Establishment of a Mini-Gene Expression Database for Bladder Tumor

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Transitional cell carcinoma (TCC) is the most common tumor of the urinary bladder, with an exceptionally high mortality rate in blackfoot disease-endemic areas of southern Taiwan [1–3]. Current understanding of the pathogenesis of bladder tumor is based on the findings of unrelated cytogenetic and molecular studies of clinical specimens and a limited number of cell lines. For example, fluorescence in situ hybridization has detected many chromosomal aberrations in bladder tumors [4–7], whereas microsatellite assay and gene expression analysis have been used to study deletions in chromosome 9 and abnormal expression of proto-oncogenes or tumor suppressor genes, respectively, that may be involved in tumorigenesis [8, 9]. However, the essential genes that are specific for tumorigenesis of TCC remain unclear. Recent functional genomic and bioinformatic research has focused on large-scale monitoring of gene expression patterns related to particular diseases, from which molecular pathogenic pathways may be elucidated.

Background and Purpose: Transitional cell carcinoma has been diagnosed mostly in urinary bladder in Southern Taiwan and has an exceptionally high mortality rate. To identify the genes associated with bladder cancer, we investigated differential gene expression. Six bladder tumor cDNA libraries were constructed and their sequences were compared and analyzed.

Methods: mRNA from bladder tumor cell lines or tissue samples were used to construct two regular cDNA libraries and four subtractive cDNA libraries after subtractive hybridization with cDNA derived from normal bladder epithelial cells. Subsequently, more than 100 cDNA inserts from each library were randomly isolated and sequenced, followed by sequence comparisons with nucleotide and protein sequence databases.

Results: After searching the Basic Local Alignment Search Tool (Blast) databases, the cDNA nucleotide sequences were grouped into novel, known, and common gene categories. Since tumor nucleotide sequences are informative and valuable for research, they were organized as a mini-gene expression database (http://bladder.nhri.org.tw). Interestingly, in one subtractive cDNA library, the ATPase 6 gene was found to be highly expressed in normal bladder epithelial cells and elevated levels of ATPase 6 mRNA were later confirmed by reverse transcription-polymerase chain reaction. However, the role of ATPase 6 in bladder tumorigenesis remains to be investigated.

Conclusions: The establishment of this database is an important step to enable systematic screening for bladder tumor-associated genes and may also be useful in developing diagnostic and/or therapeutic applications.
Construction of a cDNA library followed by sequencing of a large number of cDNA clones is a feasible and rapid approach to producing a large volume of gene expression information that can lead to the identification of relevant genes. The systematic isolation of a large volume of genes that may be involved in the tumorigenesis of bladder tumors may enable the identification of gene targets for therapeutic intervention. We have established a bladder-specific mini-gene expression database that contains nucleotide sequences derived from six bladder tumor tissue samples and cell line cDNA libraries. This research has demonstrated that expression of the mitochondrial ATPase 6 gene was suppressed in bladder tumor tissues and most bladder tumor cell lines tested, suggesting that the gene product is involved in negative control of tumor formation.

Materials and Methods

Materials, tissues, and cells
Molecular biology enzymes were mainly purchased from Stratagene (La Jolla, CA, USA) unless otherwise specified, whereas common chemicals and reagents were obtained mainly from Sigma (St. Louis, MO, USA). All reagents were used according to the manufacturers’ recommendations, if applicable.

Bladder TCC cell lines BFTC905 [10], T24 [11], and TSGH-8301 [12] and a squamous cell carcinoma cell line SCaBER [13] were all maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (tissue culture reagents from GibcoBRL, Grand Island, NY, USA) under 5% CO₂ at 37°C. Both BFTC905 and BFTC909 are TCC cell lines; BFTC909 was derived from kidney cultured under conditions described previously [10]. Tumor tissue specimens were mainly obtained from cystectomy in a 62-year-old man with grade 2/3 bladder tumor who was hospitalized in December 1998. The tissue samples from the resected bladder were handled in the Anatomical Pathology Section of the Department of Pathology of National Cheng Kung University (NCKU) Hospital. Pathologic examinations were performed to delimit the tumor areas. In addition, paired control bladder tissue specimens, morphologically normal tissue as confirmed by microscopic examination, were obtained simultaneously by the surgical removal of epithelium in areas that were far removed from tumor sites. Another bladder tumor tissue sample (TCC grade 2/3) from a patient treated in the Urology Clinics of Chi-Mei Foundation Medical Center was obtained for reverse transcription-polymerase chain reaction (RT-PCR) analysis of mitochondrial ATPase 6 gene expression. Both patients were informed about the research goals and experimental protocols and consented to all procedures.

