Characterization of 67 kD Laminin Receptor, a Protein Whose Gene Is Overexpressed on Treatment of Cells with Anti-Benzo[a]pyrene-7,8-Diol-9,10-Epoxide

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The molecular mechanisms potentially related to tumorigenesis induced by anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (anti-BPDE) were investigated by suppression subtractive hybridization of the human bronchial epithelial cells (16HBE) carcinoma induced by BPDE-transformed 16HBE cells (16HBE-C). The 67 kD laminin receptor gene (67LR1) is one of the screened overexpressed genes in 16HBE-C cells when compared with 16HBE. In order to understand the main functions of 67LR1 gene, we amplified the full length of 67LR1 gene using reverse transcription-polymerase chain reaction (RT-PCR) method. The amplified gene products were inserted into pcDNA™ 3.1 Directional TOPO expression vector. We then transfected 16HBE cells with this vector and derived stable transfected 16HBE cell lines containing the 67LR1 gene by using lipofectin and G418 selection protocols. The expression products of transfected genes were analyzed by semi-quantitative RT-PCR. Soft agar growth assay was carried out to identify the malignant features of 67LR1 gene. The stable transfected cell lines can form colonies in soft agar. Further, the transfected cells showed morphological changes compared to the control cells, such as the obvious pseudopods. These data suggest that the 67LR1 gene may be related to malignant transformation induced by the anti-BPDE. The 67LR1 protein may be related to the directionality of cell movement.

Key Words: anti-BPDE; laminin receptor; gene; carcinogenesis; pseudopod.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. They are found in the air, soil, water, and plants and also in food (Cavret et al., 2005; Kulhanek et al., 2005; Skrbic et al., 2005). PAHs represent a class of toxicological compounds, which can create a variety of hazardous effects in vivo, including cytotoxicity, genotoxicity, immunotoxicity, teratogenicity, carcinogenesis, and neurotoxicity (Abbott, 1995; Kerkvliet, 1995; Manz et al., 1991; Puga et al., 2002; Tu et al., 2004). Recently, especially in Western countries, there is increasing research interest in the health effects of PAHs, because they may enter the food chain through different sources such as the pollution of air, underground water, and soil (Cavret and Feidt, 2005; Houessou et al., 2005; Krishnamurthi et al., 2003; Lemiere et al., 2005).

Benzo[a]pyrene (B[a]P) is a representative member of PAH family, and it undergoes metabolic activation after entering the mammalian cells to highly toxic reactive metabolite intermediates, which can irreversibly damage cellular macromolecules (i.e., DNA, proteins, and lipids) (Chen et al., 2000; Pavanello et al., 1999; Rubin, 2001). Anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (anti-BPDE) is one of the many metabolites of B[a]P, and the formation of anti-BPDE–DNA adducts is considered to be critical in the carcinogenic process of B[a]P (Chen et al., 2000; Pavanello et al., 1999). Although the ultimate carcinogenic anti-BPDE reacts with nuclear DNA and becomes mutagenic (Rubin, 2001), the mechanisms for anti-BPDE-induced carcinogenesis are not fully understood. For a better understanding of cellular behaviors, the identification of genes differentially expressed in a certain type of cells exposed to carcinogens has been of great research interest. This study is carried out to investigate the differentially expressed genes and their main characteristics induced by anti-BPDE.

In our prior papers, we successfully established the malignant transformation model of human bronchial epithelial (16HBE) cells induced by anti-BPDE (Jiang et al., 2001). The 16HBE cell line was a gift kindly from Prof. Jun Xu (Guangzhou Institute of Respiratory Disease, Guangzhou, China). The 16HBE cells were treated once or several times by anti-BPDE at different doses, and the foci were observed and assessed at the different stages during the period of the whole experiment. The features of malignancy of the transformed 16HBE cells were identified by the test of soft agar culture. The results showed that the best method for malignancy transformation of 16HBE cells induced by anti-BPDE was that the 16HBE cells were treated several times with anti-BPDE at...
ThermalAce™ DNA polymerase reaction was used to obtain the full-length GAACAGCTTAAGAC-3. The specific sequences of the primers are as follows: the forward primer, 5'-GATTTC-3', the reverse primer, 5'-GATTACCGGCGCCCCAGGCACCA-3'. The length of 67LR1 and b-actin were 398 bp and 539 bp, respectively. These results were normalized using b-actin as the internal control, the gene specific primers of b-actin were 5'-GTTGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAAATGTACCGCAAG-3'. The length of 67LR1 and b-actin were 398 bp and 539 bp, respectively. The conditions of PCR were 20 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s, and extension at 72°C for 1 min.

