Subtractive screening of genes involved in cellular senescence

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Abstract

We attempted to identify the genes involved in cellular senescence, telomere maintenance and telomerase regulation through subtractive screening of cDNA libraries prepared from a human lung adenocarcinoma cell line A549 and its sublines named A5DC7, CK and AST-9. Cell phenotypes of A5DC7, CK and AST-9 are normal cell-like, cancer cell-like and intermediate, respectively. These cell lines have different phenotypes in terms of telomerase activity and telomere maintenance, and thus are thought to be useful for identifying the genes involved in cellular senescence and telomerase regulation. In this study, we identified 86 independent cDNA clones by subtractive screening. Among these cDNA clones, subtracting A5DC7 cDNAs from A549 cDNAs and CK cDNAs gave 7 and 3 cDNA clones which highly and specifically expressed in tester cell lines. Genes corresponding to these 10 cDNA clones might participate in maintaining cancer-cell phenotypes. As a result of database searching, each four of A549 specific cDNA clones are found to correspond to known cDNAs. Each two of A549 specific and two of CK specific cDNA clones have high homology to independent ESTs. Sequences having homology to each one of A549 specific and one of CK specific cDNA clones have not been deposited in the Genbank database, indicating that these two cDNA clones are part of novel genes. We anticipate that their involvement in telomerase regulation and/or senescence program can be clarified by functional analysis using each full-length cDNA.

Abbreviations: PDL, population doubling level; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; TGF- β , transforming growth factor β 1; hTERT, human telomerase reverse transcriptase; TEP1, telomerase associated protein 1

Introduction

Many studies aiming at clarifying the mechanism of cellular senescence have been conducted. Two independent cellular senescence programs have been reported, one is premature senescence, which is provoked by the ectopic expression of the Ras oncogene in normal cells within only 1 week independent of telomere shortening (Serrano et al., 1997; Wei et al., 1999). The other is replicative senescence, which depends upon telomere shortening caused by the incomplete replicative ability of DNA polymerase (End Replication Problem) and successive rounds of cell division (Hayflick et al., 1961). In spite of these studies, the complete mechanism of cellular senescence and

the gene products participating in the senescence programs are not fully understood. It has been reported that the expression profiles of several gene products such as p16^{Ink4a}, p21^{Cip1} and p53 changed concomitantly with entrance into the senescence state (Alcorta et al., 1996; Medcalf et al., 1996; Kulju and Lehman, 1995). Forced expression of *mot-1*, *hic-5*, oncogenic *ras* and *raf-1*, or treatment with phosphatidylinositol 3-kinase inhibitor and TGF- β can induce cellular senescence (Wadhwa et al., 1996; Shibamura et al., 1994; Zhu et al., 1998; Tresini et al., 1998; Katakura et al., 1999). However, the accurate roles of these molecules in the cellular senescence programs remain to be clarified.

Telomerase reverse transcriptase (hTERT), the

catalytic subunit of telomerase, is known to be specifically expressed in cancer tissue and cancer cells, but not in normal somatic cells (Kim et al., 1994). The repressed expression of hTERT is thought to be an indispensable event in normal senescent cells. Recently, several genes, cytokines, and chemical reagents have been reported to affect telomerase activity or the transcription of hTERT (Wang et al., 1998; Katakura et al., 1999). We reported that TGF- β could suppress hTERT expression by triggering two independent senescence programs (Katakura et al., 1999). Oncogene *c-myc* is reported to upregulate hTERT transcription via a cis-element of the E-box which exists in the hTERT promoter region (Greenberg et al., 1999). Further studies and identification of gene products participating in transcriptional regulation of hTERT will be needed to clarify in detail the mechanism of regulation of telomerase.

Furthermore, some researchers have reported that telomere dynamics can be dissociated from telomerase activity in some cancer cell lines and that telomerase activity does not always imply telomere maintenance (Ouellette et al., 1999). So far, three telomere binding proteins have been identified, TRF1, TRF2 and Pin2 (Wadhwa et al., 1996; Shen et al., 1997). The functions and the transcriptional regulation mechanisms of these genes, and further other molecules participating in telomere maintenance must be clarified to understand the senescence programs.

