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Full Length Research Paper

Construction and characterization of a cDNA library from human brain glioma cell line U251 with overexpressed exogenous p53 gene

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The tumor-suppressor gene p53 and its downstream genes consist of a complicated gene network, and the challenge to understand the network is to identify p53 downstream genes. In order to isolate and identify new p53 regulated genes, we constructed and characterized a normalized cDNA library from human brain glioma cell line U251 while exogenous p53 gene is overexpressed. The constructed cDNA library contained 1.3×10^6 directional recombinants, and its insert size ranged from 0.5 to 2.0 kb. Screening the cDNA library, we obtained two novel p53 downstream genes, PAP1 and PAP2. Polymerase chain reaction (PCR) analyses of the library for specific genes revealed the presence of cDNAs for p53 downstream genes such as p21, gadd45, and PCNA. These results demonstrate the sequence complexity and relatively low redundancy of our cDNA library. It is a valuable and unique resource for studying p53 gene expression, regulatory mechanisms and screening p53 downstream genes.

Key words: p53 Gene, p53 downstream gene, cDNA library, normalization.

INTRODUCTION

The tumor suppressor protein p53 is a nuclear phosphoprotein that has a key role in cellular response to several stress signals, such as DNA damage, hypoxia, viral infection, or oncogenic activation (Vousden and Lu, 2002; Shu et al., 2007; Vogelstein et al., 2000; Perwez and Harris, 2006; Horn and Vousden, 2007). p53 levels rapidly increase in response to these stressors, while they are kept low in unstressed cells (Halaby and Yang, 2007). p53 is a transcription factor, which exerts its function mainly through binding to a specific DNA responsive element (el-Deiry et al., 1992; Bourdon et al., 1997; el-Deiry, 1998). When the p53 accumulate and become activated, it can accomplish their biological functions by activating or repressing a large number of different p53 downstream genes. The alteration of p53 downstream gene expression levels is therefore a key component of the p53 response to stress, and understanding of the mechanisms leading to the alteration of p53 downstream gene expression in the cell can contribute to the understanding of p53 biological functions.

Some of the downstream genes identified so far include the muscle creatine kinase (MCK) gene, coding for the murine double minute 2 (MDM2) protein which negatively regulates p53 function; the proliferating cell nuclear antigen (PCNA) gene, coding for the PCNA which is implicated in DNA replication and nucleotide excision

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Abbreviations: LDPCR, Long distance polymerase chain reaction; **MCK,** muscle creatine kinase; **MDM2,** murine double minute 2; **PCNA,** proliferating cell nuclear antigen; **PGM,** phosphoglycerate mutase; **TIGAR,** TP53-induced glycolysis and apoptosis regulator; **SCO2,** synthesis of cytochrome c oxidase 2; **TRE,** tetracycline-response element; **Dox,** doxycycline; **FBS,** fetal bovine serum; **PBS,** phosphate buffered saline; **DSN,** duplex-specific nuclease; **ds,** double stranded; **PCR,** polymerase chain reaction.

repair *in vitro*; the GADD45 gene which codes for the protein binding to PCNA; the WAF1-CIP1 gene which codes for a 21 kDa protein that inhibits the kinase activity of the cdk2–cyclin complex required for the G1 to S phase transition in the cell cycle; the IGF-BP3 gene which codes for the binding protein of IGF1, a negative regulator of cell proliferation; the Bcl-2-associated X (BAX) gene whose product is involved in the regulation of apoptosis; and the gene coding for cyclin G whose function remains unclear (el-Deiry et al., 1992; Bourdon et al., 1997; el-Deiry, 1998; Jian et al., 1999; Sax and El-Deiry, 2003; Wang et al., 2001; Nakamura, 2004). Recent studies demonstrate that p53 can regulate the expression of several genes including phosphoglycerate mutase (PGM), TP53-induced glycolysis and apoptosis regulator (TIGAR) and synthesis of cytochrome c oxidase 2 (SCO2), each intimately linked to the processes of glycolysis and oxidative phos- phorylation (Corcoran et al., 2006; Bensaad and Vousden, 2007; Matoba et al., 2006). Although the number of identified p53 downstream genes keeps growing, it is conceivable that large fraction of the p53 downstream genes have not yet been identified.

