	ITGA2	Integrin, α 2
70	NM_020037	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
71	AU144855	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
72	AF131837	ST6GALNAC5	ST6 -N-acetylgalactosaminide α-2,6-sialyltransferase 5
73	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1
74	X57348	SFN	Stratifin
75	NM_004143	CITED1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1
76	NM_000313	PROS1	Protein S (α)
77	L25541	LAMB3	Laminin, ß 3
78	BC015881	STRA6	Stimulated by retinoic acid gene 6 homolog (mouse)
79	BC004869	C6orf168	Chromosome 6 open reading frame 168
80	AL022718	ODZ1	Odz, odd Oz/ten-m homolog 1(Drosophila)
81	NM_003483	HMGA2	High mobility group AT-hook 2
82	AW263497	SYTL5	Synaptotagmin-like 5
83	AI630979		CDNA FLJ30539 fis, clone BRAWH2001255
84	CA503291	B3GNT7	UDP-GlcNAc:ßGal ß-1,3-N-acetylglucosaminyltransferase 7
85			Full-length cDNA of Neuroblastoma Cot 50-normalized of Homo sapiens
86	NM_012413	QPCT	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
87	M18728	CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
88	BC001193	HIST3H2A	Histone cluster 3, H2a
89	M83248	SPP1	Secreted phosphoprotein 1
90	AB011133	MAST3	Microtubule associated serine/threonine kinase 3
91	NM_002658	PLAU	Plasminogen activator, urokinase
92	AK027164	LARP2	La ribonucleoprotein domain family, member 2
93	M80927	CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)
94	NM_003020	SCG5	Secretogranin V (7B2 protein)
95	AK001166	DEPDC1B	DEP domain containing 1B
96	NM_001211	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog ß (yeast)
97	NM_001394	DUSP4	Dual specificity phosphatase 4
98	BC032227	ZFYVE16	Zinc finger, FYVE domain containing 16
99	NM_002594	PCSK2	Proprotein convertase subtilisin/kexin type 2
100	AI887306		Full length insert cDNA YN63H06

Table IV. Continued.

No.	Accession no.	Gene symbol	Gene name
101	BF062804		Transcribed locus
102	AF153820	KCNJ2	Potassium inwardly-rectifying channel, subfamily J, member 2
103	AW051856	FLNA	Filamin A, α (actin binding protein 280)
104	NM_005797	MPZL2	Myelin protein zero-like 2
105	NM_005764	PDZK1IP1	PDZK1 interacting protein 1
106	AI670947	CNKSR2	Connector enhancer of kinase suppressor of Ras 2
107	NM_013271	PCSK1N	Proprotein convertase subtilisin/kexin type 1 inhibitor
108	BE383308		CDNA clone IMAGE:4067166
109	AL136588	C8orf57	Chromosome 8 open reading frame 57
110	J04152	TACSTD2	Tumor-associated calcium signal transducer 2
111	NM_016448	DTL	Denticleless homolog (Drosophila)
112	AF119873	SERPINA1	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1
113	NM_002160	TNC	Tenascin C (hexabrachion)
114	AA405456	RP13-102H20.1	Hypothetical protein FLJ30058
115	AK026776	LRRK2	leucine-rich repeat kinase 2
116	NM_030965	ST6GALNAC5	ST6 -N-acetylgalactosaminide α-2,6-sialyltransferase 5
117	U90902	TIAM1	T-cell lymphoma invasion and metastasis 1
118	AV731490	SYT1	Synaptotagmin I
119	AI608725	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
120	AW470178	SCEL	Sciellin
121	NM_000237	LPL	Lipoprotein lipase
122	AI732083		Transcribed locus
123	U42349	TUSC3	Tumor suppressor candidate 3
124	AF231124	SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1
125	AI935123	AHNAK2	AHNAK nucleoprotein 2
126	NM_004949	DSC2	Desmocollin 2
127	BF438386	RAB27B	RAB27B, member RAS oncogene family
128	AK026714	PELI1	Pellino homolog 1 (Drosophila)
129	NM_018286	TMEM100	Transmembrane protein 100
130	AW291369	FAM20A	Family with sequence similarity 20, member A
131	NM_020130	C8orf4	Chromosome 8 open reading frame 4
132	AL031664	PCSK2	Proprotein convertase subtilisin/kexin type 2
133	AA584310	CTHRC1	Collagen triple helix repeat containing 1
134	AI924046		Transcribed locus
135	NM_004795	KL	Klotho
136	AA705118		Full length insert cDNA
137	AF101051	CLDN1	Claudin 1
138	AB032261	SCD	Stearoyl-CoA desaturase (δ-9-desaturase)
139	BE218239	NLF1	Nuclear localized factor 1
140	BE796148	RNF183	Ring finger protein 183
141	AF153882	PDLIM4	PDZ and LIM domain 4
142	NM_000104	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
143	NM_002250	KCNN4	Potassium conductance calcium-activated channel, subfamily N, member 4
144	H17038	FLJ25076	Similar to CG4502-PA
145	NM_005491	CXorf6	Chromosome X open reading frame 6
146	NM_003254	TIMP1	TIMP metallopeptidase inhibitor 1
147	AI860568	FAM20A	Family with sequence similarity 20, member A
148	NM_001196	BID	BH3 interacting domain death agonist
149	AF003934	GDF15	Growth differentiation factor 15
150	AF035307	PLXNC1	Plexin C1

Table IV. Continued.