Construction of cDNA libraries and sequence comparisons
Construction of two regular and four subtractive cDNA libraries (Table) employed many commercially available reagents with protocols recommended by the manufacturers. Cellular RNA from tissue specimens or cell lines was isolated using TRIzol reagent (GibcoBRL), and mRNA was then purified using an Oligotex™ mRNA kit (Qiagen, Valencia, CA, USA). To construct a regular cDNA library, cDNA fragments were synthesized from mRNA (5 µg) using the Great Lengths cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA), and these fragments were then inserted into EcoRI-predigested bacteriophage lambda (λ ZAPII vector, Stratagene). The recombinant λ DNA molecules were subsequently packaged into bacteriophage particles using Gigapack III gold Packaging Extract (Stratagene) for the infection of E. coli host cells XL1-blue MRF’

Table. Summary of nucleotide sequence comparisons

<table>
<thead>
<tr>
<th>cDNA library*</th>
<th>Novel gene</th>
<th>Known gene</th>
<th>Vector sequence</th>
<th>Common gene</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFTC905 Reg</td>
<td>32 (18.7)</td>
<td>76 (44.4)</td>
<td>9 (5.3)</td>
<td>54 (31.6)</td>
<td>171 (100)</td>
</tr>
<tr>
<td>BFTC905 Sub</td>
<td>35 (32.4)</td>
<td>32 (29.6)</td>
<td>33 (30.6)</td>
<td>8 (7.4)</td>
<td>108 (100)</td>
</tr>
<tr>
<td>BFTC909 Sub</td>
<td>101 (68.9)</td>
<td>3 (2.2)</td>
<td>37 (27.4)</td>
<td>2 (1.5)</td>
<td>135 (100)</td>
</tr>
<tr>
<td>Tumor Reg</td>
<td>47 (30.1)</td>
<td>33 (21.2)</td>
<td>21 (13.5)</td>
<td>55 (35.3)</td>
<td>156 (100)</td>
</tr>
<tr>
<td>Tumor Sub</td>
<td>102 (55.4)</td>
<td>57 (31.0)</td>
<td>10 (5.4)</td>
<td>15 (78.2)</td>
<td>184 (100)</td>
</tr>
<tr>
<td>Tumor Rev Sub</td>
<td>20 (18.5)</td>
<td>85 (78.7)</td>
<td>3 (2.8)</td>
<td>0 (0.0)</td>
<td>108 (100)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent percentages of cDNA clones belonging to the particular categories respective to total sequences (100%) analyzed. *Six cDNA libraries were constructed in sequential order as listed. Nucleotide sequences were compared for homology with the BlastN NR database. †Of 85 cDNA clones, 72 (84.7%) are identical to those for the mitochondrial ATPase 6 gene. Reg = regular; Sub = subtractive; Rev = reversed.
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strain. The λ ZAPII vector with cDNA inserts was converted to pBlueScript SK(−) plasmid-based constructs using a commercially available in vivo excision protocol for further characterization.

Subtractive cDNA library construction was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech) with 2 μg mRNA from BFTC905 or BFTC909 cell lines or bladder normal-tumor paired tissues. Normal bladder epithelium mRNA was converted to cDNA and served as the driver sample for subtracting from the tester cDNA samples derived from cell lines and tumors (Table). To construct the reverse subtractive cDNA library, which was mainly used to identify genes for cancer suppression, the tester sample was normal epithelial cDNA whereas the driver was bladder tumor tissue cDNA. Subtracted cDNA pools were cloned individually into either λ ZAPII vector or pPCR-Script™ Amp SK(+) vector (for details see http://bladder.nhri.org.tw) using reagents, enzymes, and protocols provided by Stratagene.

