Over-expressed Genes Detected by Suppression Subtractive Hybridization in Carcinoma Derived From Transformed 16HBE Cells Induced by BPDE[1]

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Objective To screen the over differentially expressed genes in carcinoma induced by BPDE-transformed 16HBE cells (16HBE-C cells). **Methods** The suppression subtractive hybridization (SSH) method was performed to profile differentially expressed genes between 16HBE-C cells and 16HBE cells. The cDNA fragments of differentially expressed genes were inserted into TA cloning vector and transformed competent *E. coli* strain. Positive clones were randomly picked up and identified by the colony PCR method. Dot blot was used to test the same source with the tester. The differentially expressed cDNA fragments were sequenced and compared with known genes and EST database in Genbank. **Results** Eight known genes were over-expressed in 16HBE-C cells including eukaryotic translation elongation factor 1 alpha 1, HIF-1 responsive RTP801, ribosomal protein L10 (RPL10), ribosomal protein S29 (RPS29), mitochondrion related genes, and laminin receptor 1. Three differentially expressed cDNA fragments could not be matched to the known genes but to the EST database. **Conclusion** The SSH method can detect differentially expressed genes between 16HBE-C and 16HBE cells. BPDE-induced carcinogenesis may be related to alteration of at least eight known genes and three unknown genes. These expression data provide a clue to further cloning novel genes and studying functions in BPDE-induced carcinoma.

Key words: Anti-BPDE; Hybridization; Gene

INTRODUCTION

Benzo[a]pyrene (B[a]P) is a ubiquitous environment pollutant found in the air, soil, water, plants, food, and causes many adverse health effects, including immunosuppression, carcinogenesis, and endothelial cell damage. B[a]P is formed during pyrolisis and incomplete combustion of organic materials and undergoes metabolic activation after entering mammalian cells to highly toxic reactive metabolite intermediates, leading to irreversible damage to cellular macromolecules (DNA, proteins, lipids). Anti-benzo[a]pyrene-7, 8-diol-9, 10-epoxide (anti-BPDE) is the major metabolite of B[a]P and the formation of anti-BPDE–DNA adducts plays a critical role in the carcinogenic process of B[a]P[1]. Although the ultimate carcinogen anti-BPDE reacts with nuclear DNA and is mutagenic[2], the mechanism underlying anti-BPDE-induced carcinogenesis is not fully understood. For a better understanding of cellular behavior, the identification of differentially expressed genes in a certain type of cells is of a great research interest.

There are some commonly used PCR-based methods for screening differentially expressed genes between two different types of cells. Representative difference analysis (RDA), a process of subtraction coupled to amplification, is initially developed by Lisitsyn *et al.*[3] to identify differences between complex genomes. Subsequently, RDA protocols are adapted to the examination of differential gene expression between two mRNA populations[4]. Diatchenko *et al.*[5] have developed a new PCR-based cDNA subtraction method, suppression subtractive hybridization (SSH), which is essentially similar to RDA but is based on the suppression PCR effect. The SSH has overcome the problem of mRNA abundance by incorporating a hybridization step that normalizes

sequence abundance during the course of subtraction by the standard hybridization kinetics. It was also reported that the SSH method can detect differentially expressed mRNAs with a low prevalence[6-7]. The aim of the present study was to screen the differentially expressed genes in carcinoma derived from transformed 16HBE cells induced by BPDE.

MATERIALS AND METHODS

Cell Culture

Human bronchial epithelial cell (16HBE cells) and carcinoma induced by BPDE-transformed 16HBE cells (16HBE-C cells) were prepared as previously described[8]. Briefly, the16HBE cells were treated 4 times by anti-BPDE at 2.0 µmol/L and the transformed foci were observed and assessed at different stages during the whole experiment. The 16HBE-C cells were from the tumor derived from transformed 16HBE cells induced by anti-BPDE. Both cell lines were cultured in 25 cm^2 flasks with 5% carbon dioxide and 100% humidity at 37℃. The cells were cultured in MEM medium supplemented with 10% calf serum.

Isolation of Total RNA and Poly(A)$^+$ mRNA

Trizol (Gibco BRL, Carlsbad, CA) was used to extract the total RNA from cells, and the Oligotex direct mRNA kit (QIAGEN Company, Hilden, Germany) was used to isolate the poly(A)$^+$ mRNA. The procedures followed the manufacturer's instructions. The integrity of total RNA and mRNA was examined by electrophoresis on a 1% agarose gel, and the quantifty and purity were analyzed by A260 and A260/280 (Eppendorf BioPhotometer, Germany), respectively.

SSH

SSH was performed with 16HBE-C and 16HBE cells using the PCR-Select^{TM} cDNA subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The cDNA of 16HBE cells was used as the driver and 16HBE-C cells as the tester. The first-strand cDNA was synthesized from 2 µg of poly (A) RNA by oligo (dT)$_{18}$ priming using AMV reverse transcriptase, and the second-strand cDNA was generated with T4 DNA polymerase. The restriction endonulease RsaI was used to obtain shorter and blunt-ended fragments. The tester population was created with different adaptors, but no adaptors for the driver. The adaptors were used for subsequent PCR amplification. The second hybridization generated templates for the PCR

amplification from differentially expressed sequences between the tester and the driver, for which PCR was performed twice.

