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Construction of cDNA Libraries from Microdissected Benign and Malignant Thyroid Tissue

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SUMMARY: cDNA libraries were constructed from thyroid epithelial cells gained by laser capture microdissection for gene expression analysis of the progression of thyroid cancer. Six histologically diverse thyroid tissue specimens were used. A mean of 93 ng of total RNA was gained per tissue sample from a mean estimated number of 25,000 microdissected cells per sample. Analysis of randomly selected clones from six libraries showed an average insert size of 600 (range, 300–1500) bp. Preliminary sequencing of clones selected from the six libraries indicates a range of 46% to 62% known genes per library, 4% to 25% anonymous expressed sequence tags per library, and 15% to 43% novel expressed sequence tags per library. Thyroglobulin was found in normal thyroid epithelium and follicular thyroid adenoma, whereas calcitonin precursor transcripts were found in medullary thyroid carcinoma. We demonstrate production of high-quality cDNA libraries of microdissected tissue of the thyroid, which should prove useful for gene expression analysis of human thyroid tumors. (*Lab Invest 2002, 82:1707–1714*).

Assessing the gene expression profiles of benign and malignant thyroid tissue is of interest in understanding tumorigenesis, identifying specific diagnostic markers, and searching for potential targets for drug therapy of thyroid tumors. Construction and expressed sequence tag (EST) sequencing of a cDNA library can develop such a gene expression profile of a given biologic source. To optimize specificity it is of considerable interest to generate cDNA libraries directly from specific cell populations of various tissues to serve as reagents for studying in vivo gene expression.

Because bulk tissue samples invariably contain substantial cellular heterogeneity, they are not a suitable source of starting material for the generation of a representational library from a distinct cell type. Furthermore, it is not known whether gene expression profiles of in vitro cell lines necessarily reflect the expression profiles of their tissue of origin, making this source of RNA also problematic. The use of laser capture microdissection (LCM) provides the means by which defined populations of cells can be procured to obtain biomolecules specific to a given disease stage (Bonner et al, 1997; Emmert-Buck et al, 1996). Methodologies have been developed to construct cDNA libraries from isolated tumor cells of surgically resected tissue using LCM (Krizman et al, 1996; Peterson et al, 1998). Thus, cDNA libraries can be constructed from small numbers of microdissected cells and are of value in establishing gene expression profiles within a histologically defined, homogeneous population of cells (Vasmatzis et al, 1998).

In this study we selected tissue samples of normal thyroid and of benign and malignant thyroid tumors of patients with clinically well-documented course of disease. Representative cDNA libraries were constructed from the following microdissected tissues: normal thyroid, follicular thyroid adenoma (FTA), follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), medullary thyroid carcinoma (MTC), and insular (poorly differentiated) thyroid carcinoma (ITC). A small number of clones from each library were sequenced in a preliminary fashion to perform quality assurance and gain some initial insight into genes expressed in these various cell populations. Furthermore, to evaluate the ability of the libraries to recapitulate the gene expression differences already established in the literature, a semiquantitative PCR for thyroglobulin, calcitonin, oncofetal fibronectin, and HMGI(Y) was performed from the cDNA produced from the microdissected tissue (Chiappetta et al, 1998; Takano et al, 1998, 1999).

To facilitate large-scale sequencing of thousands of individual clones necessary for gene expression profiling, these libraries have been submitted to the Tumor Gene Index of the Cancer Genome Anatomy

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Project (CGAP) (Strausberg et al, 1997) (http://www.ncbi.nlm.nih.gov/ncicgap/).