No.	Accession no.	Gene symbol	Gene name
151	AU154504	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
152	NM_016629	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21
153	BF110534		Transcribed locus
154	N95414	ITGA2	Integrin, α 2
155	NM_002120	HLA-DOB	Major histocompatibility complex, class II
156	AI819198	KISS1R	KISS1 receptor
157	AB014609	MRC2	Mannose receptor, C type 2
158	AA565141	EHF	Ets homologous factor
159	NM_005764	PDZK1IP1	PDZK1 interacting protein 1
160	BF434846	TNC	Tenascin C
161	D43968	RUNX1	Runt-related transcription factor 1
162	AW271106		Transcribed locus
163	AI254547	PDLIM4	PDZ and LIM domain 4
164	AW962511	C11orf80	Chromosome 11 open reading frame 80
165	AI431730	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9
166	AV702357		Transcribed locus
167	AC004010	AMIGO2	Adhesion molecule with Ig-like domain 2
168	BF515888	ABCC3	ATP-binding cassette, sub-family C
169	AI250910		CDNA FLJ30378 fis, clone BRACE2007953
170	AW117368	PSD3	Pleckstrin and Sec7 domain containing 3
171	BF723605		CDNA FLJ11892 fis, clone HEMBA1007281
172	M34064	CDH2	Cadherin 2, type 1, N-cadherin (neuronal
173	NM_003064	SLPI	Secretory leukocyte peptidase inhibitor
174	AF255647	TMEM163	Transmembrane protein 163
175	NM_003236	TGFA	Transforming growth factor, α
176	NM_015310	PSD3	Pleckstrin and Sec7 domain containing 3
177	AU158871		CDNA FLJ13810 fis, clone THYRO1000279
178	NM_004887	CXCL14	Chemokine (C-X-C motif) ligand 14
179	AW183080	GPR92	G protein-coupled receptor 92
180	BC001120	LGALS3	Lectin, galactoside-binding, soluble, 3
181	AF131799	SDK1	Sidekick homolog 1,
182	BF218922	VCAN	Versican
183	NM_004304	ALK	Anaplastic lymphoma kinase (Ki-1)
184	AL353944	RUNX2	Runt-related transcription factor
185	BF515959		CDNA clone IMAGE:5286005
186	BC001288	CD55	CD55 molecule
187	AI246687	CTSC	Cathepsin C
188	NM_004199	P4HA2	Procollagen-proline, 2-oxoglutarate 4-dioxygenase
189	BC003143	DUSP6	Dual specificity phosphatase 6
190	NM_004834	MAP4K4	Mitogen-activated protein kinase 4
191	BE221212	COL1A1	Collagen, type I, α 1
192	BC005047	DUSP6	Dual specificity phosphatase 6
193	AW471145	PRSS23	Protease, serine, 23
194	NM_007173	PRSS23	Protease, serine, 24
195	H48516	DLEU2	Deleted in lymphocytic leukemia, 2
196	AA775177	PTPRE	Protein tyrosine phosphatase, receptor type, E
197	NM_020651	PELI1	Pellino homolog 1 (Drosophila)
198	BF111326		Transcribed locus
199	BC003143	DUSP6	Dual specificity phosphatase 6
200	AB018289	KIAA0746	KIAA0746 protein

Table IV. Continued.

No.	Accession no.	Gene symbol	Gene name
201	AA643687	SLC28A3	Solute carrier family 28
202	NM_004994	MMP9	Matrix metallopeptidase 9
203	AI189753	TM4SF1	Transmembrane 4 L six family member 1
204	NM_000574	CD55	CD55 molecule
205	NM_017423	GALNT7	UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7
206	AW044663		Transcribed locus
207	AL390172	BCAT1	Branched chain aminotransferase 1, cytosolic
208	AA552969	ST8SIA4	ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 4
209	AI028661	MTUS1	Mitochondrial tumor suppressor 1
210	U79292	MMP16	Matrix metallopeptidase 16
211	AF132203	SCD	Stearoyl-CoA desaturase (δ-9-desaturase
212	BC002710	KLK10	Kallikrein-related peptidase 10
213	AU145865	LOC653135	Similar to Nucleosome-binding protein 1
214	AU151483	CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)
215	AI743727		Transcribed locus
216	NM_000358	TGFBI	Transforming growth factor, ß-induced, 68 kDa
217	U16996	DUSP6	Dual specificity phosphatase 6
218	NM_003081	SNAP25	Synaptosomal-associated protein, 25 kDa
219	AL137725	EPPK1	Epiplakin 1
220	AA579773		Transcribed locus
221	AW007080	IL17RD	Interleukin 17 receptor D
222	NM_014020	TMEM176B	Transmembrane protein 176B
223	AB011537	SLIT1	Slit homolog 1
224	CA448665	CD55	CD55 molecule
225	AA460299	MLF1IP	MLF1 interacting protein
226	U90905		Clone 23574 mRNA sequence
227	NM_005010	NRCAM	Neuronal cell adhesion molecule
228	BF224052	MYEF2	Myelin expression factor 2
229	AW802645		CDNA FLJ26187 fis,
230	AI675453	PLXNA3	Plexin A3
231	NM_024767	DLC1	Deleted in liver cancer 1
232	AI763378	EHF	Ets homologous factor
233	AK002075	MYEF2	Myelin expression factor 2
234	W72527	SLC1A4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
235	AI826125		Transcribed locus
236	NM_018584	CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor
237	AF144103	CXCL14	CXCL14
238	NM_007267	TMC6	Transmembrane channel-like 6
239	NM_005756	GPR64	G protein-coupled receptor 64
240	AI732305		Transcribed locus
241	NM_018422	PSD3	Pleckstrin and Sec7 domain containing 3
242	NM_003151	STAT4	Signal transducer and activator of transcription 4
243	AK025615	BCAT1	Branched chain aminotransferase 1, cytosolic