For screening novel p53 downstream genes, we established a cell line U251-pTet-p53 carrying a wild-type p53 transgene whose expression can be induced under the control of the tetracycline-response element (TRE). In this cell line, exogenous p53 gene can be overexpressed in doxycycline (Dox) medium but not in the medium without Dox. It was proved that exogenous p53 gene overexpression could lead to a great number of differential p53 downstream genes expression, some up-expressed and others down-expressed (Shu et al., 2002; Shu et al., 2006a).

In this study, we described the construction of cDNA library of human brain glioma cell line U251 while exogenous p53 gene is overexpressed together with the screening of p53 downstream genes. Here, we used the switching mechanism at 5' end of RNA transcript (SMART) and normalized technique to construct a normalized cDNA library, which provided a resource for the investigation of the molecular mechanism of p53 overexpression and screening of p53 downstream genes. Finally, we screened the library and gained two novel p53 downstream genes, whose structures and functions need further investigation.

MATERIALS AND METHODS

Cell lines and cell culture

The human brain glioma cell line U251 and U251-pTet-p53 carrying a wild-type p53 transgene were described previously (Shu et al., 2002; Shu et al., 2006a). U251 was maintained in Roswell Park Memorial Institute 1640 (RPMI1640) (Gibco) containing 10% fetal bovine serum (FBS). U251-pTet-p53 cells were maintained in complete culture medium containing RPMI1640, 10% FBS, G418 (100 mg/ mL) and hygromycin (100 mg /mL). All the cells were cultured at 37℃ and 5% CO_2. To induce p53 expression, Dox was added to the medium every 2 days to a final concentration of 2 μg/mL (that is, Dox+). To maintain U251- pTet-p53 cells in a repressed state with respect to p53 expression, U251-p53 cells were first rinsed three times with phosphate buffered saline (PBS) (without Ca^{2+} or Mg^{2+}) and then switched to culture media lacking Dox (that is, Dox–).

RNA isolation and cDNA synthesis

Total RNA from cells was isolated by using Trizol (Invitrogen). Samples were treated with RNase-free DNase, extraction procedure (Liu et al., 2005; Wang et al., 2005) performed according to the manufacturer's instructions. Isolation of mRNA was carried out according to the protocol of Quick Prep™ Micro mRNA Purification (Amersham Biosciences). The quantity and integrity of mRNA were detected by ultraviolet spectrometer and electrophoresis on a denaturing formaldehyde agarose gel.

First-strand cDNA was synthesized by using SMART™ cDNA Library Construction Kit (Clontech). The procedure was performed according to the manufacturer's instructions. 3 μl of mRNA sample, 1 μl of SMART IV Oligonucleotide (5'-AAGCAGTGGTATCAA CGCAGAGTGGCCATTACGGCCGGG-3'), 1μl of CDS III/3′ polymerase chain reaction (PCR) Primer (5'-ATTCTAGAGGCC GAGGCGGCCGACATG-d(T)-3') were incubated at 72℃ for 2 min, then at 0℃ for 2 min immediately. 2 μl of 5×First-Strand buffer, 1 μl of dithiothreitol (DTT) (20 mmol /L), 1 μl of dNTP mix (10 mmol/ L) and 1 μl of MMLV reverse transcriptase (200 U/μl) were added and then incubated at 42℃ for 1 h. Long distance (LD) PCR was performed according to the manufacturer's instructions. The PCR mixture consisted of 2 μl of first-strand cDNA, 10 μl of advantage 2 PCR buffer, 2 μl of 50×dNTP mix, 2 μl of 5′ PCR primer (5'-AAG CAGTGGTATCAACGCAGAGT-3'), 2 μl of CDS III/3′ PCR primer, 2 μl of 50×advantage 2 polymerase mix and 80 μl of deionized H_2O. The amplification program was as follow: 95℃ for 20 sec, followed by 24 cycles of 94℃ for 30 sec, 68℃ for 6 min. 5 μl of the PCR products were analysed on 1.2% agarose/ethidium bromide (EtBr) gel. The PCR products of the double stranded (ds) cDNA was purified and stored at -20℃

cDNA Normalization

Hybridization reaction

cDNA Normalization was performed by utilizing TRIMMER-DIRECT kit according to the manufacturer's instructions (Evrogen). The hybridization reaction mixture was precipitated with the following reagents: 6 μl of Duplex-specific nuclease (DSN), 4 μl of 800 ng template, 4 μl of 4×hybridization buffer. Sterile water was added to a total volume of 16 μl, mixed and incubated in a thermal cycler at 98℃ for 2 min, then 68℃ for 5 h