Results

Clinical and Histologic Features of Thyroid Tissue Samples

We selected thyroid tumor samples and normal tissue samples from patients in the files of the Study Group for Surgical Endocrinology, University of Vienna, Medical School. After operation all patients were seen regularly at the outpatient clinics of the Department of Surgery, University of Vienna, and their course of disease was recorded. Tumor entities were selected according to patient ages and gender, as well as stage at initial diagnosis and course of disease, as listed in Table 1. Normal thyroid tissue was taken from the contralateral side of a male patient with unilateral PTC. The FTA was of the macrofollicular type, with isoprismatic to flat follicular epithelial cells and a thin fibrous capsule without evidence of capsular or vascular invasion. FTC tissue was taken from the operated material of the first recurrence of disease 3 years after initial radical surgery and radioiodine treatment. Histology displayed a widely invasive microfollicular and trabecular tumor. Tumor cells showed an oxyphilic cytoplasm and atypical nuclei with large nucleoli (Fig. 1). The PTC was of the common variant, with complex papillae and isoprismatic cells with typical nuclear criteria such as nuclear crowding, nuclear grooves, and ground glass nuclei. Tumor tissue of a female patient with multiple endocrine neoplasia IIa syndrome was selected to represent MTC. The tumor cells grew in solid nests and trabeculae with polygonal shape, amphophilic cytoplasm, and medium-sized nuclei separated by a highly vascular stroma and hyalinized collagen. The ITC was grossly invasive and consisted of insular nests of small uniform tumor cells and high mitotic activity.

Amount of RNA and cDNA Insert Sizes

The average number of LCM shots per tissue sample was approximately 5,200, and the mean estimated number of cells obtained was roughly 26,000 per sample. The individual numbers of shots, the estimated numbers of cells, and the amount of total RNA obtained for cDNA library construction per sample is given in Table 2. An average amount of 61 ng of total RNA was used to generate each cDNA library. After PCR amplification the products were run on 1.2% agarose gel and a continuous smear ranging from 400 to 1500 bp was observed (data not shown). The mean amount of cDNA after PCR amplification was 144 $\pm$ 17.4 ng. Libraries were subcloned into pAMP 10 cloning vector and transformed into DH10B cells. A total of 12 colonies per library were randomly picked for PCR analysis to obtain an average insert size of 600 bp, with a range from 300 to 1500 bp (Fig. 2).

Library Analysis

To further assess the quality of the library, 192 individual colonies of each library were randomly picked and expanded for sequencing. Individual sequences were analyzed by BLAST, with the combined characterization of all inserts shown in Tables 2 and 3. Sequence analysis of the six libraries demonstrated that 46% to 62% (82–146) of the sequenced inserts derived from known genes. Anonymous ESTs were found in 4% to 25% (8–44) and unknown ESTs in 15% to 43% (13–76) of the sequenced clones. These libraries contain small numbers of clones without inserts or inserts too short to sequence. The number of background clones, as defined by clones containing either mitochondrial sequence, ribosomal RNA sequence, or sequence derived from *Escherichia coli* was nearly nonexistent (Table 2). A list of genes that were common to several libraries or for which sequences were found in more than one clone of each library, respectively, is given in Table 3. Many of these genes are presumably expressed to a large extent in most if not all thyroid-derived epithelium.

Semiquantitative PCR

The results of the semiquantitative PCR are shown in Figure 3.

Table 1. Clinical Data of Patients from Whom Tissue Samples Were Collected for LCM Microdissection

Histologic diagnosis	Patient age	Patient gender	Stage at initial diagnosis	Course of disease
Normal thyroid tissue	24a	Male		
Follicular thyroid adenoma	58a	Female		
Papillary thyroid carcinoma	86a	Female	T4bN1bM0	No recurrent disease after 1 yr
Follicular thyroid carcinoma	73a	Female	T2aN0M0	Recurrence of disease after 3 and 5 yr; alive after 10 yr
Medullary thyroid carcinoma	48a	Female	T4bN1b	MEN IIa, no recurrent disease
Poorly differentiated (insular) thyroid carcinoma	43a	Male	pT4aN0M1	Died of recurrent disease