those commonly down-regulated (Table V). The up-regulated genes included genes associated with signal transduction (*CEACAM6*, *RGS4*, *KISS1R*, *PSD3*, *CXCL14*, *GPR92*), regulation of transcription (*NFE2L3*, *CDH3*, *CITED1*, *EHF*, *RUNX1*), regulation of cell growth and cell cycle (*PDGFA*, *GPNMB*, *SFN*, *DUSP6*), apoptosis (*PMAIP1*, *PRUNE2*, *TNFRSF10C*), proteolysis (*TMPRSS4*, *PRSS23*, *MMP9*, *KLK10*, *CTSC*, *ADAMTS9*), cell adhesion (*FN1*, *CDH6*, *ITGA2*, *TNC*, *AMIGO2*, *TGFBI*) and immune response (*CD55*). Among the up-regulated genes on our list, *CH3L1*,

Table V. Genes commonly down-regulated in thyroid cancer.

No.	Accession no.	Gene symbol	Gene name
1	NM_000792	DIO1	Deiodinase, iodothyronine, type I
2	AI758950	SLC26A7	Solute carrier family 26, member 7
3	U53506	DIO2	Deiodinase, iodothyronine, type II
4	AW166711	PIP3-E	Phosphoinositide-binding protein PIP3-E
5	AB014737	SMOC2	SPARC related modular calcium binding 2
6	M10943	MT1F	Metallothionein 1F
7	AI038059	DIO2	Deiodinase, iodothyronine, type II
8	BC037315		Hypothetical protein LOC286002
9	BF246115	MT1F	Metallothionein 1F
10	W63754	GLT8D2	Glycosyltransferase 8 domain containing 2
11	NM_003759	SLC4A4	Solute carrier family 4, member 4
12	NM_004105	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1
13	NM_000441	SLC26A4	Solute carrier family 26, member 4
14	AF063606	TCEAL2	Transcription elongation factor A (SII)-like 2
15	NM_022783	DEPDC6	DEP domain containing 6
16	AW364675		Transcribed locus
17	NM_004669	CLIC3	Chloride intracellular channel 3
18	NM_005950	MT1G	Metallothionein 1G
19	AI826799	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1
20	AB056106	ABI3BP	ABI gene family, member 3
21	BF002046	ANGPTL1	Angiopoietin-like 1
22	Z98443	WDR72	WD repeat domain 72
23	AI967987	MUM1L1	Melanoma associated antigen (mutated) 1-like 1
24	NM_002167	ID3	Inhibitor of DNA binding 3
25	NM_013989	DIO2	Deiodinase, iodothyronine, type II
26	NM_001449	FHL1	Four and a half LIM domains 1
27	NM_012082	ZFPM2	Zinc finger protein, multitype 2
28	AF074331	PAPSS2	3'-Phosphoadenosine 5'-phosphosulfate synthase
29	AI690433	COL23A1	Collagen, type XXIII, α
30	D13889	ID1	Inhibitor of DNA binding 1,
31	AI989706	TDRD9	Tudor domain containing 9
32	BF982174	SDPR	Serum deprivation response
33	AL136566	C9orf58	Chromosome 9 open reading frame 58
34	NM_002380	MATN2	Matrilin 2
35	BE856341	LAYN	Layilin
36	NM_005951	MT1P2	Metallothionein 1 pseudogene 2
37	U16153	ID4	Inhibitor of DNA binding 4
38	AK023852		CDNA FLJ13790 fis, clone THYRO1000026
39	AL022726	ID4	Inhibitor of DNA binding 4
40	AF138300	DCN	Decorin
41	AV733266	IGJ	Immunoglobulin J
42	NM_018371	ChGn	Chondroitin ß1,4 N-acetylgalactosaminyltransferase
43	BE856336	C8orf13	Chromosome 8 open reading frame 13
44	AA701657	LIFR	Leukemia inhibitory factor receptor α
45	AI474666		Transcribed locus
46	R73030	LRP2	Low density lipoprotein-related protein 2
47	AA934610		Transcribed locus
48	BF431199	IYD	Iodotyrosine deiodinase
49	NM_002222	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1
50	U89281	HSD17B6	Hydroxysteroid (17-ß) dehydrogenase 6

Table V. Continued.