In this study, we employed the PCR-Select subtraction method using cDNA libraries prepared from human lung adenocarcinoma-derived cell lines (A549, A5DC7, CK and AST-9 cells) which have quite different phenotypes in terms of cellular senescence, telomerase regulation and telomere maintenance to identify the genes involved in the senescence programs, which resulted in acquisition of A549 and CK specific cDNA clones.

Materials and methods

Cell lines and culture conditions

A5DC7, CK and AST-9 cell lines were sublines derived from the human lung adenocarcinoma cell line A549. A5DC7 cells were established by selecting major histocompatibility complex antigen class II positive cells by panning from IFN- γ -treated A549 cells (Kawamoto et al., 1995). CK cells were established by culturing A5DC7 cells senesced in 5% serum-containing medium in 10% serum-containing medium,

conditions under which the cells show vigorous proliferation (Katakura et al., 1997). AST-9 cells were obtained by treating A549 cells with 200 μ M hydrogen peroxide (H_2O_2) for 4 days and subsequent limited dilution cloning (manuscript in preparation). All these cell lines were cultured at 37 °C in a 95% air/5% CO_2 atmosphere. A549 and CK cells were cultured in ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Whittaker Bioproducts, Inc., Walkersville, MD, USA). A5DC7 and AST-9 cells were cultured in ERDF medium supplemented with 5% FBS.

Assay of telomerase activity

Telomerase activity was measured by the TRAP (Telomeric Repeat Amplification Protocol) assay with some modifications (Kim et al., 1994; Katakura et al., 1997). Briefly, 10^6 cells were pelleted and lysed in lysis buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM β -ME, 0.1 mM AEBSF). The cell lysates were centrifuged at 15,000 rpm for 20 min at 4 °C, and the supernatants were stored at -80 °C. Cell extract (2 μ l) was mixed with a reaction mixture (48 μ l) containing 50 μ M dNTP, 1 $\times$ PCR buffer, 1.0 μ g of T4 gene 32 protein, 0.1 μ g of TS primer (5'-AATCCGTCGAGCAGAGTT-3') and 2.0 U of Taq DNA polymerase. The extension reaction was performed at 20 °C for 30 min, then stopped by heating at 90 °C for 3 min. CX primer (0.1 μ g 5'-CCCTTACCCTTACCCTTACCCTAA-3'), used to amplify the extended telomeric sequences obtained using the TS primer, was then added to the mixture. The mixture was subjected to 30 PCR cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min. The PCR products were analyzed by electrophoresis using a 10% polyacrylamide non-denaturing gel in TBE buffer. Gels were stained with SYBR Green I (TAKARA, Shiga, Japan), and analyzed by means of a LAS1000 image analyzer (FUJIFILM, Tokyo, Japan).

DNA extraction and telomere length analysis

Genomic DNA was prepared using the DNA Extractor WB Kit (Wako, Kyoto, Japan) according to the manufacturer's protocol. Three μ g of DNA was digested simultaneously with *Eco*RI and *Hinf*I at 37 °C for 1 h. The digested DNA was loaded onto a 0.8% agarose gel. The length of the terminal restriction fragment

Table 1. Phenotypic feature of A549-derived cell lines

Phenotypes	Cells			
	A549	A5DC7	CK	AST-9
Telomerase activity	+	–	+	+
Telomere shortening	–	+	–	+
Senescence marker (β -galactosidase)	–	+	–	–
hTERT expression	+	–	+	+
TEP 1 expression	+	+	+	+

(TRF) was determined by Southern blot analysis using a telomeric sequence probe [(TTAGGG)₄], and we then estimated the length of the telomere as described previously (Katakura et al., 1997).

RNA preparation

Total RNA was extracted from A549, A5DC7, CK and AST-9 cells using TRIzol reagent (GIBCO BRL, Gaithersburg, MD, USA). Poly (A)⁺ RNA was then prepared from approx. 100 μ g of total RNA by two sequential rounds of affinity selection using oligo d (T) cellulose (Collaborative Biomedical Products, Bedford, MA, USA). The integrity of the poly (A)⁺ RNA was confirmed by electrophoresis under denaturing conditions.