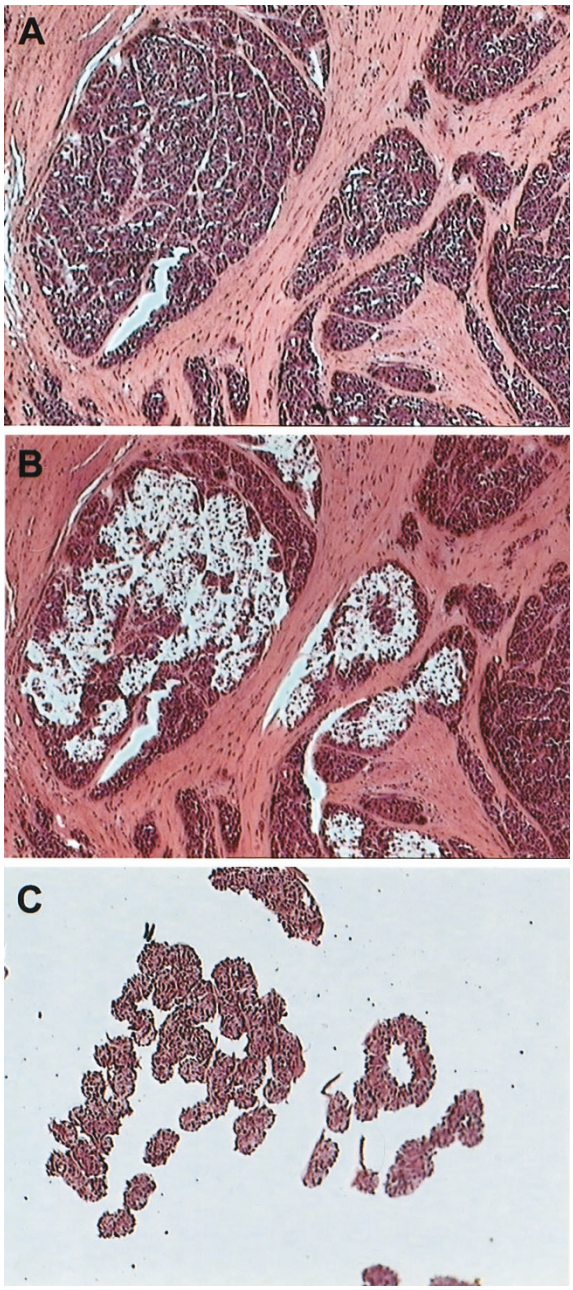

Figure 1.
Microdissection of follicular thyroid carcinoma (FTC). A, Before microdissection; B, after microdissection; C, microdissected cells attached to the cap.

Discussion

Construction of cDNA libraries from microdissected tissue samples has been successful in generating gene expression profiles of tumors of distinct histology and tumor stages. Here, we report the generation of a series of cDNA libraries constructed from microdissected benign and malignant thyroid tumor cells. The tissue samples selected were chosen according to stage and clinical course. All invasive tumors selected had either metastasized or recurred. With regard to FTA and FTC, tumor samples of patients whose clinical course confirmed the histologic diagnosis were selected. This was done because doubts about the validity of the histologic criteria in differentiating follicular adenomas from carcinomas were raised (Greenebaum et al, 1985). The MTC patient is known to manifest Pheochromocytoma as well as a germ-line mutation of the RET oncogene, indicating multiple endocrine neoplasia IIA syndrome.

The quality of a cDNA library can be determined by a combination of the average length of the inserts, the percentage of empty or short clones, and the relative amount of contaminating background clones. Our libraries showed an insert size of 600 bp on average (range, 300–1500 bp), up to 4% short clones, and background contaminating clones of 1%. These results indicate that high-quality cDNA libraries from microdissected tissue were successfully constructed.

To assess whether the libraries are able to represent gene expression differences of genes known to be expressed differentially in various tumor entities or in normal thyroid tissue, a semiquantitative PCR was performed from the cDNA produced from the microdissected tissue material. In accordance with data in the literature, the expression of calcitonin was restricted to the cDNA of MTC, the expression of oncofetal fibronectin to the cDNA of PTC and ITC, and the expression of HMGI(Y) to the cDNA of FTC and PTC (Chiappetta et al, 1998; Takano et al, 1998, 1999). The expression of thyroglobulin was reduced in the cDNA of FTC and PTC compared with normal thyroid tissue and absent in ITC and MTC. These results indicate a high specificity of the cDNA that was used for cloning.