No.	Accession no.	Gene symbol	Gene name
51	AW292765		CDNA FLJ42786 fis, clone BRAWH3006761
52	AI743534	ARHGAP24	Rho GTPase activating protein 24
53	AI928242	TFCP2L1	Transcription factor CP2-like 1
54	NM_022969	FGFR2	Fibroblast growth factor receptor 2
55	NM_005544	IRS1	Insulin receptor substrate 1
56	AF138302	DCN	Decorin
57	NM_006033	LIPG	Lipase, endothelial
58	NM_002885	RAP1GAP	RAP1 GTPase activating protein
59	NM_003890	FCGBP	Fc fragment of IgG binding protein
60	AI817041	CXCR7	Chemokine (C-X-C motif) receptor 7
61	AI281593	DCN	Decorin
62	AI680541	LIFR	Leukemia inhibitory factor receptor α
63	NM_001554	CYR61	Cysteine-rich, angiogenic inducer, 61
64	M87789	IGHG1,2,3,4	Immunoglobulin heavy locus
65	AI650848	TBC1D4	TBC1 domain family, member 4
66	NM_021069	SORBS2	Sorbin and SH3 domain containing 2
67	NM_005952	MT1X	Metallothionein 1X
68	NM_014214	IMPA2	Inositol(myo)-1(or 4)-monophosphatase 2
69	BF060767	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5
70	AI659533	SORBS2	Sorbin and SH3 domain containing 2
71	AI217416		MRNA; cDNA DKFZp564B213
72	AV699825	LOC145786	Hypothetical protein LOC145786
73	S55735	IGHA1,2	Immunoglobulin heavy constant α 1, α 2
74	NM_005084	PLA2G7	Phospholipase A2
75	NM_006829	C10orf116	Chromosome 10 open reading frame 116
76	AL049977	CLDN8	Claudin 8
77	AI948472	PAX8	Paired box 8
78	T16544		Transcribed locus
79	AA464273	SHANK2	SH3 and multiple ankyrin repeat domains 2
80	NM_007038	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5
81	NM_004165	RRAD	Ras-related associated with diabetes
82	BF304996	RGS16	Regulator of G-protein signaling 16
83	U94829	RGS16	Regulator of G-protein signaling 16
84	BF433902	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b
85	AF333388	MT1P2	Metallothionein 1 pseudogene 2
86	NM_002450	MT1X	Metallothionein 1X
87	NM_016725	FOLR1	Folate receptor 1 (adult)
88	L29008	SORD	Sorbitol dehydrogenase
89	NM_003104	SORD	Sorbitol dehydrogenase
90	NM_006633	IQGAP2	IQ motif containing GTPase activating protein 2
91	AW058459	TMEM171	Transmembrane protein 171
92	AA912476	LOC145786	Hypothetical protein LOC145786
93	AW299226	CD36	CD36 molecule (thrombospondin receptor)
94	AI823572	MGC45438	Hypothetical protein MGC45438
95	NM_018476	BEX1	Brain expressed, X-linked 1
96	AI095542		Transcribed locus
97	AW299958	PAPSS2	3'-Phosphoadenosine 5'-phosphosulfate synthase 2
98	NM_001674	ATF3	Activating transcription factor 3
99	AV699883	LOC653381	Similar to Sorbitol dehydrogenase
100	BE932011	C8orf79	Chromosome 8 open reading frame 79

Table V. Continued.

No.	Accession no.	Gene symbol	Gene name
101	NM_006208	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
102	NM_005044	PRKX	Protein kinase, X-linked
103	AW157094	ID4	Inhibitor of DNA binding 4,
104	BF217861	MT1E	Metallothionein 1E
105	NM_000633	BCL2	B-cell CLL/lymphoma 2
106	AL021786	ITM2A	Integral membrane protein 2A
107	AI221950	LRRN3	Leucine rich repeat neuronal 3
108	BE048514	SDC2	Syndecan 2
109	BF973568	SELM	Selenoprotein M
110	NM_004417	DUSP1	Dual specificity phosphatase 1
111	AL573851	ESAM	Endothelial cell adhesion molecule
112	M63438	IGKC,IGKV1-5	Immunoglobulin κ constant,variable 1-5
113	NM_013231	FLRT2	Fibronectin leucine rich transmembrane protein 2
114	AF109161	CITED2	Cbp/p300-interacting transactivator, 2
115	NM_022748	TNS3	Tensin 3
116	NM_014059	C13orf15	Chromosome 13 open reading frame 15
117	NM_024508	ZBED2	Zinc finger, BED-type containing 2
118	BC004490	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
119	AV646610	ID4	Inhibitor of DNA binding 4,
120	NM_002023	FMOD	Fibromodulin
121	NM_005461	MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
122	AW149846	GPX3	Glutathione peroxidase 3 (plasma)
123	AL136805	TSHZ3	Teashirt zinc finger homeobox 3
124	U63917	GPER	G protein-coupled estrogen receptor 1
125	NM_024512	LRRC2	Leucine rich repeat containing 2
126	AA758751	DPY19L2	Dpy-19-like 2
127	NM_002228	JUN	Jun oncogene
128	NM_001174	ARHGAP6	Rho GTPase activating protein 6
129	NM_005953	MT2A	Metallothionein 2A
130	AU159390		CDNA FLJ13886 fis, clone THYRO1001559
131	AU154891		CDNA FLJ10919 fis, clone OVARC1000347
132	AI459140	GSTM3	Glutathione S-transferase M3 (brain)
133	AL136550	TMEM47	Transmembrane protein 47
134	AW575927	IGKC,IGKV1-5	Immunoglobulin κ constant,variable 1-5
135	N63377		CDNA FLJ13569 fis, clone PLACE1008369
136	BC001060	PAX8	Paired box 8
137	NM_003944	SELENBP1	Selenium binding protein 1
138	AI301859		Transcribed locus

TUSC3, *TIMP1*, *FN1* and *TMPRSS4* were previously reported to be genes that were activated in thyroid carcinoma (20,21).