The first PCR was performed at 94℃ for 10 seconds, at 66℃ for 30 seconds and at 72℃ for 2 minutes using Watman Biometra TGRADIENT PCR System. One microliter of diluted primary PCR product was used for secondary PCR, in which the test was performed at 94℃ for 10 seconds, at 68℃ for 30 seconds and at 72℃ for 1.5 minutes.

Construction of Subtractive Library

The second PCR products were inserted into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and transferred into one shot TOP10 chemically competent E.coli cells (Invitrogen, Carlsbad, CA). The transformed bacteria were plated onto LB agar plates containing ampicillin followed by overnight incubation at 37℃. The cDNA inserts of positive clones were picked up randomly and amplified under the PCR conditions with T3/T7primer in the vector. Each subtracted clone was used as a PCR template. Most of the cDNA inserts analyzed with PCR were sequenced. All the sequencing was performed commercially in the Bioasia Company (Shanghai, China) using an automatic fluorescent DNA sequencer (ABI PRISM100, Model 377, USA).

Dot Blot Analysis

Dot blot hybridization was performed to confirm that the subtraction products were from the tester. The PCR products in the analysis of positive clones were prepared for the dot blot hybridization. The cDNA of 16HBE-C cells was used as the probe, labeled with DIG-high prime (Roche company, Penzberg, Germany).

BLAST With Genbank

The program BLAST was used to compare the sequences of isolated clones with all sequences in the National Center for Biotechnology Information (NCBI) nucleic acid database or EST database.

RESULTS

Identification of Total RNA and Poly(A)$^+$ mRNA Quality

Total RNA exhibited two bright bands, which corresponded to 28S and 18S RNA, respectively, with a ratio of intensities of about 1.5-2.5:1 (Fig. 1). Poly (A)$^+$ mRNA appeared as a smear with weak ribosomal RNA bands (Fig. 1). The ratio of A260/

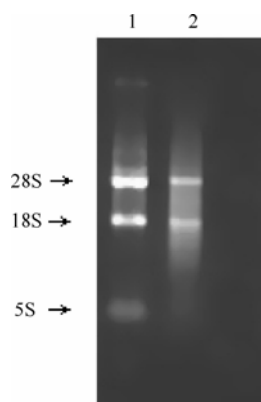280 of total RNA and mRNA was greater than 2.1.

FIG. 1. Electrophoresis of total RNA and mRNA on a 1% agrose gel. Lane 1: total RNA from normal 16HBE cells. Lane 2: poly (A)$^+$ mRNA from normal 16HBE cells.

SSH

The second hybridization products were amplified by the outside primers. The products presented unclear smear on 2% agarose/EB gel. After the second PCR with the inside primers, several bands could be seen clearly on the smears. Based on the manual instructions, the experiment was successful (Fig. 2).

Identification of Inserted Fragments in Library Plasmids by PCR

PCR products of library plasmids amplified with T3 and T7 primers on a larger scale showed that each plasmid included one inserted fragment ranging from 200-1000 bp (Fig. 3). Most of the up-regulated genes in carcinoma 16HBE cells were confirmed by the same source with the tester by the dot blot.

DNA Sequencing and Analysis

All differentially expressed clones were subseq-

uently analyzed by DNA sequencing and BLAST search. Of the clones, 8 sequences matched to 8 known genes with over 99% identity (Table 1). Three differentially expressed cDNA fragments could not match to the known genes but to the EST database.
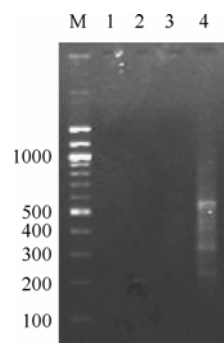

FIG. 2. Results of SSH. M: 100 bp DNA ladder; lane 1: first PCR products from unsubtracted cDNA population; lane 2: secondary PCR products from unsubtracted cDNA population; lane 3: first PCR products from subtracted cDNA population; lane 4: secondary PCR products from subtracted cDNA population.
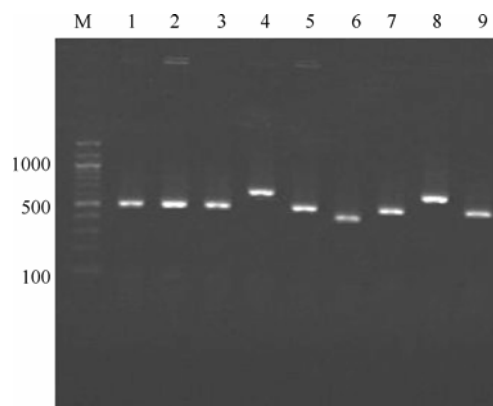

FIG. 3. Identification of inserted fragments by PCR using T3/T7 primers of vector. M: 100 bp DNA ladder. Lanes 1-9: PCR products from cDNA inserted fragments.