Preliminary sequencing of the inserts revealed ranges of 42% to 66% known genes, 4% to 25% known ESTs, and 15% to 43% novel ESTs found for the various libraries. Some ESTs were found in several clones of one library, indicating a high expression of previously unknown genes in these histologic entities. These genes may play an important role in thyroid tumorigenesis and should be further characterized. Among the known genes, we found transcripts of 40S and 60S ribosomal protein genes in all libraries. Furthermore, transcripts of the monocyte chemotactic protein-3 gene were found in clones of all libraries but in substantially higher numbers in libraries of malignant tumors. Monocyte chemotactic proteins are chemokines involved in macrophage recruitment during inflammation and cancer (Opdenakker et al, 1994).

Transcripts of cytokeratins of low molecular weight known to be expressed in benign and malignant follicular cells of the thyroid were found in the libraries of normal thyroid and of follicular and papillary cancer (Henzen-Logmans et al, 1987). Sequences of thyroglobulin precursor, known to be exclusively expressed in follicular epithelial cells, were found in 10 clones of normal thyroid cells and in 3 clones of FTA (Davila et al, 1988). The α-type calcitonin precursor was identified in six clones of the MTC library. Calcitonin is a specific marker of MTC and was expressed highly in this sample, as shown by immunohistochemistry at the time of diagnosis (data not shown). Transcripts of vimentin were found in one clone of the normal thyroid tissue and in two clones of the ITC. Although generally

Table 2. Results of LCM, RNA Isolation, and Library Sequencing

Histologic diagnosis	Number of LCM shots	Estimated cell number	Total RNA amount	Clones sequenced	Diversity	% Unknown ESTs	% Background clones	Short clones
Normal thyroid tissue	2,317	11,585	65 ng	182	75%	20%	1%	4%
Follicular thyroid adenoma	2,281	11,405	28 ng	179	63%	43%	1%	0%
Follicular thyroid carcinoma	8,026	40,130	63 ng	175	71%	31%	1%	3%
Papillary thyroid carcinoma	12,550	62,750	226 ng	186	56%	7%	1%	0%
Medullary thyroid carcinoma	3,178	15,890	155 ng	183	76%	29%	1%	4%
Poorly differentiated thyroid carcinoma	3,057	15,285	22 ng	168	68%	15%	1%	4%

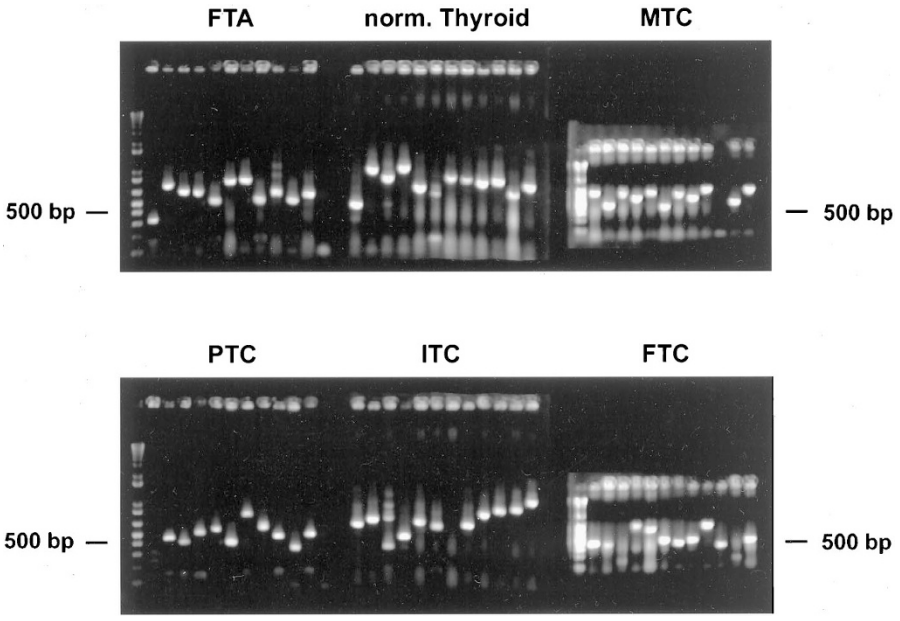

Figure 2.
Insert size distribution after PCR amplification of 12 randomly selected clones of the various libraries.

indicating mesenchymal differentiation of a cell, vimentin expression can be found in low amounts in normal thyroid tissue and in higher levels in malignant thyroid tumors as shown by Henzen-Logmans et al (1987). Taken together these data indicate that this series of cDNA libraries are representative of the various histologic entities investigated.