RT-PCR

cDNA was synthesized from 5 μ g of total RNA using 200 U of SuperScript II Rnase H[–] Reverse Transcriptase (GIBCO BRL) and 0.5 μ g of oligo (dT)₂₀ as an antisense primer according to the manufacturer's protocol. The reverse transcription reaction mixture was diluted 10-fold with distilled water, and then 1 μ l was used for the PCR reaction according to the procedure of Nakamura et al. (1997). To amplify the hTERT (human telomerase reverse transcriptase) mRNA, the mixture was subjected to 31 rounds of PCR (94 °C for 45 s, 60 °C for 45 s and 72 °C for 90 s) using the primers LT5 (5'-CGGAAGAGTGTCTGGAGCAA-3') and LT6 (5'-GGATGAAGCGAGTCTGGA-3').

Construction of cDNA libraries and subtractive hybridization

cDNA libraries were constructed using poly (A)⁺ RNA from each of the cell lines derived from A549 cells, and tester and driver cDNA were synthesized

using specific primers included in the PCR-Selected cDNA Subtraction Kit (CLONTECH Lab., Palo Alto, CA, USA). We then subtracted driver cDNA from tester cDNA in all combinations of cell lines and specifically amplified the subtracted cDNA fragments with the primers used for preparation of the tester and driver cDNA. About the detailed schematic protocols we referred to the instruction manual supplied with the PCR-Selected cDNA Subtraction Kit.

Dot blot hybridization

Secondary differential screening was performed with four replicate dot-blotted membranes (Zeta-Probe membrane, Bio-Rad, Richmond, CA, USA) with 1 μ g of subtracted cDNA fixed on each. The four membranes were probed separately with [³²P]-labeled cDNA synthesized from total RNA obtained from A549, A5DC7, CK and AST-9 cells.

Nucleotide sequence analysis

The subtracted cDNAs were cloned into the pGEM-T or pGEM-T Easy vector (Promega, Madison, WI, USA). Sequences of plasmid DNAs were determined by means of an ALF express DNA sequencer (Pharmacia, Uppsala, Sweden) using a Thermosequenase fluorescent-labelled primer cycle sequence kit with 7-deaza-dCTP (Amersham, Buckinghamshire, UK) according to the manufacturer's protocol. A homology search against the Genbank database was done by using the BLAST program.

Results and discussion

Cell phenotypes of A549-derived cell lines

We previously established three cell lines named A5DC7, CK and AST-9, all of which are derived from the human lung adenocarcinoma cell line A549. A5DC7 cells were isolated from IFN- γ -treated A549 cells and showed normal senescent cell phenotypes with restored contact inhibition ability and anchorage-dependent growth as well as reduced tumorigenicity (Kawamoto et al., 1995). We also established CK cells from senesced A5DC7 cells at 106 PDL by raising the serum concentration from 5 to 10%. CK cells showed vigorous growth when cultured in 10% serum-containing medium, but not in 5%, and was also found to have regained telomerase activity and corresponding telomere maintaining ability as well as resumed