Furthermore, we identified 138 transcripts whose expression ratios were <0.2 in ≥50% of the informative thyroid cancers (Table V). Among these down-regulated genes, 98 transcripts had been functionally characterized to a certain extent. They included *ID3* (inhibitor of DNA binding 3), *ID4* (inhibitor of DNA binding 4) and *TFCP2L* (Transcription factor CP2-like 1), which have been implicated in the growth suppression or regulation of transcription (22-24). In particular, *ID3* and *ID4* were significantly down-regulated in each of the 18 thyroid cancer cases. *TFCP2L* was also significantly down-regulated in 17 of the 18 cases, indicating down-regulation of those genes may be related to thyroid cancer tumorigenesis.

Identification of RGS4 as a molecular target gene for thyroid cancer therapy. To validate the expression data obtained by microarray analysis, we performed semi-quantitative RT-PCR experiments for a total of 20 representative genes, which were frequently overexpressed in thyroid cancer cases,

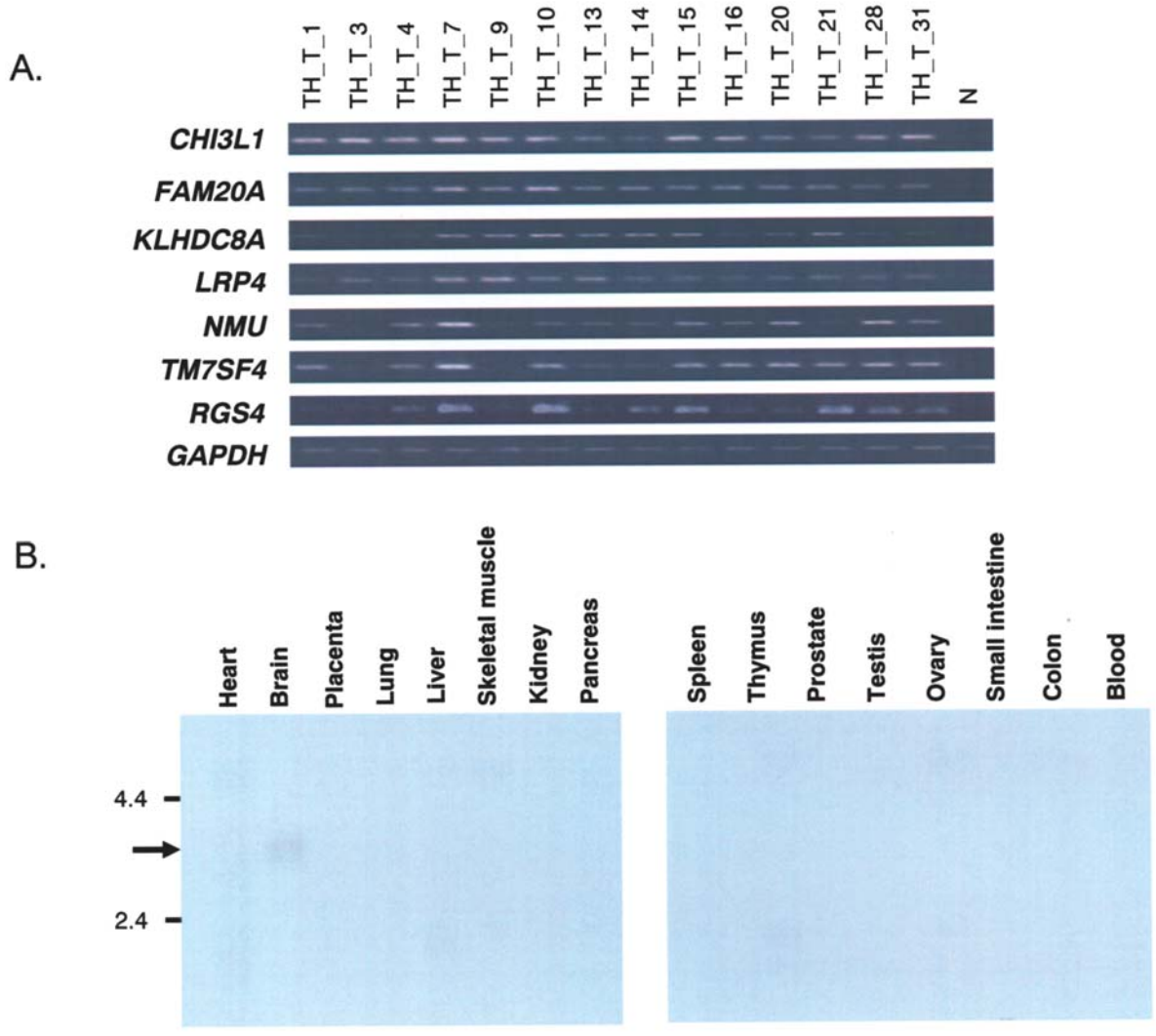

Figure 2. (A) Semi-quantitative RT-PCR analysis of representative up-regulated genes. Expression of seven representative up-regulated genes in microdissected papillary thyroid cancer cells (TH_T_1, 3, 4, 7, 9, 10, 13, 14, 15, 16, 20, 21, 28, 31) and normal thyroid follicular epithelial cells (N) examined by semi-quantitative RT-PCR. GAPDH was used as a quantitative control. (B) Northern blot analysis of the RGS4 expression in normal human tissues.