Only a fraction of the generated inserts were sequenced. The specificity and role of genes found expressed in these libraries will need to be investigated for their relevance in thyroid tumor pathology. The identification of more expressed genes from these libraries needs to occur, followed by immunohistochemical and hybridization studies to confirm the role of these genes in thyroid cancer disease. These libraries have been submitted for large-scale sequencing through the Cancer Genome Anatomy Project effort, and this additional sequencing information should lend a vast amount of gene expression data to the field of gene expression analysis of thyroid cancer histology and progression and should aid in establishing a gene expression profile of human thyroid tumors.

Materials and Methods

Tissue Samples and LCM

Fresh tumor tissue samples from patients undergoing surgery for thyroid tumors were immediately snap-frozen in liquid nitrogen. Hematoxylin and eosin sections were subsequently analyzed and confirmed by a certified pathologist. Single sections representing each of the following histologies were used for library construction: normal thyroid tissue, FTA, FTC, PTC, MTC, and ITC (Table 1). Tissue samples were processed as previously described (Bonner et al, 1997; Emmert-Buck et al, 1996). Briefly, empty cryomolds were placed on dry ice for 1 minute before immobilizing the frozen tissue sample in a thin layer of OCT embedding medium (Sakura Finetek, Torrance, Cali-

Table 3. Genes Found in More Than One Clone and Common in Libraries of Several Tissue Samples

Gene	Normal thyroid tissue	Follicular thyroid adenoma	Follicular thyroid carcinoma	Papillary thyroid carcinoma	Medullary thyroid carcinoma	Poorly differentiated thyroid carcinoma
Known EST	40	8	33	27	44	36
Unknown EST	44	76	53	13	51	25
40 S ribosomal protein	S15A, 17, 20, 24, 26	S11, 16	S3A, 8, 26	S4, 10, 13, 19, 21, 24, 28	S4, 11, 14, 19	S13, 14, 15A, 21, 24, 26
58AA long hypothetical protein	1 Clone		6 Clones			1 Clone
60 S ribosomal protein	Acidic P0, L6, 9, 26, 31, 34, 35A, 37A, 44	L6, 26, 31, 37A	L28, 34, 37A, 38, 44	Acidic P1, L3, 4a, 8, 9, 11–13, 17, 21, 24, 26, 28, 29, 31, 34, 35, 35A, 37A, 39, 44	L3, 8, 13A, 35, 37A	Acidic P0, L15, 19, 34, 37, 37A
ATP synthase A chain			2 Clones	2 Clones	1 Clone	4 Clones
α-Type calcitonin precursor					6 Clones	
Breast basic conserved protein		1 Clone				1 Clone
Cyclin 1			2 Clones			
Cytochrome C oxidase polypeptide	I, II, VIB	I, II	I	I, II, VIIC precursor	I	II, VIIA liver precursor, VIIC precursor
Elongation factor 1-α 1	2 Clones			1 Clone		1 Clone
Enoyl CoA hydratase			2 Clones			
Ferritin light chain					1 Clone	1 Clone
Glutathion S transferase	1 Clone		1 Clone			
HLA II γ prec.			2 Clones			
Hypothetical protein	1 Clone	1 Clone	1 Clone	4 Clones		
κ Casein prec.				3 Clones		1 Clone
Keratin	I Cytoskeletal 19		II Cytoskeletal 56KD, 8	II Cytoskeletal 7		
Laminin receptor					1 Clone	1 Clone
MCP3 prec.	2 Clones	1 Clone	4 Clones	23 Clones	7 Clones	8 Clones
NADH ubiquinone oxidoreductase	AGGG subunit prec., B15, chain 1, 4	Chain 4	Chain 3, 4, 4L	B17, chain 4	AGGG subunit prec., chain 3, 4	Chain 1, 4, 4L, 5
QM protein	3 Clones		1 Clone	1 Clone		
RAS-related protein				1 Clone	1 Clone	1 Clone
RT 14 protein	2 Clones					
Thyroglobulin precursor	10 Clones	3 Clones				
Translationally controlled tumor protein	1 Clone				2 Clones	
Ubiquitin					2 Clones	1 Clone
Ubiquitin-like protein fubi				2 Clones		
Vimentin	1 Clone					2 Clones

fornia). Cryomolds containing the tissues were then filled to the top with optimal cutting temperature compound and allowed to harden at −70° C, after which 8-μm thick sections were cut using a cryostat. Sections were placed onto RNAse-free glass slides, fixed in 70% ethanol, and stained with hematoxylin and eosin before microdissection, as recommended. A standard LCM microscope was used for microdis-