DSN treatment

Two sterile tubes were prepared 15 min before the end of the hybridization procedure. In the first tube, 1 μl of DSN storage buffer and 1 μl of DSN solution (in storage buffer) were added which was labeled as 1/2 DSN enzyme. In the second tube, 3 μl of DSN storage buffer and 1 μl of DSN solution was added and labeled as 1/4 DSN. Then the two tubes were placed on ice, and 5 μl of the preheated DSN master buffer, respectively were added, incubated at 68℃ for 25min and finally on ice.

Analysis of normalization efficiency

18S rRNA quantitative PCR was used to evaluate the normalization efficiency by comparing the abundance of known cDNAs before and

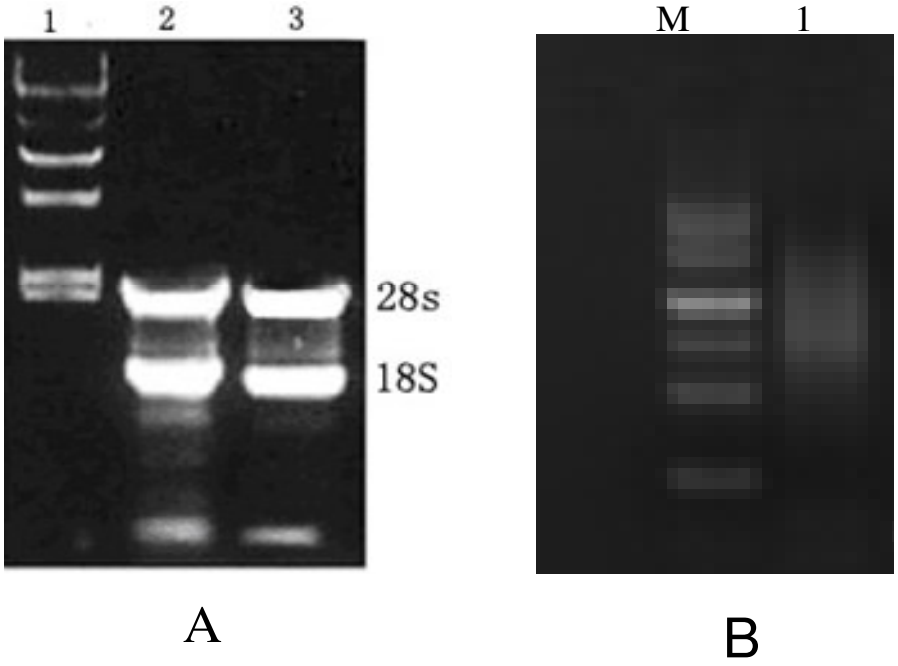


Figure 1. Analysis of RNA separation and purification. A, 1.1% Agarose gel electrophoresis of total RNA; B, 1.1% agarose gel electrophoresis of mRNA.

after normalization. Primers were designed with primer 3. The sequences of 5' PCR primer: 5'-TCGTAGTTGGACTTAGGGTGGG -3', 18S rRNA 3' PCR primer: 5'-CAAATGCTTTCGCAGTTGTTCG-3' were used. 3' PCR primer(10 mM) 1µl, 5' PCR primer(10 mM) 1µl, 10× PCR reaction buffer 2.5 µl, $MgCl_2$ (25mM) 2 µl, dNTP (2.5mM) 2.0 µl, ddH_2O 17.25 µl, TaKaRa Ex Taq 0.25 µl was properly mixed and the PCR reaction condition were: 75℃ for 5 min; 94℃ for 30 sec; 20×: 94℃ for 10 sec; 60℃ for 30 sec; 72℃ for 2.5 min.

