

Discovery of Differentially Overexpressed Genes in Immortalized Cells and Human Pulmonary Non-small Cell Carcinomas

Purpose: Our aim of research is to find novel genes that overexpressed in various samples such as cell lines and tissues that infinitely proliferate; so-called immortalized cells, cancer cells and tissues. In this study, we obtained a gene from immortalized cell line (WI-38 VA13) then identified it from various cell lines and human lung tissues. **Materials and Methods:** Using suppressive subtractive hybridization (SSH) method, we obtained many genes and selected a novel gene of them. And then the novel gene fragment was amplified by PCR and ligated in T easy vector for sequencing. And finally we found a differentially expressed gene in cell lines and tissues when it was performed by reverse transcriptase-PCR (RT-PCR). **Results:** As the result of transformation of genes that we gained using SSH, we obtained about 150 clones. And then we certificated several genes by DNA prep and confirmed it by sequencing. We examined whether the gene sequences are novel or known genes by genome homology search and we confirmed the gene expressions by RT-PCR. As a result, we identified a differentially overexpressed gene (named "clone 58") in immortalized cells, cancer cell lines and lung squamous cell carcinomas. **Conclusion:** The "clone 58" mRNA was significantly up-regulated in various human cell lines and also human lung cancer tissues compared to the normal. We suppose that this gene can carry out a specific role related to the induction of cancer and/or the mechanism of the changeover of a normal cell to an immortalized cell. In short, the discovery of our gene has an importance in the point that they are thought to have a connection with immortalization and carcinogenesis of human cells and tissues. (*J Lung Cancer* 2006;5(2):96 – 101)

Key Words: Lung cancer, Immortalization, Carcinogenesis, Suppression subtractive hybridization

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INTRODUCTION

Lung cancer is the growth of malignant in the lung. It is the most frequent cause of cancer death in several countries among both men and women. Despite aggressive approaches to therapy and new lung cancer medication, survival rates have changed little in the last decade(1). There are two major types of lung cancer. Depending on how cells look under a microscope, they are classified as either small cell lung cancer or non-small cell lung cancer. Small cell lung cancer and non-small cell lung cancer grow and spread in different ways. The more common

type of lung cancer is non-small cell lung cancer. This type of cancer grows and spreads slowly. Other names for non-small cell lung cancer are squamous cell carcinoma (also called epidermoid carcinoma), adenocarcinoma, and large cell carcinoma. The less common type of lung cancer is small cell lung cancer(2,3). Diagnosis is made through imaging studies (X-rays) and biopsies. Treatment depends on the type of lung cancer and includes chemotherapy, radiotherapy, and surgery(4).

Despite this cancer is very serious disease, it has not been studied enough. For this reason, in this study, we tried to identify a gene related to immortalization and/or carcinogenesis.

Many oncogenes and tumor suppressor genes, such as FGFR3, LMO3, Rb, p53 and p27, have been known to be involved in the development of this cancer. However, we believe that many unknown novel genes still exist in the multistep carcinogenesis of lung cancer. Thus, we focused on that point and infinite proliferation of the cancer(5~7).

For the research of new molecules that may play a role in various developmental processes, a number of subtractive hybridization techniques have been employed in recent years. Suppression subtractive hybridization (SSH) is easily applied to obtain clones of genes that are expressed in one population but not in the other as comparing the two populations of mRNA(8~10). Also, it is a potentially faster method for identifying differentially expressed genes associated with a particular disease state. In addition, by SSH-PCR, genes with low-abundance mRNA transcripts can be readily identified and characterized(11~13). Using this technique, we isolated novel genes that are overexpressed in WI-38VA13, the SV40-immortalized WI-38 fibroblast, as compared to WI-38, characterized by RT-PCR and sequenced.