using 14 clinical thyroid cancer cases. We confirmed that these genes were highly expressed in all or most of the thyroid cancer cases examined, although their expression was undetectable in normal thyroid follicular epithelial cells (7 genes are shown in Fig. 2A). The results of RT-PCR experiments using thyroid cancer materials were concordant to those of the microarray analysis.

Among the up-regulated genes in the thyroid cancer shown in Fig. 2A, we focused on the regulator of G-protein signaling 4, *RGS4*, for further biological analysis since it was overexpressed very commonly in clinical thyroid cancer samples, though not expressed in any of the normal human tissues examined except the brain by the microarray data (data not shown). Subsequent Northern blot analysis using an *RGS4* cDNA fragment as a probe confirmed that an ~3 kb transcript was hardly detectable in normal human tissues except the brain (Fig. 2B). Expression of this gene was elevated in a very small subset of breast, esophagus, lung, colon, bladder, pancreatic and prostate cancers through our previous expression profile analysis (data not shown).

Inhibition of growth of thyroid cancer cells by specific small interfering RNA against RGS4. To assess whether *RGS4* is essential for the growth and survival of thyroid cancer cells, we constructed plasmids to express siRNAs against *RGS4* (si#1 and si#2) as well as control plasmids (siRNA for Scramble and Mock vector) and transfected each of them into the FTC-133 thyroid cancer cell line, which endogenously expressed a high level of *RGS4* mRNA. Among the siRNA constructs we tested, si#2 effectively knocked-down the expression of *RGS4* mRNA, compared with control siRNAs (si-Scramble and si-Mock) (Fig. 3A). We observed a significant decrease in the number of colonies (Fig. 3B) and in the number of viable cells measured by MTT assay for the FTC-133 cells treated with si#2 (Fig. 3C), indicating an important role of *RGS4* in thyroid cancer cell survival and/or growth.

Discussion

Recent progress in genomic and molecular analysis has improved our understanding of the genesis of a wide range of human neoplasms. For thyroid carcinoma, several groups