Amplification of normalized cDNA

The reaction mixture was precipitated with the following components in the tube: 40.5 µl water, 5 µl 10 × advantage 2 PCR buffer, 1 µl 50 × dNTP mix, 1.5 µl evrogen PCR primer M_1, 1 µl 50 × advantage 2 polymerase mix. PCR was then carried out according to the following program: 95℃ for 3 min; 94℃ for 30 s, 55℃ for 30 s, 72℃ for 2 min for 30 cycles; 72℃ for 7 min.

Construction of cDNA library

After purifying the amplified cDNA with PCR purification kit , the cDNA was digested with 100 µl of Sfi I restriction enzyme at 50℃ for 2 h, fractionated by CHROMA SPIN-400 column (Clontech) to collect larger than 800 bp cDNA. Approximately, 250 ng of cDNA was ligated into 100 ng of Sfi I digested and dephosphorylated pDNR-LIB vector (Clontech) and electroporated into *E. coli* JM109 cells using the Bio-RAD Gene Pulser under standard conditions (Gou et al., 2001; Chu et al., 2003). To calculate the titer of un-amplification library, the library was plated directly on selective medium (containing 30 µg/ml of chloramphenicol), which were inverted and incubated at 37℃ overnight. 15 clones were selected randomly and amplified by PCR according to the following program: 94℃ for 5 min; 94℃ for 1 min, 56℃ for 1 min, 72℃ for 1 min for 29 cycles; 72℃ for 10 min.

Library screening and PCR amplification of p53 downstream genes

To screen the p53 downstream genes, the library was plated at 5000 colonies/plate and transferred to Hybond nylon membrane in duplicate. The membranes were treated according to the manufacturer's guidelines. Gel-purified DNA for PAP1 and PAP2 gene (Shu et al., 2006b) to be used as probes was labeled using Digoxinum-dUTP and a *in situ* hybridization system (Boster, Wuhan, China). Hybridization was performed according to the manufacturer's instructions.

Known p53 downstream genes PCR was performed in 25 µl reaction mixture containing 5 µl of amplified cDNA sample, 10 µl of dNTP, 2.0 µl of each primer, 2.5 µl of PCR buffer (Mg^{2+}Plus) and 0.25 µl (1 unit) of Taq DNA polymerase (TaKaRa). Temperature conditions typically consisted of an initial denaturation at 94℃ for 3 min followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 45 s and elongation at 72℃ for 1 min, and then a final elongation step at 72℃ for 10 min. The primer sequences are shown in Table 1.

RESULTS

Analysis of total RNA and mRNA

High quality isolation of RNA is a critical step for constructing a cDNA library. In this study, total RNA is described as a long smear with clear bands of 28 and 18 S (Figure 1A). The ratio of OD_{260}/OD_{280} to the total RNA was 1.94, and the concentration was 3.90µg/µl. These indicated that the total RNA obtained from human brain glioma cell line U251 while exogenous p53 gene over-expressed did not degrade obviously and that the purity was high. Figure 1B shows that the smear of mRNA was

so long which indicated that the integrity of mRNA had been achieved.

Synthesis and normalization of cDNA

We used the SMART cDNA synthesis, LD-PCR amplification (Barnes, 1994) and cDNA normalization strategy (Patanjali et al., 1991; Soares et al., 1994; Sasaki et al., 1994; Tanaka et al., 1996) to ensure the amplification of long cDNA fragments. The results showed that the concentration of ds cDNA was 0.27 μg/μl and the size of the cDNA ranged from 200 bp to 4 kb, with an average size of 1000 bp, which indicated that LD-PCR was successfully use for synthesis (Figure 2). It showed that a sharp decrease in the representation levels of abundant transcripts was observed in normalized cDNA samples compared with non-normalized samples indicating that the effect of normalization was obvious (Figure 2). The efficiency of normalization was demonstrated by comparison of normalized and non-normalized SMART amplified cDNA. Figure 3 shows that the bands corresponding to abundant transcripts (18S rRNA), clearly visible in the pattern of the non-normalized cDNA, and having a dramatic decrease in the normalized cDNA sample indicated that the amount of these highly abundant transcript was sharply reduced after normalization.