MATERIALS AND METHODS

1) RNA isolation

Cells grown in monolayer lysed directly in a culture dish. Media poured off, 1 ml TRI REAGENT (Molecular Research Center, Ohio, USA) per 10 cm² of culture dish area added and the cell lysate passed several times through a pipette. The homogenate stored for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. The homogenate supplemented 0.2 ml chloroform per 1 ml TRI REAGENT and shaken vigorously for 15 seconds. The resulting mixture stored at room temperature for 5 minutes and centrifuged at 12,000 g for 14 minutes at 4°C. The aqueous phase transferred to a fresh tube and mixed with 0.5 ml of isopropanol. The sample was stored at room temperature for 10 minutes and centrifuged at 12,000 g for 8 minutes at 4°C. The supernatant was removed and RNA pellet washed with 1 ml of 70% ethanol and subsequent centrifugation at 12,000 g for 5 minutes at 4°C. After the ethanol was removed, RNase-free water by diethyl pyrocarbonate (DEPC) treatment was added.

2) cDNA synthesis

1 ug RNA sample, 1 ul 3'BD SMART CDS PrimerIIA (12 uM) and 1 ul BD SMARTIIA Oligonucleotide (12 uM) were placed together in 0.2 ml reaction tube. The tube incubated at 72°C for 2 minutes and cooled for 2 minutes on ice. After that, 2 ul 5×First-strand Buffer, 1 ul DTT (20 mM), 1 ul dNTP Mix (10 mM of each dNTP) and 1 ul BD PowerScript Reverse Transcriptase were mixed together. The tube incubated at 42°C for 1 hr and placed on ice to terminate first-strand synthesis.

3) Suppression subtractive hybridization

The amplified cDNA by long distance PCR (LD PCR) as described (BD biosciences clontech, CA, USA) were cut using the restriction enzyme, Rsa I. For the differentially expressed gene fragments between Normal (driver) and Immortalization (tester), the adaptor ligated only in tester and hybridization with driver. The differentially expressed gene fragment were amplified by PCR and ligated in T easy vector (Promega, USA) for sequencing.

4) Patients and tissue specimens

Lung tissue specimens from Korean patients with four adenocarcinoma and three squamous cell carcinomas were donated by the Korea Lung Tissue Bank, Seoul, Korea.

5) Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on normal lung and non-small cell lung carcinomas with 1 ug total RNA using SuperscriptTM First-Strain Synthesis (Invitrogen, USA). Other cell lines used were WI-38 (lung normal, fibroblast), WI-38 VA13, WI-26 VA4 (immortalized, SV40-transformed), CCD-986sk (skin normal, fibroblast), WM-266-4 (skin, melanoma), CCD-18Co (colon normal), KM1214 (colon, carcinoma), RWPE-1 (prostate, normal), DU-145 (prostate, adenocarcinoma), SK-N-SH (brain, neuroblastoma, metastasis to bone marrow), A172 (brain, glioblastoma), HeLa (cervix, adenocarcinoma) and NCL-H596 (lung, carcinoma). To normalize mRNA amounts of "clone 58" between tissue samples, 18srRNA was used as the reference gene. Primers used were "clone 58" sense, 5'-AAATGAA TACAGCGAGGAAA-3', and anti-sense, 5'-AAACCACAGC-

AAATGCTTAT-3', leading to 321 bp PCR product, 18srRNA sense, 5'-TACCTACCTGGTTGATCCTG-3', and anti-sense, 5'-GGGTTGGTTTGGATCTGATA-3', leading 255 bp PCR product. PCR was performed using the TaKaRa Ex Taq™ under the following PCR conditions: first 5 min at 94°C, then 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C for 35 cycle, 5 min 72°C. The product of the PCR amplification was analyzed on 1.0% agarose gel.

6) Serum stimulation

WI-38 normal lung fibroblast cell was routinely maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), essential amino acids, non-essential amino acids. WI-38 was seeded at a density of 1×10^6 cells/100 mm dishes. When the cells were 60% confluent, cells arrested with medium supplemented with 0.1% FBS for 48 hr and then supplied with fresh 10% FBS medium. After 10% FBS treatment, cells were harvested at different time points of 0, 0.5, 1, 2, 4, 8, 12 and 24 hr.