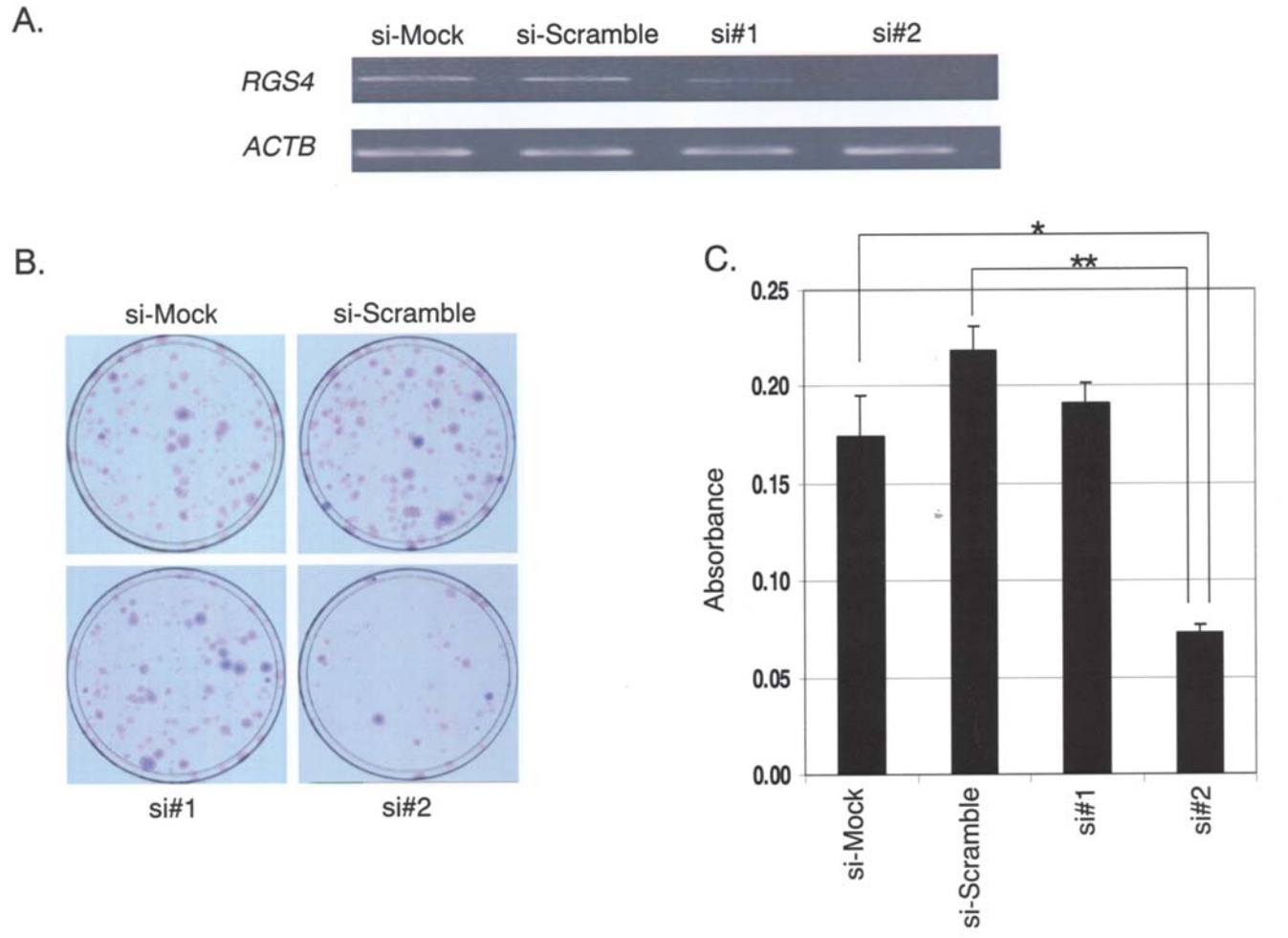

Figure 3. Knockdown effect of *RGS4* siRNA on thyroid cancer cell growth and viability. Two *RGS4* siRNA expression vectors (si#1 and #2) and control vectors (si-Scramble and si-Mock) were transfected into thyroid cancer FTC-133 cells. (A) RT-PCR experiments for examining the *RGS4* transcript level in the cells treated with siRNA. *ACTB* expression level was used as a quantitative control. The si#2 vector reveals a knockdown effect; si#1, si-Scramble, and si-Mock failed to show any effect on the level of the *RGS4* transcript. Transfection with the si#2 vector resulted in a reduction of the numbers of colonies (B) and the number of viable cells (C), compared with the cells transfected with si#1, si-Scramble and si-Mock ($^{**}p<0.005$ and $^{*}p<0.01$, respectively; unpaired t-test).

reported results of microarray-based expression profile analysis and demonstrated candidate genes that might be useful as diagnostic markers (4,7,9,10,21,25-29). However, tumor tissues are mixtures of various cell types such as inflammatory cells, stroma cells, endothelial cells and fibroblasts in addition to cancer cells and the proportions of each cell type vary significantly from one individual to another. Hence, the expression profile data using mRNAs isolated from bulk tumor tissues are very unlikely to reflect the exact changes during the course of thyroid carcinogenesis. In addition, normal thyroid is a mixture of different cell types. Since thyroid carcinomas except a medullary type of carcinoma are considered to originate from thyroid epithelial cells in follicles of the thyroid gland, it is better to use normal epithelial cells as a control to know the difference in expression levels related to the process in thyroid carcinogenesis. Therefore, in this study we performed LMM to enrich populations of papillary thyroid cancer cells as well as control normal thyroid follicular epithelial cells. A comparison of our data and one of the previous expression profile data of thyroid cancer using RNAs from bulk tumor tissues by means of microarray (30), only 71 of the 243 transcripts, that we identified to be commonly up-regulated in >50% of informative thyroid cancer cases shown in Table IV, overlapped to the bulk-expression profile data. We assume that these discrepancies were attributed mostly to the difference in the sample preparation processes and are confident that our data demonstrated more precisely the expression changes during the carcinogenesis process from thyroid epithelial to malignant cells. For example, genes highly expressed in parafollicular cells, though not in thyroid follicular epithelial cells, may be selected as down-regulated or unchanged genes in expression profiles from bulk tissues. To evaluate the purity of microdissected cell populations, we analyzed the expression of the *CALCA*, which is expressed abundantly in parafollicular C cells, in our gene expression profiling. By applying the microdissection procedure carefully, we were able to minimize the proportion of contaminating parafollicular C cells to as little as 2.6% in preparations of normal thyroid follicular epithelial cells. Indeed, a comparison of our data with the previous expression profile data of thyroid cancer by means of microarray (6,10,31,32), only 46 of the 138 transcripts, that we identified to be commonly down-regulated in >50% of informative thyroid cancer cases shown in Table V, overlapped to the bulk-expression profile data. Therefore, 92 genes, which had

not been identified as down-regulated genes in previous reports, were identified as down-regulated genes in this study. In a list of the 92 down-regulated genes, which were identified in this study, we found *CLIC3* (chloride intracellular channel 3), *SDPR* (serum deprivation response) and *BEX1* (brain expressed, X-linked 1), which are reported as a regulator of cell growth, a regulator of the survival of cancer cells and a tumor suppressor, respectively (33-35). This might suggest that a great majority of the down-regulated genes including genes with a tumor-suppressive function may be undetected in previous studies. We believe that it is crucial to perform the LMM system to purify as much as possible the populations of cancerous and normal epithelial cells obtained from surgical specimens to better understand genes, which are involved in the carcinogenesis.