E. coli clones randomly picked from all six tumor cDNA libraries were subjected to mini-plasmid minipreparations[14], followed by automated sequencing analysis (ABI Prism 377 DNA Sequencer, Perkin Elmer; Foster City, CA, USA). Nucleotide sequences obtained from these experiments were subjected to sequence homology searches against the Basic Local Alignment Search Tool (Blast)X NR, BlastN NR, and BlastN EST databases.

RT-PCR confirmation of gene expression
To quantify mRNA expression of the mitochondrial ATPase 6 gene, RT-PCR was employed with total cellular RNA isolated from bladder tumor tissue, a normal-tumor tissue pair, and four bladder tumor cell lines. RNA (1 μg) isolated as described above was treated with 1 U RQ1 DNase (Promega, Madison, WI, USA). Reverse transcription was then performed using SuperScript™ II reverse transcriptase and random hexamer primers according to the protocol provided by GibcoBRL. One-twentieth of the synthesized cDNA was then subjected to PCR with primers specific for the ATPase 6 gene [15] (sense primer: 5′-ATCATGTTTGAGACC-TTCAA-3′; anti-sense primer: 5′-TATGCACAGGTT-GGAACAAGAT-3′) in a PCR system (GeneAmp® PCR System 9600, Perkin Elmer) programmed with a regular thermocycling program. The DNA products, which were taken from PCR reactions after 15, 17, 19, and 21 cycles, were visualized after 2% agarose gel electrophoresis. This allowed the exponential stage of PCR amplification, in terms of the number of thermocycles, to be determined. The amount of mRNA from genes in cells was positively proportional to the amount of RT-PCR products. In parallel, the β-actin gene (sense primer: 5′-ATCATGTTTGAGACC-TTCAA-3′; anti-sense primer: 5′-TATGCACAGGTT-GGAACAAGAT-3′) was amplified under identical conditions to normalize the amount of RNA used in all PCR experiments. The Figure shows the results of experiments employing 17 and 21 thermocycles for the amplification of the ATPase 6 and β-actin gene fragments, respectively.

Results

Construction of bladder cDNA libraries
Over a 2-year period, our laboratories have constructed seven regular and subtractive cDNA libraries using mRNA from bladder tumor tissues and cell lines. Among

Figure. Expression of the mitochondrial ATPase 6 gene in bladder tissues and cell lines. Genespecific primers for ATPase 6 or β-actin were used in the reverse transcription-polymerase chain reaction (RT-PCR) with 0.05 μg cellular RNA isolated from two peripheral blood mononuclear cell (PBMC) samples, two bladder tumor tissues, one normal bladder epithelium, and four bladder tumor cell lines. Tumor 1 and Normal represent RNA samples isolated from a single resected bladder organ and are indicated by the designation pair. In order to compare the amount of PCR products synthesized in the exponentially increasing ranges, 17 and 21 thermocycles were chosen to amplify the ATPase 6 and β-actin gene fragments, respectively. Under these experimental conditions, RT-PCR produced relatively equal amounts of β-actin DNA fragments in all RNA samples, whereas only Normal and TSGH-8301 samples yielded increased amounts of the ATPase 6 gene fragment. The 100-bp molecular-ladder is indicated at the right edge of the figure with the arrows pointing to the 500-bp bands.