**Soft agar growth assay of the transformed cells.** The ability of cell lines to grow in soft agar was measured by using the 16HBE cell line transfected with the 67LR1 gene. The 16HBE and 16HBE cell lines with transfected vector were used as negative controls. The 16HBE cell line with the transfected gene encoding the eukaryotic translation elongation factor 1 (eTEF1α) was used as a positive control. eTEF1α gene is another differentially expressed genes in

**MATERIALS AND METHODS**

**Cell culture, isolation of total RNA, and mRNA suppression subtractive hybridization.** The human bronchial epithelial cells (16HBE cells), a carcinoma cell line derived from transformed 16HBE cells (16HBE-C cells) induced by the anti-BPDE and suppression subtractive hybridization (SSH), were established in our laboratory as previously reported (An et al., 2005; Jiang et al., 2001). Briefly, The 16HBE cells were treated four times with anti-BPDE at a concentration of 2.0 μmol/l and passaged 15 times. Then the malignant features of the cells were assessed by soft agar culture and tumorigenesis in nude mice. The 16HBE-C cells come from the carcinoma derived from transformed 16HBE cells induced by anti-BPDE. Both of the cell lines were cultured in 25-cm² flasks with 5% carbon dioxide and 100% humidity at 37°C. The cells were grown in the MEM medium supplemented with 10% calf serum. 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)⁺ RNA. The integrity of total RNA and mRNA were examined by electrophoreses of the samples on a 1% agarose gel, and the quantification and purity were analyzed by A260 and A260/280 (Eppendorf BioPhotometer, Germany), respectively. Suppression subtractive hybridization (SSH) was performed with 16HBE-C and 16HBE cells using the PCR-Select™ cDNA Subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer’s recommendations. The cDNA of 16HBE cells was used as the driver and 16HBE-C cells as the tester. After two times of subtractive hybridization and two times of PCR, the products of second nested PCR were inserted into TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and transferred into One Shot TOP10 Chemically Competent E. coli cells (Invitrogen, Carlsbad, CA). All of the positive clones were selected, sequenced, and compared with all sequences available in the National Center for Biotechnology Information (NCBI) nucleic acid database or EST database.

**Full-length amplification of 67LR1 gene.** Based on the screened nucleotide sequence from the suppression subtractive hybridization, the full-length sequence of Laminin receptor 1 (67 kDa) (67LR1) gene was downloaded from GenBank. In order to match the overhang (GTGG) in the cloning vector pDNA™ 3.1 Directional TOPO, the nucleotide sequence 5’-CACC-3’ was added to the forward primer, and the stop codon was removed. The gene-specific sequences of the primers are as follows: the forward primer, 5’-CACCATGTCGGCAGCCCTTGATG-3’; the reverse primer, 5’-TGGCAGAAACAGCTTAAGAC-3’. The product length of the amplification is 904 bp. ThermalAce™ DNA polymerase reaction was used to obtain the full-length gene. In order to get more purified products, gel purification of the PCR products was carried out according to the instructions of the manual (NucleoTrap Gel Extraction Kit, Clontech, Palo Alto, CA).

**Construction of the expression vector and transfection of 16HBE cells.** The products of the gel purification of the gene were inserted into expression vector pcDNA™ 3.1 Directional TOPO (Invitrogen, Carlsbad, CA) and transformed into One Shot TOP10 chemically Competent Cells. Five colonies were randomly picked up and verified by PCR and DNA sequence analysis. Plasmid DNA of the positive colonies prepared using the QIAGEN Plasmid Purification Kit (QIAGEN Company, Hilden, Germany) was used to transfect 16HBE cells by the lipofectin transfection procedure. Stable transfected cell lines were selected using G418 (200 μg/ml). Overexpression of 67LR1 gene was determined by semiquantitative RT-PCR. The sequence of the gene specific primers used were 5’-AAACCCTGTGATGTGCGA-3’ and 5’-ACAGATCGGACGATGACCTC-3’. These results were normalized using b-actin as the internal control, the gene specific primers of b-actin were 5’-GTTGGGCGCCCCAGGCACCA-3’ and 5’-CTCCTTAAATGTACCGCAAGCCATGGGGAC-3’. The length of 67LR1 and b-actin were 398 bp and 539 bp, respectively. The conditions of PCR were 20 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s, and extension at 72°C for 1 min.

**FIG. 1.** The full-length amplification of 67LR1 gene from the RNA of 16HBE-C cells. M: DNA marker; 1 and 2: the results of 67LR1 gene (904 bp) amplified from the RNA of 16HBE-C cells.
16HBE-C cells. Cells were seeded in 60-mm diameter dishes (1000 cells/dish) in 0.35% agar in complete medium on a base of 0.6% agar. There were five parallel culture dishes in every group. Colonies were counted 14 days later by microscope and by eyes, respectively.

RESULTS

Identification of Total RNA and Poly (A)⁺ mRNA Quality and the Results of SSH

Total RNA exhibited two bright bands, which correspond to 28S and 18S RNA, respectively, with a ratio of intensities of about 1.5–2.5:1. Poly (A)⁺ mRNA appears as a smear with weak ribosomal RNA bands (An et al., 2005). The ratio of A260/280 of total RNA and mRNA was greater than 2.1. The results of SSH revealed that 67LR1 gene is one of the overexpressed genes in 16HBE-C cells compared with 16HBE (An et al., 2005).

