[CANCER RESEARCH 61, 4536–4540, June 1, 2001]

Detection of Differentially Expressed Genes in HeLa × Fibroblast Hybrids Using Subtractive Suppression Hybridization[1]

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ABSTRACT

To understand genetic differences and similarities between tumorigenic and nontumorigenic HeLa × fibroblast hybrid cells, subtractive suppression hybridization (SSH), based on suppression PCR and a combination of normalization and subtraction in a single procedure, was used. Using the nontumorigenic CGL1 and tumorigenic CGL3, forward (CGL1-CGL3) and reverse (CGL3-CGL1) subtracted libraries were constructed. Among 192 clones, seven were identified as differentially expressed genes specific for either CGL1 or CGL3. All seven were not reported previously as differentially expressed genes in this hybrid system. In the forward subtraction, *p16* was isolated, indicating the involvement of the loss of tumorigenic phenotype. Subsequent transfection of wild-type *p16* to the tumorigenic CGL3 showed growth suppression in colony formation assay; however, no tumor suppression was observed when the transfectant was inoculated into nude mice. These results indicate that: (*a*) SSH is a suitable method to identify differentially expressed genes in two types of cells; and (*b*) although p16 plays some roles in growth suppression, the p16-transfected CGL3 is still capable to proliferate *in vivo*.

INTRODUCTION

Somatic cell hybrids, generated by fusing together two different cells of the same species, have been extremely useful in the study of genetic analysis of their malignant potential (1). Fusions between tumorigenic cells, such as HeLa, and normal cells, such as human diploid fibroblasts, result in hybrids that are genetically stable and behave as transformed cells but are suppressed with respect to their tumorigenic potential (2). Previous studies have revealed that extensive correlation exists between the ectopic expression of the cell surface marker IAP[4] and their tumorigenicity (3). In addition, cytogenetic studies have shown that chromosomes 11 and 14 were potential sites of tumor suppressor loci, and microcell-mediated transfer of a chromosome 11 into the tumorigenic segregants suppressed tumorigenicity (4, 5). This evidence suggests that at least some necessary genetic events for controlling their tumorigenic phenotypes may exist. However, comprehensive genetic mechanisms responsible for the phenotypic change still remain to be clarified.

To isolate novel differentially expressed genes, SSH, DD, and serial analysis gene expression are the most commonly used techniques. In a previous study, as the first attempt for the identification of genes differentially expressed between the nontumorigenic CGL1 and tumorigenic CGL3, we used DD (6). Although we isolated 17 differentially expressed genes by DD, it has also been speculated that there were substantial number of genes that cannot been detected by DD because we failed to detect *IAP* gene, the ectopic expression of which is invariably associated with the tumorigenic phenotype of these hybrid cells. It has been reported that the genes detected by DD are not necessarily detected by the other methods (*e.g.*, SSH) as well because the genes found by DD are dependent upon the primer pair used (7). In the context of identification of the genes differentially expressed between CGL1 and CGL3, we used a PCR-based cDNA subtraction technique, SSH, to construct differential gene expression libraries from the hybrid cells. The SSH is a combination of subtraction and kinetic enrichment coupled to subsequent amplification, which enables one to compare two different populations of mRNA and obtain clones of genes that are expressed in one population but not in the other (8). This technique has proven to be useful in identifying tissue-specific and low abundance transcripts because it can achieve >1000-fold enrichment for differentially expressed cDNA populations through a process that normalizes sequence abundance during the course of subtraction by standard hybridization kinetics (7, 8). Recent study revealed that rare mRNAs (fewer than five copies/cell) comprise the majority of total species of transcripts, and they constitute only a small portion of the total number of transcripts, whereas a small number of mRNAs expressed at a high level represent a large portion of the total transcripts (9). To increase the representation of the rare mRNA species for the identification, an effective method, such as normalization and subtraction, is needed. Thus, SSH can be applied for the detection of phenotype-restricted differentially expressed genes and those that have not been detected by DD between the nontumorigenic CGL1 and tumorigenic CGL3. In fact, we have isolated *p16*, which has not been reported as a differentially expressed gene in the hybrid system in the literature, including our previous DD analysis. Inactivation of the *p16* gene in many tumors strongly suggests a role in the regulatory programs disturbed during cellular transformation (10). We selected p16 for a functional assay to understand the significance of differentially expressed gene. Here we describe the usefulness of SSH, and a possible role of the *p16* gene in this hybrid system in the context of the tumorigenic phenotype will also be discussed, based on evidence obtained by means of colony formation and tumorigenicity assay of p16-transfected CGL3.

MATERIALS AND METHODS

Cell Lines and Culture. The derivation of CGL1 and CGL3 from a hybrid of the HeLa variant D98/AH2 and a normal fibroblast cell strain GM77, ESH5, was described previously (4). CGL1 is not tumorigenic when inoculated s.c. into nude mice, and CGL3 is a spontaneous tumorigenic segregant of CGL1. Restriction fragment polymorphism analysis had revealed that CGL1 has four copies of chromosome 11, whereas CGL3 has lost one copy of them (11). Both cell lines were grown in DMEM supplemented with 10% calf serum and l% glutamine and were regularly monitored for *Mycoplasma* contamination and found to be negative. Cells at ~80% confluence were harvested.