Table 2. Sequences of the subtracted cDNA clones

Tester	Driver	Tester/driver	Acc. #	Gene name (Clone name)
A5DC7	CK	0.5	M11167	Human 28S ribosomal RNA gene (7K-1)
		0.8	D50525	Human mRNA for TI-227H (7K-2)
		0.7	U25411	Human mitochondrial DNA regione (7K-3)
AST-9	CK	0.9	U76549	Human cytokeratin 8 mRNA, complete cds (QK-2)
		1.1	AF081484	Homo sapiens alpha-tubulin isoform 1 mRNA (QK-3)
		1.0	AF146651.1	Homo sapiens glyoxalase-I gene (QK-4)
		1.3	NM_000296.1	Homo sapiens polycystic kidney disease 1 (QK-5)
		1.3	NM_002018.1	Homo sapiens flightless I (Drosophila) homolog mRNA (QK-6)
		0.9	NM_001614.1	Homo sapiens actin, gamma 1 mRNA (QK-7)
		1.1	AW161411	EST sequence (QK-8)
		1.0	M59830	Human MHC class III HSP70-2 gene (QK-9)
		1.0	J04611	Human lupus p70 (Ku) autoantigen protein mRNA (QK-10)
		1.0	NM_006009.1	Homo sapiens Tubulin, alpha, brain-specific mRNA (QK-11)
		1.3	AI151262	EST sequence (QK-14)
		1.0	AI064986	EST sequence (QK-17)
		1.0	C17315	EST sequence (QK-18)
		1.0	NM_006263.1	Homo sapiens proteasome activator subunit 1 mRNA (QK-19)
		1.0	NM_00819.1	Human phosphoribosylglycinamide formyltransferase mRNA (QK-20)
		0.9	AC007078	EST sequence (QK-21)
		1.1	AF033950	Homo sapiens phospholipase D2 mRNA (QK-22)
		1.4	AC005522	EST sequence (QK-23)
		0.9	AC022221	EST sequence (QK-24)
AST-9	A549	0.7	X00351	Human mRNA for beta-actin (QA-3)
		0.8	X04106	Human mRNA for calcium dependent protease (QA-4)
		0.6	X16869	Human mRNA for elongation factor 1-alpha (QA-5)
		0.7	M16247	Human gamma-actin mRNA (QA-6)
		0.7	AB023165.1	Homo sapiens mRNA for KIAA0948 protein (QA-7)
		0.5	NM_004792.1	Homo sapiens Clk-associating RS-cyclophilin mRNA (QA-8)
		0.5	NM_005035.1	H. sapiens polymerase (RNA) mitochondrial mRNA (QA-9)
		0.8	D78345	Human DNA for Ig gamma heavy-chain (QA-10)
		1.0	NM_000988.1	Homo sapiens ribosomal protein L27 mRNA (QA-12)
		0.6	NM_007207.1	Homo sapiens dual specificity phosphatase 10 mRNA (QA-13)
AST-9	A5DC7	0.8	S42658	S3 ribosomal protein (human, colon, mRNA, 826 nt) (Q7-1)
		0.9	X54629	Human pHL-1 gene (Q7-2)
		0.5	NM_005556.1	Homo sapiens keratin 7 mRNA (Q7-5)
		0.3	NM_005538.1	Homo sapiens inhibin beta C mRNA (Q7-6)
		0.4	AC021342	EST sequence (Q7-7)
		0.4	U09196	Human mRNA upregulated in retinoic acid treated HL-60 cells (Q7-10)
CK	A5DC7	1.0	AA932850	EST sequence (K7-1)
		1.3	NM_001961.1	Homo sapiens eukaryotic translation elongation factor 2 mRNA (K7-2)
		1.5	AJ001443.1	Homo sapiens mRNA for SAP 130 spliceosomal protein (K7-3)
		1.2	NM_006115.1	Homo sapiens preferentially expressed antigen in melanoma mRNA (K7-4)
		1.1	U48252	Human protein kinase C-binding protein RACK8 mRNA (K7-5)

Table 2. (continued)