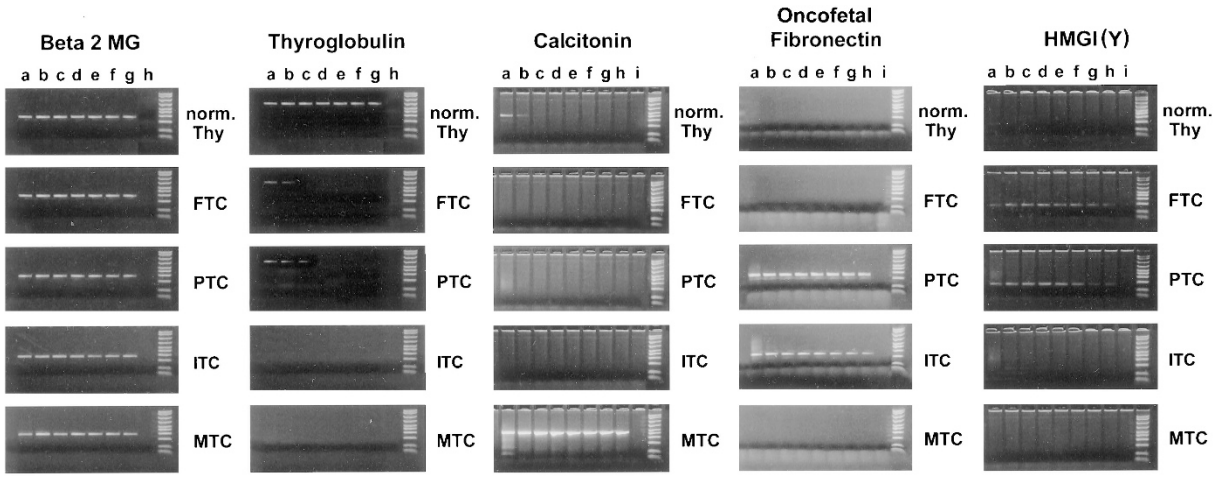

Figure 3.

Results of the quantitative PCR from the cDNA produced from microdissected tissue. For β2-microglobulin and thyroglobulin, the dilutions of the PCR template were as follows: *a*, 1:10; *b*, 1:20; *c*, 1:50; *d*, 1:100; *e*, 1:200; *f*, 1:500; and *g*, 1:1000. *Lane h* represents the negative control. For calcitonin, oncofetal fibronectin, and HMGI(Y) template dilutions were as follows: *a*, 1:5; *b*, 1:10; *c*, 1:20; *d*, 1:50; *e*, 1:100, *f*, 1:200; *g*, 1:500; and *h*, 1:1000. *Lane i* represents the negative control. β2-microglobulin reached the plateau phase in all dilutions of the various samples, indicating highly abundant cDNA in all library products. Expression of thyroglobulin was significantly reduced in FTC and papillary thyroid carcinoma (PTC) compared with normal thyroid tissue, as indicated by a loss of the signal at dilutions of 1:50 and 1:100, respectively. No thyroglobulin expression was detected in the library products of insular (poorly differentiated) thyroid carcinoma (ITC) and medullary thyroid carcinoma (MTC). Calcitonin was highly abundant in MTC, in which the signal intensity of the PCR products reached the plateau phase in all dilutions. Weak signals of the calcitonin products were also seen in normal thyroid at dilution of 1:5 and 1:10 of the template, indicating the presence of a low number of c-cells in the tissue material used for the generation of the cDNA libraries. Signal detection of oncofetal fibronectin was restricted to library PCR products of PTC and ITC, with a slightly lower concentration in the library product of ITC indicated by a decrease in signal intensity at a dilution of 1:200. PCR products of HMGI(Y) were detected in the libraries of FTC and PTC.

section (Arcturus Engineering, Mountain View, California). Using this procedure approximately 5000 cells can be gained after 1000 shots. The caps containing the procured cells were subsequently transferred to a 0.5-ml RNAse-free microfuge tube containing the buffer for total RNA extraction. Caps coated with ethylene vinyl acetate for use with LCM were supplied by Arcturus Engineering.