The Full-Length Amplification of 67LR1 Gene, Construction of the Expression Vector, and Transfection of 16HBE Cells

The full-length 67LR1 gene (904 bp) was successfully amplified from the RNA of 16HBE-C cells (Fig. 1), and the amplified gene was inserted into the pcDNA™ 3.1 Directional TOPO vector. The positive transformants were analyzed by PCR, using the T7 and BGH priming site in the vector, and were further confirmed by the sequencing analysis of the transcription initiation region in the gene (Fig. 2). Semi-quantitative RT-PCR analysis of gene expression showed that the gene is overexpressed in 67LR1-transfected 16HBE cells compared with the controls (Fig. 3). Furthermore, the overexpression of the gene resulted in morphological changes comparing to the control (Figs. 4A and 4B), such as the obvious pseudopod.

Results of Soft Agar Growth Assay

In the soft agar experiments, we first did one primary experiment in a small number of samples. The results in the experiment showed differences from each other in the different groups. Then we increased the number of replicates in order to avoid the experiment bias and did the experiments. The 67LR1-transfected 16HBE cells showed anchorage-independent growth in semisolid medium (Fig. 4D) and also formed pseudopod in soft agar compared with 16HBE (Fig. 4A) and transfected eTEF1α gene control (Fig. 4C). The average
results ± SD of the experiments are reported in Table 1. Statistical analysis revealed a significant increase ($p < 0.001$) in 67LR1-transfected 16HBE cells compared with the two negative control groups.

**DISCUSSION**

The identification and characterization of genes that are differentially expressed during carcinogenesis or exposure to carcinogens provide important information with regard to understanding of the mechanisms responsible for malignant transformation (Joseph et al., 2002). In the current investigation, we have used the 16HBE transformation system and SSH analysis of gene expression to understand the molecular mechanisms of carcinogenesis induced by anti-BPDE.

Analysis of differentially expressed genes by SSH technique demonstrated overexpression of the 67LR1 gene in 16HBE-C cells compared with 16HBE cells. The 67LR1 gene was reported to be frequently detected in both carcinoma and stromal cells in solid tumors (Givant-Horwitz et al., 2003). This might explain abundant clinical and experimental data suggesting a key role for the 67LR1 gene in the interaction between cancer cells and the basement membrane glycoprotein during tumor invasion and metastasis (Magnifico et al., 1996). Several studies have clearly demonstrated an increase in 67LR1 expression in tumors compared with normal tissues and a correlation between 67LR1 expression, invasive phenotype of the tumor, and poor prognosis (Magnifico et al., 1999). But the main characterization of the 67LR1 gene is not very clear.

Both 16HBE cells transfeected with the 67LR1 gene and colonies formed in soft agar from such cells showed morphological changes compared to the control 16HBE cells. One of these changes is the presence of pseudopods. Stabilization of

**FIG. 3.** Transfection-mediated overexpression of 67LR1 gene in 16HBE cells. M: DNA marker. 1, 2, and 3 are the results of semiquantitative PCR of 16HBE group, 16HBE + vector group, and 16HBE + 67LR1 group, respectively.

**FIG. 4.** (A) The human bronchial epithelial cells ($\times 250$). (B) 16HBE cells transformed with 67LR1 gene. Arrows demonstrate the pseudopods. (C) Colony formed in 16HBE + eTEF1α1 gene group in semisolid agar. The colony did not show morphological changes (such as pseudopods). (D) Colony formed in 16HBE + 67LR1 gene group in semisolid agar. Arrow demonstrates the pseudopods.
these pseudopods determines the directionality of cell movement (Nabi, 1999) and may be related to cell adhesive ability and cell spreading ability (Zhao et al., 2005). Our results suggest that overexpression of 67LR1 gene could be a biomarker of malignant transformation. Overexpression of the 67LR1 gene could also predict tumor metastasis. Further work needs to be done to substantiate this hypothesis. The significance of increased message expression of 67LR1 gene awaits confirmation of the gene product at the protein level.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


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**TABLE 1**

The Frequency of Colony Formation in Semisolid Agar

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of parallel culture dishes</th>
<th>Number of colony formation X ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>16HBE</td>
<td>5</td>
<td>1.33 ± 0.516</td>
</tr>
<tr>
<td>16HBE + vector</td>
<td>5</td>
<td>1.57 ± 0.535</td>
</tr>
<tr>
<td>16HBE + 67LR1 gene</td>
<td>5</td>
<td>20.98 ± 1.966*</td>
</tr>
</tbody>
</table>

*Compared with 16HBE or 16HBE + vector, p < 0.001.