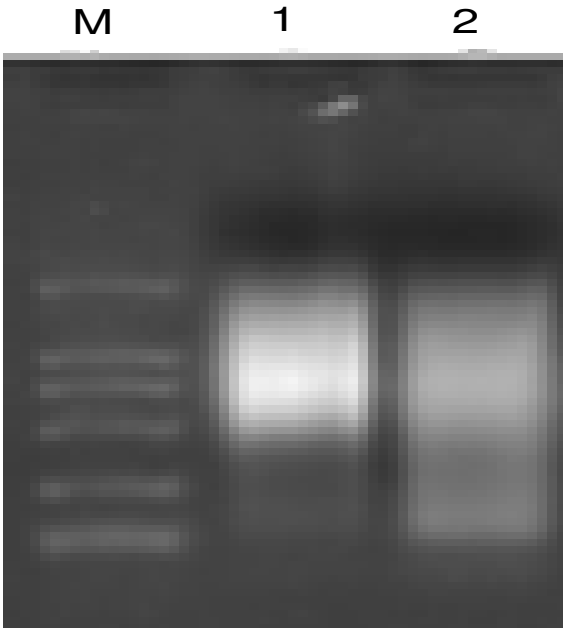


Figure 2. Analysis of cDNA normalization. 5 μl of the PCR products were electrophoresesed on a 1.1% agarose gel. M, DNA marker; Lane 1, cDNA by LD-PCR (unnormalization); Lane 2, cDNA from normalization, efficient normalization was achieved.

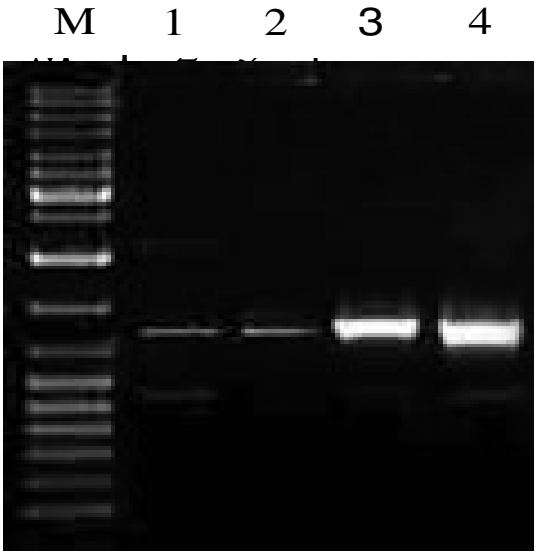


Figure 3. Analysis of abundant 18S rRNA gene transcripts in normalized and non-normalized cDNA by PCR. M, DNA marker; Lanes 1 and 2, normalized samples; Lanes 3 and 4, non-normalized samples. A dramatic decrease in the normalized cDNA sample was observed.

Construction of a human cDNA library from cell line with overexppression of p53

Using the SMART cDNA synthesis, LD-PCR amplification (Barnes, 1994) and cDNA normalization strategy (Patanjali et al., 1991; Soares et al., 1994; Sasaki et al., 1994; Tanaka et al., 1996), a cDNA library was constructed from mRNA of human brain glioma cell line U251 while exogenous p53 gene is overexpressed. The titer of the unamplified constructed cDNA library was approximately 1.3 $\times 10^6$pfu/ ml and the amplified was approximately 1.1 $\times 10^8$ pfu/ml. To determine the modal insert size, 15 cDNA clones were randomly isolated and inserts were amplified by PCR. The distribution of the insert length was between 0.5 and 2.0 kb (Figure 4).

In conventional cDNA library construction, each clone represents an independent mRNA molecule that has been converted into cDNA. This is not the case for a cDNA library generated by PCR amplification, since it might favor certain sequences over others (Corrick et al., 1996). It was therefore critical to ascertain whether the cDNA library we had constructed was proportionally representative of the mRNA population present in the human brain glioma cell line U251 while exogenous p53 gene overexpressed. To this end, we used two different approaches: (1) The library was screened with two human p53 downstream genes' DNA probes which were newly identified by our group to ensure the quality of the library; (2) PCR with specific primers were used to test for the presence of the following p53 downstream genes : p21, gadd45 and PCNA. The results showed that our library contains PAP1 and PAP2 clones (Figure 5A-D), which will