RNA Extraction and Determination of RNA Integrity

Three slides of each tissue sample were microdissected using three caps each. Each cap containing the captured cells of one slide was digested in 130 μl of guanidine-isothiocyanate buffer (Stratagene Inc., La Jolla, California) and 1 μl of β-mercaptoethanol for 30 minutes at room temperature. All three tubes from a given tissue microdissection were pooled in a single 1.5-ml RNAse-free microfuge tube. After addition of 40 μl of 2 M sodium acetate, 440 μl of saturated phenol, and 120 μl of chloroform-isoamyl alcohol, each tube was vortexed vigorously and placed on ice for 15 minutes followed by centrifugation at 14,000 rpm at 4° C for 30 minutes. The upper layer was transferred to a fresh RNAse-free microfuge tube, and the RNA was precipitated with 400 μl of cold isopropanol and 2 μl of glycogen (10 μg/μl; Gene Hunter, Nashville, Tennessee) at −80° C for 1 hour. The RNA was precipitated by centrifuging at 14,000 rpm at 4° C for 30 minutes. After washing the pellet with 70% ethanol, the RNA was resuspended in 7 μl of RNAse-free H_2O, 1 μl of 10× reaction buffer (GenHunter, Nashville, Tennessee), and 2 μl of DNase (10 U/μl; GenHunter). The reaction was incubated at 37° C for 2 hours. The DNase was inactivated at 65° C for 10 minutes followed by RNA precipitation as described. The RNA was resuspended in 5 μl of RNAse-free H_2O and 1 μl of RNAse inhibitor (20 U/μl; GenHunter) for subsequent cDNA synthesis. Total cellular RNA was quantitated by the VersaFluor Fluorometer system according to the manufacturer's recommendations (BioRad Laboratories, Hercules, California) using RiboGreen dye (Molecular Probes, Inc., Eugene, Oregon).

cDNA Synthesis and Library Construction

Double-stranded cDNA was synthesized from total RNA using a modified version of the Superscript Choice System (Life Technologies Inc., Gaithersburg, Maryland). Briefly, total RNA was mixed with 500 ng/μl oligo d(T) in a volume of 5 μl. This mixture was heated at 65° C for 10 minutes, placed directly at 42° C for 5 minutes, and then mixed with 15 μl of a preheated (42° C) mixture consisting of 4 μl of 5× first-strand buffer, 2 μl of 100 mM dithiothreitol, 1 μl of 10 mM dNTPs, and 1 μl of Superscript II RT. This reaction was incubated at 42° C for 30 minutes, after which it was placed on ice. The second-strand replacement reaction was performed in a 150-μl volume by the addition of 91 μl of diethylenepyrocarbonate water, 30 μl of 5× second-strand buffer, 3 μl of 10 mM dNTPs, 1 μl of *E. coli* DNA ligase, 4 μl of *E. coli* DNA Pol I, and 1 μl of *E. coli* RNAse H. This reaction was incubated at 16° C for 2 hours, and then 2 μl of T4 DNA polymerase was added and incubated for an additional 10 minutes. The tubes were placed on ice to stop the reaction, and the cDNA pellet was extracted with phenol/chloroform and precipitated with ethanol. The final pellet was resuspended in 13.5 μl of sterile ddH_2O. Adapter

ligation reaction was performed in a 25-μl reaction volume (5 μl of 5× adapter buffer, 1 μg/μl *Eco*RI adapters, 3.5 μl of 0.1 M dithiothreitol, 2 μl of T4 DNA ligase), with incubation at 16° C overnight. The adapter-ligated cDNA was purified in 1% low melting agarose, and products from 0.4 to 2 Kb were excised with a scalpel blade. The agarose was melted at 65° C for 15 minutes and digested overnight at 37° C with β-agarose enzyme according to the manufacturer's recommendations (New England Biolabs, Beverly, Massachusetts). The cDNA pellet was extracted with phenol/chloroform, precipitated with isopropyl alcohol plus 1 μl of 20 μg/ml glycogen at −20° C for 15 minutes, and resuspended in 83.5 μl of sterile ddH_2O.