- RT = no reverse transcriptase added during RT-PCR reactions to confirm that RNA preparations were free of genomic or mitochondrial DNA. + = RT-PCR positive control; − = RT-PCR negative control.
these, six cDNA libraries were further subjected to nucleotide sequence analysis in order to obtain gene sequence information. At least 100 cDNA clones from each library were analyzed. The nucleotide sequences for the cDNA clones were then subjected to sequence homology searches against the BlastN NR, BlastN EST, and BlastX NR databases. The Table categorizes the nucleotide sequences from all cDNA libraries into novel (unreported or expression sequence tag, EST), known (already characterized), vector (cloning vector), and common gene (house keeping/ multi-copy genes) groups. From homology searches, we observed that the percentage of cDNA in the common gene category was smaller in subtractive libraries than in regular libraries, indicating that the procedure of deleting common transcripts in the tester mRNA pools during the construction of subtractive cDNA libraries was effective (Table). The reverse was also true that, in general, percentages of novel sequences were increased in the subtractive cDNA libraries. Since we were also interested in identifying genes that are associated with blackfoot disease, mRNA from a kidney TCC cell line BFTC909, which was derived from a blackfoot disease patient [10], was used to construct cDNA libraries (BFTC909 Sub and another unpublished library). The most striking finding came from the sequencing data of Tumor Rev Sub in which 72 of 85 (84.7%) cDNAs in the known gene category coded for the mitochondrial ATPase 6 gene, a subunit of the F1F0 ATP synthase complex. The abundance of this gene fragment in the library may reflect greater expression of the gene in the normal bladder than in the paired tumor tissue, or be due to an artifact of nonspecific enrichment of cDNA arising during subtractive library construction.

Characterization of ATPase 6 expression

To investigate the reason for the frequent detection of the mitochondrial ATPase 6 gene in the reversed subtractive cDNA library, we employed RT-PCR to analyze the expression levels of the gene in a variety of tissues and cell lines. As shown in the Figure, a stronger PCR signal for the ATPase 6 gene was detected in mRNA isolated from normal bladder epithelial tissue, which was used to construct the reverse subtractive cDNA library, as compared to its paired tumor tissue and another sample from a tumor of the same grade. Furthermore, three (BFTC905, ScABER, T24) of the four bladder tumor cell lines examined, including transitional and squamous cell carcinoma, were found to express less ATPase 6 mRNA. This suggests that the ATPase 6 gene was transcribed more in normal bladder epithelial cells than in tumor tissues and most of the cell lines, suggesting that this gene may be involved in the negative regulation of tumor formation. However, probably due to the decreased expression in primary cells, no diagnostic ATPase 6 DNA fragment was detected in two PCR control experiments with mRNA isolated from peripheral blood mononuclear cells under these amplification conditions (Figure), while it could be readily detected after 25 cycles of PCR (data not shown). In these experiments, the β-actin gene served as a control target for RT-PCR normalization of the amounts of all RNA samples used. In parallel, reverse transcriptase was purposely left out during PCR amplifications of both ATPase 6 and β-actin genes to detect any contamination by mitochondrial or genomic DNA, respectively, in RNA preparations. As shown in the Figure, no such contamination was found.

Database establishment

A total of 862 cDNA sequences obtained in this research were organized as a mini-bladder tumor gene expression database, the first nucleotide sequence database specific for human bladder tumor. Since the revealed cDNA nucleotide sequences may be informative and valuable for bladder tumor research, the database is now posted in the public domain (http://bladder.nhri.org.tw/) and is supervised by the National Health Research Institutes of Taiwan for all interested scientists.

Discussion

This paper summarizes the findings of a 3-year effort to construct cDNA libraries and to set up a mini-gene expression database for bladder tumor. We performed more than 850 random nucleotide sequence determinations and BLAST database searches, as well as information sorting and organization to set up the bladder mini-gene expression database. The database allows scientists worldwide who are interested in conducting research related to bladder cancer to search for sequence homology electronically, a procedure called electronic Northern. We believe that the establishment of this database is an important first step towards systematic screening for bladder tumor-associated genes. Identification of these genes may be useful in developing diagnostic and/or therapeutic applications. From one of the subtractive cDNA libraries, we further found that the expression of mitochondrial ATPase 6 gene was upregulated in normal bladder epithelium compared to that in tumor tissues and most of the cell lines tested.

In order to successfully find the genes of interest from cDNA library screening, it is essential to have good-quality starting materials. Therefore, we carefully examined the quality of every cDNA library we constructed in this study. By judging the recombinant rate
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