Tester	Driver	Tester/driver	Acc. #	Gene name (Clone name)
CK	A5DC7	1.0	AC023119	EST sequence (K7-7)
		1.0	X77584	H. sapiens mRNA for ATL-derived factor/thioredoxin (K7-8)
		1.0	M31013	Human nonmuscle myosin heavy chain mRNA, 3' end (K7-9)
		5.4	AC021854	EST sequence (K7-10)
		1.1	AC021028	EST sequence (K7-11)
		1.4	D78130	Homo sapiens mRNA for squalene epoxidase (K7-12)
		6.4		Novel gene (K7-13)
		9.1	AB019563.1	EST sequence (K7-14)
		1.3	M121575	Human cytochrome c oxidase COX subunit IV mRNA (K7-15)
		1.1	AA326035	EST sequence (K7-16)
		1.3	Z11531	H. sapiens mRNA for elongation factor-1-gamma (K7-17)
		1.1	W21593	EST sequence (K7-18)
		1.1	AA307843	EST sequence (K7-19)
		1.3	NM_005184.1	Homo sapiens calmodulin 3 (phosphorylase kinase, delta) mRNA (K7-20)
		1.4	M14630	Human prothymosin alpha mRNA (K7-21)
		1.5	NM_001398.1	Homo sapiens enoyl Coenzyme A hydratase 1 mRNA (K7-22)
		1.5	AF135266.1	Homo sapiens p8 protein homolog mRNA (K7-23)
		1.2	AI267521	EST sequence (K7-24)
		1.2	AI267770	EST sequence (K7-25)
A549	AST-9	1.2	AI267767	EST sequence (AQ-1)
		1.2	AF043739	Synthetic construct human telomerase catalytic subunit mRNA (AQ-2)
		1.0	AI123796	EST sequence (AQ-3)
		1.4	X05236	Human fibroblast mRNA for aldolase A (AQ-4)
		1.5	X57398	Human mRNA for pM5 protein (AQ-5)
		1.4	AC10246	EST sequence (AQ7-6)
		1.4	AC021038	EST sequence (AQ-7)
		1.4	AF159295.1	Homo sapiens serine/threonine protein kinase mRNA (AQ-8)
		1.7	AF092132.1	Homo sapiens PAK mRNA (AQ-10)
		1.6	Z48501	H. sapiens mRNA for polyadenylate binding protein II (AQ-11)
		1.6	AF035119	Homo sapiens deleted in liver cancer-1 mRNA (AQ-13)
		1.6	AB017114.1	Homo sapiens mRNA for AD 3 (AQ-14)
A549	A5DC7	2.7		Novel gene (A7-2)
		2.4	AC026668	EST sequence (A7-3)
		2.3	L33075	Homo sapiens ras GTPase-activating-like protein mRNA (A7-4)
		2.2	AF132973.1	Homo sapiens CGI-39 protein mRNA (A7-5)
		1.5	HSM800551	EST sequence (A7-6)
		2.0	AF091871	H. sapiens molybdopterin-synthase small and large subunit mRNA (A7-7)
		1.7	L10379.1	EST sequence (A7-10)
		1.8	AB002304	Human mRNA for KIAA0306 gene (A7-11)
		2.4	AI564998	EST sequence (A7-12)
		1.8	K10378	Human (clone CTH-B43a) mRNA (A7-14)
		2.3	AF005654	Homo sapiens actin-binding double-zinc-finger protein mRNA (A7-15)
		1.5	AF117958.1	Homo sapiens ferritin L subunit (A7-16)

proliferation (Katakura et al., 1997). Furthermore, we established AST-9 cells from A549 cells treated with 200 μ M of hydrogen peroxide for 4 days. Although AST-9 cells express almost the same level of telomerase activity as A549 cells, AST-9 cells showed passage number-dependent telomere shortening (manuscript in preparation). These cell lines are thought to be useful for identifying the genes involved in cellular senescence programs, maintenance of telomere length and telomerase regulation.

Firstly, we compared cell phenotypes of A549, A5DC7 (106 PDL), CK (129 PDL) and AST-9 (144 PDL) from the viewpoint of cellular senescence, that is, telomerase activity, the ability to maintain the telomere length during the culture, and expression of the senescence marker of β -galactosidase. Consequently, higher telomerase activity was detected in A549, CK and AST-9 cells, but not in A5DC7 cells. Telomere length estimated from the TRF length analysis of A5DC7 and AST-9 cells shortened as compared to that of parental cell line of A549 during the long-term culture, and that of CK cells lengthened as compared to that of senesced A5DC7 cells at 106 PDL in a manner dependent upon the number of passages. Furthermore, we analyzed the expression of mRNAs for telomerase components (hTERT and TEP1) by RT-PCR. hTERT is the catalytic subunit of human telomerase, and hTERT mRNA expression is known to be correlated with the telomerase activity (Nakamura et al., 1997). TEP1 is the homologue of the *Tetrahymena* telomerase-associated protein p80 (Harrington et al., 1997), and it is known to be constitutively expressed in both normal cells and cancer cells. Corresponding to the higher telomerase activity described above, hTERT mRNA was expressed at a high level in A549, CK and AST-9 cells but not in A5DC7 cells. TEP1 mRNA was constitutively expressed in all of these A549-derived cell lines (Table 1). These results demonstrated that the A549 cells and its sublines, A5DC7, CK and AST-9 cells have quite different phenotypes in terms of telomerase regulation, telomere length maintenance and cellular senescence programs, suggesting that specific gene products participating in regulation of these processes can be obtained by subtractive screening by using these cell lines.