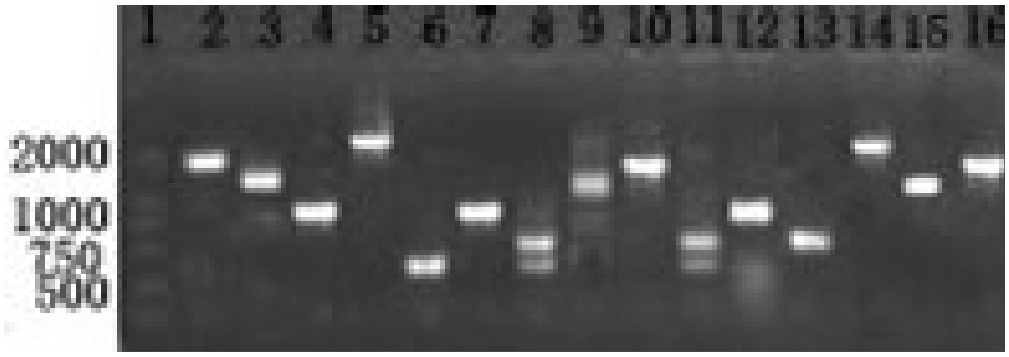
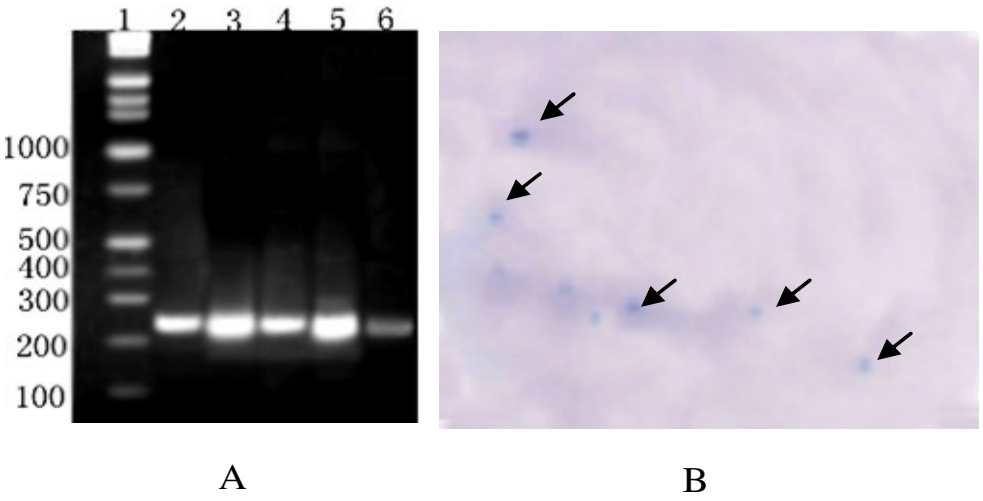


Figure 4. Gel electrophoresis of size-identification of the inserted ds cDNA. Lane 1, DNA marker; Lane 2-16, inserted ds cDNA, respectively.