The entire volume of cDNA (83.5 μl) was used for PCR amplification to amplify the library for cloning purposes. The PCR reaction was performed as described previously (Krizman et al, 1996). A reaction containing no library template was subjected to PCR cycling as a negative control. A total of 10 μl from both the library PCR reaction and the negative control was analyzed by 1.2% agarose gel electrophoresis. Library PCR product was purified by phenol:chloroform extraction and alcohol precipitation using isopropanol. The pellet was resuspended in 11 μl of sterile H_2O. A total of 1 μl of the cDNA was used for determination of the concentration of the PCR product by fluorometer reading as described previously, but with PicoGreen dye for DNA quantification (Molecular Probes).

A total of 0.5 μl of the amplified cDNA was nondirectionally cloned into the UDG cloning vector pAMP10 according to the CloneAmp cloning kit (Life Technologies). A total of 1 μl of UDG cloning reaction was transformed into 100 μl of DH10B cells according to the manufacturer's recommendations (Life Technologies).

Library Analysis and Sequencing

Twelve clones were picked at random and used in 50-μl PCRs with M13f and M13r primers to amplify inserts for cDNA insert size determination. A total of 10 μl from each reaction was analyzed on 1.2% agarose gel, and product size was determined by comparison to a 100-bp ladder marker (Life Technologies). A total of 96 recombinant clones were randomly picked from each library for sequencing on an ABI3700 automatic sequencer. Individual clone sequences were compared with the sequence databases GenBank and dbEST by the BLAST program accessed through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Semiquantitative RT-PCR

Semiquantitative PCR was performed from the cDNA to evaluate the expression levels of β2-microglobulin, thyroglobulin precursor, calcitonin, oncofetal fibronectin, and HMGI(Y) among the various carcinomas. Primer sequences, product length, and annealing temperatures are listed in Table 4. Five microliters of the cDNA were diluted into 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, and 1:1000 solutions with ddH_2O. One microliter of each dilution was used as template for PCR in a total reaction volume of 30 μl, running for 30 cycles. Primer concentrations were 1 pmol/μl; $MgCl_2$ concentrations ranged from 1.5 thru 3.0 mM. For each gene a negative control using 1 μl of ddH_2O instead of a template was also run under the same conditions.

After running PCR, 15 μl of the reaction volume was loaded on a 2% agarose gel stained with ethidium bromide to visualize the PCR products. The level of expression of the various factors was assessed by estimating the differences in the decrease in the signal intensity with increasing dilutions and comparing to the total abundance of cDNA as assessed by β2-microglobulin.

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Table 4. Primer Sequences, Product Length, and Annealing Temperature Used for Quantitative RT-PCR

Gene	Primer sequence	Product length	Annealing temperature
β2-microglobulin	S 5′ATT TCC TGA ATT GCT ATG TG 3′ AS 5′GAA TTC ACT CAA TCC AAA TG 3′	287 bp	55° C
Thyroglobulin	S 5′GTT GGC AAC CTC ATC GT 3′ AS 5′AAT TCT GCA GTG CCT GGT 3′	663 bp	55° C
Calcitonin	S 5′CCT TCC TGG CTC TCA GCA TC 3′ AS 5′GAG TTT AGT TGG CAT TCT GG 3′	407 bp	60° C
Oncofetal fibronectin	S 5′TCT TCA TGG ACC AGA GAT CT 3′ AS 5′TAT GGT CTT GGC CTA TGC CT 3′	215 bp	60° C
HMGI(Y)	S 5′AAC CAC CAC AAC TCC AGG AAG G 3′ AS 5′AAA GCT GTC CAG TCC CAG AAG G 3′	164 bp	62° C

S, sense; AS, antisense.

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