RESULTS

1) Identification of genes overexpressed in human immortalized cell using SSH

Using SV40-immortalized cell line (WI-38VA13) compared to normal cell line (WI-38, lung fibroblast cell), we obtained subtracted individual clones by suppression subtractive hybridization. In this study, WI-38VA13 was used as tester and WI-38 was used as driver. To ascertain the efficiency of the result, both subtracted cDNA and unsubtracted cDNA have been amplified by PCR with GAPDH primers for different cycles: 18, 23, 28, and 33 cycles (Fig. 1). As shown in Fig. 1, expression of GAPDH was observed in unsubtracted cDNA after 18 cycles and in subtracted cDNA after 33 cycles. As the

result, the abundance of GAPDH decreased significantly after subtraction. It was important to confirm that individual clones indeed represent differentially expressed genes. It indicates that a drastic reduction of highly abundant cDNAs was achieved. The subtracted nested PCR products, from the SSH, have been cloned in T/A vectors and plated on ampicillin agar plates including X-gal and IPTG. With this experimental design, 150 plasmid clones were obtained. We digested them with EcoRI restriction enzyme to evaluate the multiplicity of genes. The digested cDNA clones were searched for high sequence homologies by the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST/>). Some of the digested 150 clones detected high homology to known genes in the public database of the 5 kinds (Table 1). We identified 40 novel genes by BLAST of digested 150 clones. "clone 58" sequences were found to be homologous to regions of chromosome 12. In this study, we selected that this 676 bp cDNA of "clone 58" was not homologous to any of the known genes in the GenBank database. The sequence of "clone 58" is

Table 1. Identification of Known Tumor Specific Genes from SSH Libraries

GeneBank Accession no.	Matching gene
NM_021105	Home sapiens phospholipid scramblase 1 (PLSCR1), mRNA
NM_003870	Home sapiens IQ motif containing GTPase activation protein 1 (IQGAP1), mRNA
NM_004336	Home sapiens BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast) (BUB1), mRNA
NM_012325	Home sapiens microtubule-associated protein, RP/EB family, member 1 (MAPRE1), mRNA
NM_004083	Home sapiens DNA-damage-inducible transcript 3 (DDIT3), mRNA

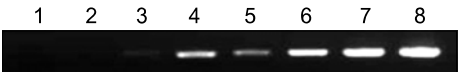


Fig. 1. Reduction of GAPDH abundance by PCR-select subtraction. Normal cDNA was prepared from WI38 (human lung fibroblast cell) total RNA and immortalization cDNA was prepared from WI38 VA13 (transformed WI38 by SV40). PCR was performed on subtracted (lanes 1~4) or unsubtracted (lanes 5~8) secondary PCR product with the GAPDH 5' and 3' primers. Lanes 1, 5: 18 cycles; lanes 2, 6: 23 cycles; lanes 3, 7: 28 cycles, lanes 4, 8: 33 cycles.

ACCTAGTGTGTAACATTCGTCATGACAGCCCTAGGAAATGAATACAGC
GAGGAAATCCTACACAGCACAAAGGCATGGAGGTGCCAGGATGCTCC
TCTGCGTGAAGAGTAGATGTAGATGAGGCTGGAATTATCTATCCTAGCT
GCCAGACCCATGTGCCCTTTGTTTATGATAGTTACAGCACCTATGATACA
TATTTGTACCATGTATGTCACATGAACTTCCTCTGAGGATGGAGAAG
TCAATACCTTAATTATTCACACAAAGCTTGCGTGAAACGATATCATC
ACTACGAGTATATTGTGCTGCTGTAAGCACACGCCGTGAGATAAGCAT
TTGCTGTGTTTGAATGTCCTCCAAAGCTCATGCTAAATTTAATTGC
CATTGCAACAGTGTGCAAGGTGAGAACTTTAAGAGATGATCAGGTCAT
GAGAAGCTGCGCTCGTAATGGATTAAATCCCTATCGCAGTAGTGGA
CCCCCTTTTCTCTGCTGCTGCTCTCATGTAGCTTGTGCTTCTCC
TTTCACCATGGGATAACACAGCAAGAAAGCCCTCACCAGATGCTGGCA
CCTGCTATTGGACTTCCGGCCCTCAGAACTGAGAAATACATGCTCTTTC
CTTATTAATAAAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 2. Sequence of "clone 58".