Subtractive screening of genes participating in the cellular senescence programs

In this study, we have sought to identify the genes

overexpressed and/or specifically expressed either in A549, A5DC7 (96 PDL), CK (129 PDL) or AST-9 (144 PDL) cells by the PCR-select subtraction method, and obtained 86 independent cDNA clones (Table 2). Then, we performed homology search analysis against Genbank database by using BLAST program with regard to those cDNA clones obtained by subtractive screening. As a result, 58 cDNA clones were found to have high homology to known sequences of cDNAs or genes deposited in the Genbank database. 26 cDNA clones had homology to sequences deposited as EST in the Genbank database. Furthermore, the cDNA clones named K7-13 and A7-2 did not show any significant homology to the sequences deposited in the Genbank database including the EST database.

Expression profile analysis of subtracted clones by DNA macroarray analysis

We compared expression levels of subtracted cDNA clones by dot blot hybridization to select for clones for further analysis. As a result of quantitative analysis, 10 cDNA clones such as K7-10, K7-13, K7-14 (tester: CK, driver: A5DC7), A7-2, A7-3, A7-4, A7-5, A7-7, A7-12 and A7-15 (tester: A549, driver: A5DC7) were found to be more than 2-fold higher expressed in tester cells than in driver cells (Table 2). Considering together with that both tester cells have cancer cell phenotypes and that driver cells of A5DC7 cells have normal cell phenotypes, these specifically expressed cDNA clones code for genes whose products play an important role in maintaining cancer cell phenotypes. For example, Ras GTPase-activating-like protein corresponding to A7-4 interacts with Cdc 42, which plays an important role in cell adhesion (Kuroda et al., 1998) and actin-binding double-zinc-finger-protein corresponding to A7-15 is involved in maintaining cellular structure (Roof et al., 1997). Their up-regulation in A549 cells may be linked to maintenance of cancer cell phenotypes. However, the participation and/or the roles of the remainder of the corresponding cDNA or genes in cellular senescence programs and/or maintenance of cancer cell phenotypes have not been clarified. We might identify the novel functions of these known cDNA or gene products in cellular senescence by functional analysis of these cDNA or gene. Furthermore, specifically expressed cDNA clones (K7-10, K7-13, K7-14, A7-2, A7-3 and A7-12) corresponding to 2 novel genes and 4 ESTs may have novel functions in telomerase regulation, telomere maintenance and

cellular senescence, which might be clarified by acquisition of the corresponding full-length cDNA and subsequent functional analysis.

Although there are several discrepancies between the expression profiles and the origins of these subtracted cDNA clones, that is, the case that the ratio of tester to driver is below 1.0, this was thought to be due to the highly expression of the cDNA clones and their overloading to the subtractive screening. Furthermore, although cDNA clones corresponding to genes playing an important or indispensable roles in cancer cells, cellular senescence, telomerase regulation and/or telomere maintenance, for example TI-227H (7K-2), Ku70 (QK-9), elongation factor 1 α (QA-5), preferentially expressed antigen in melanoma (K7-4) and hTERT (AQ-2) (Ishiguro et al., 1996; Jeng et al., 1999; Welle et al., 1997; Ikeda et al., 1997; Kim et al., 1994) were obtained by subtractive screening, their expression profiles did not change against our expectations. This might be due to our use of origin-identical cells for expression profile analysis. More accurate estimation of expression profiles of these cDNA clones might be possible by using origin-different normal senescent cells of TIG-1 cells, thereby we believe that we can obtain more cDNA clones which specifically express in senescent cells or cancer cells and play an important roles in cellular senescence, telomerase regulation and/or telomere maintenance.

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