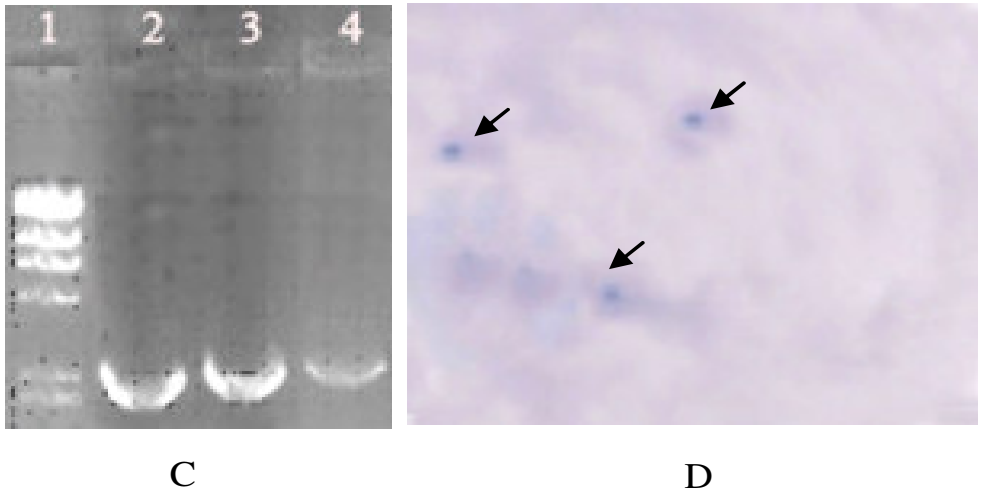


Figure 5. Screening the library. A, The possible clones were detected by PCR: Lane 1, DNA Marker; 2-6, five possible clones. The results indicated that the five clones all contained PAP1 cDNA; B, PAP1 cDNA screening by colony *in situ* hybridization. Arrowheads showed the possible clones; C, the possible clones was detected by PCR: Lane 1, DNA Marker; 2-4, three possible clones. The results indicated that the five clones all contained PAP2 cDNA; D, PAP2 cDNA screening by colony *in situ* hybridization. Arrowheads showed the possible clones.

Figure 6. Human p53 downstream genes was amplified from the cDNA library by PCR. Lane 1, DNA marker; Lane 2, β-actin gene (as control); Lane 3, gadd45 gene; Lane 4, p21gene; Lane 5, PCNA gene.

be further research into. Human p53 downstream genes, p21, gadd45 and PCNA, was amplified from this cDNA library by PCR using specific primers (Figure 6). These results indicate that our cDNA library offers a good source for finding p53 downstream genes and should be able to contribute to the under- standing of the p53 network (Shu et al., 2007).

DISCUSSION

p53 and its downstream genes consist of a complicated gene network. It is very important to understand the p53 gene regulatory network in order to know the p53 physiological functions. The ultimate challenge to define the complete p53 gene regulatory network is to identify p53 downstream genes. Although the number of identified p53 downstream genes keeps growing, it is conceivable that large fractions of the p53 downstream genes have not yet been identified (Vousden and Lu, 2002; Shu et al., 2007; Vogelstein et al., 2000; Perwez and Harris, 2006). The exploitation and utilization of genetic resources play an important role in research on structure and function of p53 downstream genes. A cDNA library constructed from human brain glioma cell line U251 while exogenous p53 gene overexpressed will be of help in acquiring the expression spectrum of the human brain glioma cell line U251 while exogenous p53 gene is overexpressed and finding p53 downstream genes.

SMART technique is a novel and useful method for constructing cDNA libraries. Its important characteristic is that it provides a method for producing high-quality and full-length cDNA libraries that preserve the complete 5′ terminal sequence of mRNA. Conventionally-generated cDNA libraries contain a high percentage of 5'-truncated clones, but library construction methods that enrich for full-length mRNA are laborious, and involve several enzymatic steps performed on mRNA, which renders them sensitive to RNA degradation. The SMART technique is suitable to generate full-length enriched cDNA libraries with large average insert sizes in a straightforward and robust way. The representation of full-coding clones is high also for large cDNAs, when high-quality starting mRNA is used (Wellenreuther et al., 2004; Suzuki et al., 1997; Draper et al., 2002).

We adopted the pDNR vector, which provided a rapid and simple method for constructing high-quality cDNA libraries that are ready for identification, amplification and analysis of the clones. But there was another problem associated with gene identification, which the researchers might be faced to make efficient high-throughput analysis of the library, with a view to the differential abundance of various transcripts in the cell. For rare mRNA sequence, it was likely to have presented difficultly in the identification of cDNA library because cDNAs of high abundance were sequenced repeatedly, and then the rare transcripts were relatively low. It was a huge waste when this type of cDNA library was used for gene screening. Normalized cDNA library was developed to overcome the problem caused by different expression frequencies of genes and it was an efficient tool for gene identification (Weissman, 1987; Shagin et al., 2007). The normalization method may be effectively used for samples enriched with full-length cDNA sequences.

Conclusion

A high quality cDNA library from human brain glioma cell line U251 while exogenous p53 gene overexpressed was successfully constructed.

ACKNOWLEDGMENTS

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