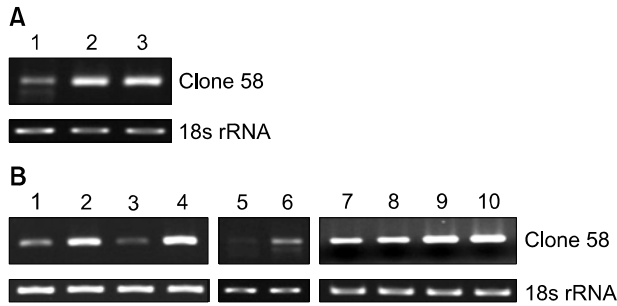


Fig. 3. Expression of clone 58 in WI38, WI38 VA13 (A) and various cell lines (B). (A) Lane 1: WI38 (lung fibroblast normal cell), lane 2: WI38 VA13 (SV-40 transfected cell), lane 3: WI26VA4 (SV-40 transfected cell). (B) Lane 1: skin normal cell, lane 2: skin tumor cell, lane 3: colon normal cell, lane 4: colon tumor cell, lane 5: prostate normal cell, lane 6: prostate tumor cell, lane 7: SK (SK-N-SH): brain neuroblastoma, metastasis to bone marrow, lane 8: A172: brain, glioblastoma, lane 9: HeLa, lane 10: Lung cancer. 18 s rRNA was used as the reference gene.

identified as shown figure (Fig. 2).

2) Verification of 58 gene in immortalized cell, cancer cell lines and some lung cancer tissues by RT-PCR

In this study, to confirm the cancer-specific expression of the identified “clone 58”, we performed RT-PCR analysis. Immortalized cell, cancer cell lines and tissues were used for validation by RT-PCR with housekeeping gene, 18 s rRNA, as control. The 7 pairs of human normal and lung cancer tissues were provided by Korea Lung Tissue Bank. RT-PCR products of “clone 58” were analyzed on 1.0% agarose gel. The “clone 58” mRNA expressions in immortalized, cancer cell lines (Fig. 3) and squamous cell carcinoma tissues (Fig. 3) were significantly higher than normal cell lines and tissues. This result indicates a specific up-regulation of the “clone 58” identified by SSH. In cancer cell lines, “clone 58” very strongly expressed in the SV40-immortalized cell lines (Fig. 3A), skin melanoma, colon carcinoma, prostate adenocarcinoma, brain neuroblastoma, brain, glioblastoma, HeLa, Lung carcinoma (Fig. 3B). 18s rRNA was used as the reference gene. And also, squamous cell carcinoma tissues showed differentially expressed “clone 58” than normal tissues (Fig. 4A) whereas it didn’t seem to be steadily upregulated in adenocarcinoma tissues (Fig. 4B).

3) Serum stimulation

To determine the association with serum stimulation conditions, normal WI-38 fibroblast cell was cultured at a density

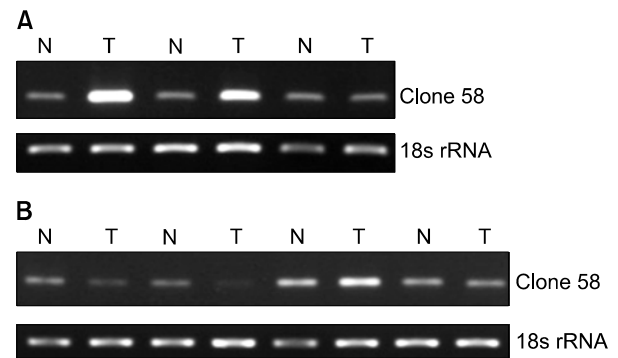


Fig. 4. Clone 58 expression in non-small cell lung carcinoma tissues. (A) N: normal lung tissues, T: squamous cell carcinoma. (B) N: normal lung tissues, T: adenocarcinoma. 18s rRNA was used as the reference gene.