Some of the genes with an altered expression in most of thyroid cancers may play causal roles in thyroid carcinogenesis and also serve as molecular diagnostic markers and candidate targets for the development of novel therapeutic drugs for thyroid cancer. Among the up-regulated genes, *FN1* (fibronectin 1), *TNC* (Tenascin C), *TIMP1* (TIMP metallopeptidase inhibitor 1), *TGFA* (transforming growth factor α), *LGALS3* (*Galecin3*; lectin, galactoside-binding, soluble, 3) were previously reported as genes up-regulated in thyroid cancer (6,10,21,26,31-32,36-38). The expression of the FN1 protein was indicated to be associated with malignant transformation because of its much higher expression in thyroid carcinomas than adenomas (39). Most of papillary and medullary thyroid carcinomas demonstrated a strong TNC positivity in immunohistochemical analysis although its biological role in thyroid carcinoma is still unclear (40). *TIMP1* was reported as a diagnostic marker in distinguishing benign from malignant thyroid neoplasms and predicting the extent of this disease (41). TGFA stimulates invasion and growth of thyroid cancer cells by binding to the EGFR and the TGF-α-mediated growth-promoting effect could be suppressed by an antagonist to the EGF receptor (42). Moreover, TGFA was suggested to be involved in the regulation of thyroid cancer cell proliferation in an autocrine manner (43). *LGALS3* was shown to be a useful marker in the diagnosis of thyroid cancer and was likely to be involved in the invasion and metastasis of cancer cells (44,45).

However, among the down-regulated genes, *ID3* (inhibitor of DNA binding 3), *ID4* (inhibitor of DNA binding 4) and *TFCP2L1* (Transcription factor CP2-like 1) were implicated in the growth suppression or regulation of transcription. ID3 and ID4 proteins constitute a family of helix-loop-helix (HLH) transcription factors that are important regulators of cellular differentiation and proliferation (46-49). ID4 is suggested to modulate various key developmental processes and is involved in cell cycle control and cellular senescence (48). Epigenetic silencing of this gene was found in gastric cancer and suggested its functions as a tumor suppressor (50). Immunoblot and quantitative real-time (RT) polymerase chain reaction revealed a marked down-regulation of *CRYAB* (αB-crystallin), whose gene silencing was caused by the down-regulation of *TFCP2L1* in malignant anaplastic thyroid carcinomas (ATC) and the ATC-derived cell line (51). Table V included a large number of down-regulated genes, which were reported as down-regulated genes in previous microarray data using thyroid cancer samples (10,21,26,31,32,36-38). Among them, the down-regulation of *DUSP1* (Dual specificity phosphatase 1), *BCL2* (B-cell CLL/lymphoma 2), *PAX8* (Paired box 8), and *MT1G* (Metallothionein 1G) were reported in multiple studies (10,52). The expression of *DUSP1* was found to be reduced in thyroid cancer cell lines and down-regulated in a large fraction of thyroid carcinomas compared with normal tissue (53-56). Immunohistochemistry with an anti-bcl-2 monoclonal antibody on paraffin sections showed its high expression in normal follicular cells, while it appeared to be down-regulated in papillary, follicular and undifferentiated thyroid carcinomas (57). *PAX8* has a high expression in benign thyroid tissues and no or weak expression in thyroid cancers (57,58). Metallothionein 1G (*MT1G*) is a well-known gene whose protein is associated with the protection against DNA damage, oxidative stress and apoptosis (59). Down-regulation of *MT1G* in thyroid cancer has been reported by many authors (53-55). In addition, the 92 down-regulated genes we identified here included *EFEMP1* (EGF-containing fibulin-like extracellular matrix protein 1), *ANGPTL1* (angiopoietin 1), and *LIFR* (leukemia inhibitory factor receptor α), which were implicated in growth suppression or apoptosis. It was reported that *EFEMP1* had the capability of reducing tumor angiogenesis and tumor growth *in vivo* (60). High frequency of the down-regulation of *ANGPTL1* in tumor tissues, which is a pro-angiogenic factor, was also reported suggesting a role for this protein in tumor inhibition (61). It was recommended that LIFR is required for differentiation and growth arrest of myeloid leukemic cells (62).

Among the up-regulated genes in our list, in this study we further focused on Regulator of G-protein signaling 4 (*RGS4*) as a possible molecular target for thyroid cancer therapy due to frequent transactivation in thyroid cancers and its undetectable level of expression in any of the normal human adult tissues except the brain. We demonstrated that the knock-down of its expression level with siRNA resulted in the significant suppression of thyroid cancer cell growth, suggesting its essential role in the enhancement of cell growth. *RGS4* belongs to the family of RGS proteins, which are regulatory molecules that act as GTPase activating proteins (GAPs) for G-α subunits of heterotrimeric G proteins. RGS4 contains an RGS domain, which is a conserved 120-amino acid sequence among each of the RGS family members. The expression of *RGS4* in neurons is essential for embryonic neuronal development. More recently, RGS4 has been implicated in the involvement in tumor motility (63).

Taken together, the up-regulated genes, including *RGS4*, identified through our precise expression profiles of thyroid cancers should shed light on a better understanding of thyroid carcinogenesis and provide useful information in discovering possible novel molecular targets for the development of thyroid cancer treatment and diagnostic tumor markers.

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