TABLE 1

Results of Sequence and Blast Search

Clon No.	Length of Inserted Fragments	Isogenous Gene	Identity
14	343	HIF-1 Responsive RTP801	41 (99%)
A2	370	Ribosomal Protein L10 (RPL10)	370 (100%)
A21	119	Ribosomal Protein S29 (RPS29)	118 (99%)
A24	353	Mitochondrion	353 (100%)
A28	310	Mitochondrion	309 (99%)
A37	405	Laminin Receptor 1	404 (99%)
A38	346	Mitochondrion	345 (99%)
A41	418	Translation Elongation Factor 1 Alpha 1	418 (100%)

DISCUSSION

B[a]P has been used as a prototype of carcino-genic polycyclic aromatic hydrocarbon since its isolation from coal tar in the 1930s. One of its diol epoxides, anti-BPDE, is considered as an ultimate carcinogen on the basis of its ability to bind to DNA, mutagenicity, and extreme pulmonary carcino-genicity[2]. However, the molecular basis of its carcin-ogenesis is not clear.

Differentially expressed genes between the corresponding normal and cancer tissues can help us understand the molecular basis of malignancy and potentially serve as biomarkers or prognostic markers of malignancy. The identification and character-rization of human genes expressed exclusively or preferentially in the cells of tumor will hopefully shed light on the mechanisms of tumor development and provide useful genetic markers for screening, diagnosis, prognosis, therapeutic monitoring, and development of therapeutic vaccines. The SSH method allows identification of both over-expressed genes (designated forward -SSH) and under-expressed genes (designated reverse -SSH) by exchanging the driver and tester populations during the procedure (Clontech, Palo Alto, USA). Since this technique was established by Diatchenko, many new genes have been separated from almost all kinds of tissues, such as renal cell cancer, lung cancer, and liver cancer[9-16].

Our results revealed a number of both novel and known genes that responded to the treatment with anti-BPDE. One of these genes is eukaryotic translation elongation factor 1 (eEF1). The eEF1 complex plays a central role in protein synthesis, delivering aminoacyl-tRNAs to the elongating ribosome. During protein synthesis, eEF1A forms a ternary complex with aminoacylated tRNA and GTP (eEF1A-GTP-aatRNA), and delivers aminoacylated RNA to the ribosome after GTP hydrolysis. Inactive eEF1A-GDP and deacylated tRNA are released from the ribosome and must be recycled[17]. The eEF1A subunit, a classic G-protein, also performs a role aside from protein synthesis[18]. It has been reported that constitutive expression of eEF1A causes fibroblasts to become highly susceptible to transformation[19]. In addition, a truncated form of eEF1A, encoded by the PTI-1 gene, has been identified in prostate cancers, thus inhibiting PTI-1 expression with PTI-1 antisense can result in the suppression of tumorigenic potential of the PTI-1 gene[17].

Ribosomal proteins are a major component of ribosome and play a critical role in protein biosynthesis. Recently it has been shown that ribosomal proteins also function during various cellular processes that are independent of protein biosynthesis, therefore having so called extra-ribosomal functions[20]. In the present study, two ribosomal proteins, RPL10 and RPS29, were up-regulated in the 16HBE-C cells, suggesting that RPL10 and RPS29 may be involved in the carcinogenesis induced by BPDE.

Laminin receptor 1 (67-kDa), another up-regu-lated gene in the 16HBE-C cells, is frequently detected in both carcinoma and stromal cells in solid tumors[21]. This might explain the abundant clinical and experimental data suggesting a key role of the 67-kDa laminin receptor in the interaction between cancer cells and basement membrane glycoprotein laminin during tumor invasion and metastasis[22]. Several studies investigating this receptor's role in tumor progression have clearly demonstrated that 67LR expression increases in tumors compared with that in normal tissues and there is a correlation between 67LR expression, invasive phenotype of the tumor, and poor prognosis[22].

HIF-1 responsive RTP801, a novel hypoxia-inducible factor 1(HIF-1) gene, has a complex type of involvement in the pathogenesis of ischemic diseases[23]. Whether this gene plays a role in the carcinogenesis warrants further investigations.

In the postgenome era, the identification of genes expressed under various conditions or phenotypes will become a challenge. In summary, we could expect that gene expression profiling identified by the SSH method will help decipher cell-specific or low-abundance and yet unidentified genes, but further *in vivo* studies are still necessary to elucidate the biological functions of these differentially expressed genes.

In conclusion, the expression of a number of novel and known genes, is regulated by BPDE in cells at the mRNA level, and this type of study may provide the basis and rationale for further cloning novel genes. These screened genes are only the primary results, further studies are still needed to elucidate their functions and relations among them.

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