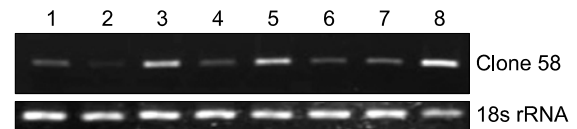


Fig. 5. Serum stimulation. Clone 58 expressions have no significant on time dependent manner. Lung fibroblast cell line (WI38) was used. Lane 1: arrest stage, lane 2: 0.5 hr, lane 3: 1 hr, lane 4: 2 hr, lane 5: 4 hr, lane 6: 8 hr, lane 7: 12 hr, lane 8: 24 hr after serum stimulation. 18 s rRNA was used as the reference gene.

of 60% cells, and then treated medium containing 0.1%FBS for 48 hr. During 48 hr treated, WI38 cells achieved an induction of G1-phase arrest of the cell cycle. After that, we added fresh medium containing 10%FBS to return normal cell cycle and harvested cells after 0, 0.5, 1, 2, 4, 8, 12 and 24 hr. We expected “clone 58” expression would be significantly increased by cell cycle. However, after serum stimulation, the expression was not regulated by the time points (Fig. 5).

DISCUSSION

There are several causes of lung cancer. Most of these causes relate to the use of tobacco. Smoking is the cause of lung cancer for about 85% of lung cancer cases in the world(14,15). Cigarette smoking, asbestos exposure, air pollution, other lung diseases, diet, and personal history are all risk factors for lung cancer(4,16). Various kinds of oncogenes or tumor suppressor genes are suggested for the lung cancers. But it is still uncertain and it is not sufficiently studied yet. So it is still a very important thing that we should find and identify novel genes.

Because it is believed that there are still many novel genes to be not found yet in vivo.

There are several methods to identify differentially expressed gene such as, suppression subtractive hybridization(17), differential display polymerase chain reaction(18), expressed sequenced tags (EST) analysis(19), cDNA library array technology(20) and RNA fingerprinting(21). To identify new novel genes involved in carcinogenesis of lung cancer, we performed the PCR-based subtractive hybridization method gene between normal lung fibroblast cell line and immortalized cell line. Using this method, we isolated a novel gene and known genes that are overexpressed. Therefore, we have obtained a highly expressed novel gene in immortalized cell line than normal lung fibroblast cell line.

We compared the "clone 58" expression by RT-PCR between human normal lung tissues and non-small cell carcinoma tissues (adenocarcinoma, squamous cell carcinoma). We could not find any special feature in adenocarcinoma tissue pairs. However, "clone 58" tends to be overexpressed in squamous cell carcinomas rather than in normal lung tissues. These findings suggest that "clone 58" may play a role in carcinogenesis for squamous cell carcinoma.

To determine whether this novel gene is associated with cell cycle regulation, we studied a serum stimulation in normal lung fibroblast cells. In general, cell cycle machinery is itself regulated by the growth factors that control cell proliferation, allowing the division of individual cells to be coordinated with the needs of the organism as a whole. Not surprisingly, defects in cell cycle regulation are a common cause of the abnormal proliferation of cancer cells, so studies of the cell cycle and carcinogenesis have become closely interconnected(22,23).

Accordingly, we tried to investigate whether this gene is involved in the mechanisms of growth inhibition and cell cycle arrest using time course stimulation study. A typical eukaryotic cell cycle is illustrated by human cells in culture, which divide approximately every 24 hours. For a typical rapidly proliferating human cells with a total cycle time of 24 hours, the G1 phase might last about 11 hours, S phase about 8 hours, G2 about 4 hours, and M about 1 hours. During G1, the cell is metabolically active and continuously grows. In the present study, we induced G1 arrest using 0.1% FBS and stimulated using 10% FBS after 48 hr. Therefore, we tested the time dependent activation of this novel gene. The nucleotide sequence

of this clone identified in our study using Rapid Amplification cDNA ends (RACE) PCR and other functional studies are continuing investigated.

CONCLUSION

The "clone 58" mRNA was significantly up-regulated in various human cell lines and also human lung cancer tissues compared to the normal. We suppose that this gene can carry out a specific role related to the induction of cancer and/or the mechanism of the changeover of a normal cell to an immortalized cell. In short, we think that the discovery of our gene has an importance that they are thought to have a connection with immortalization and carcinogenesis of human cells and tissues.

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