SSH. SSH was performed between CGL1 and CGL3 using the PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer's recommendations. In the forward subtraction, CGL1 was used as tester and CGL3 as driver (CGL1-CGL3), whereas in the reverse subtraction, CGL1 was used as driver and CGL3 as tester (CGL3-CGL1). Total RNA was extracted by the conventional acid guanidinium thiocyanate:phenol:chloroform method, and poly(A)$^+$ RNA was then isolated with FastTrack 2.0 (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 2 μg of poly(A)$^+$

Received 10/26/00; accepted 4/27/01.

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[1] This work was supported by National Cancer Institute Grant CA 19104.

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[4] The abbreviations used are: IAP, intestinal alkaline phosphatase SSH, suppressive subtractive hybridization; DD, differential display; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; CMV, cytomegalovirus; EST, expressed sequence tag; RDA, representational difference analysis.

RNA by oligo(dT) priming using AMV reverse transcriptase, and second-strand cDNA was generated with T4 DNA polymerase. The restriction endonuclease *Rsa*I was used to obtain shorter and blunt-ended fragments. Two tester populations were created with different adaptors, but no adaptors with driver; the adaptors were used for subsequent PCR amplification. The first hybridization was carried out with each tester population and the driver. Hybridization kinetics led to equalization and enrichment of differentially expressed sequence. The second hybridization generates templates for PCR amplification from differentially expressed sequences between tester and driver. We carried out 27 cycles of primary PCR and 11 cycles of secondary PCR with the Advantage cDNA polymerase mix (Clontech). To evaluate the efficiency of cDNA subtraction, we compared the expression levels of G3PDH by reverse transcription-PCR in subtracted and unsubtracted cDNA populations. In addition, cDNA Southern blot hybridization was performed to confirm that the subtraction was done successfully with subtracted and unsubtracted control cDNA populations using *collagen I-*α_2 and *H19* probes, which had already been known as differentially expressed genes in CGL1 and CGL3, respectively (6). The subtracted fragments were then inserted into the T/A cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). Individual transformants carrying subtracted cDNA fragments were isolated from white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/isopropyl-1-thio-β-D-galactopyranoside agar plates.

Differential Screening. A total of 192 (96 each for forward and reverse subtracted library) individual recombinant clones were picked and used to inoculate 96-well microtiter plates containing 100 μl of LB medium and 50 μg/ml of ampicillin at 37°C for 4 h with shaking. One μl of the growing culture was transferred to 0.2 ml of PCR reaction tube containing the master mix with secondary PCR primers and used as the PCR template. After appropriate cycles of PCR amplification, the PCR products were electrophoresed on a 1.0% agarose/ethidium bromide gel to confirm that each recombinant had the proper insert. For denaturing purposes, each PCR product was then mixed with equivalent volume of 0.6 N NaOH. Two μl of denatured PCR product were transferred to a nylon membrane. A set of two identical membranes consisting 96 dots was prepared with forward/reverse subtracted libraries and then hybridized with ^{32}P-labeled forward/reverse and subtracted/unsubtracted cDNA probes. The dot blot hybridization was carried out according to the manufacturer's protocol (PCR-Select Differential Screening kit; Clontech). The results of the blot was divided into four categories: (*a*) clones that hybridized to the forward subtracted probe but not to the unsubtracted probe; (*b*) clones that hybridized equally to both probes; (*c*) clones that hybridized to both probes but the intensity of the hybridization signals was different; and (*d*) clones that did not have a detectable hybridization signal for either of the probes. Groups of (*a*) and (*c*) were regarded as positives, and these corresponding clones were prepared for Northern blot.

Northern Blot and Direct Sequencing. Thirty μg of total RNA or 2 μg of poly(A)$^+$ RNAs were fractionated on 1% agarose formaldehyde gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The probes were generated by PCR using secondary primers from the colonies corresponding to the dots shown to be differentially expressed by dot blot differential screening. After PCR amplification, fragments were extracted from the gel and purified for labeling. The membranes were hybridized overnight to ^{32}P-labeled probes generated with Strip-EZ DNA Random Primed Probe Synthesis kit (Ambion, Austin, TX). After selecting true-positive clones by Northern blot with PCR fragments as probes, the isolated PCR fragments were then sequenced with an automated DNA sequencer, ABI Prism model 377 (Applied Biosystems, Foster City, CA).

Stable Transfection of Wild-Type p16. The CGL3 cells were seeded into 10-cm Petri dishes at a density that resulted in ~60% confluence after 24 h. Structure of expression vector pCMV.p16.CITE.IAP was described previously (12). The CGL3 cells were stably transfected with either pCMV.CITE.IAP or pCMV.p16.CITE.IAP after *Not*I digestion using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Twenty-four h after transfection, cells were passaged for selection at a dilution of 1:5 into medium containing Hygromycin at the concentration of 450 units/ml. After selection, plasmid-containing colonies were pooled, and stable transfectants were generated. Colonies were then isolated, placed in basal medium, and propagated prior to analysis.

Western Blot. Cells were lysed in a buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0), and 1 mM phenylmethylsulfonyl fluoride. Samples were electrophoresed on a 10% SDS-polyacrylamide gel, which was then transferred onto Immobilon-P nylon membrane (Millipore, Bedford, MA), incubated with p16 c-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution, followed by peroxidase-conjugated antirabbit HRP IgG (Santa Cruz Biotechnology), and developed with the SuperSignal Chemiluminescent detection system (Pierce, Rockford, IL) according to the manufacturer's protocol. As an internal control, β-actin expression was used with the anti β-actin antibody (Sigma, St. Louis, MO) at a 1:5000 dilution. To confirm equal loading of proteins, a second identical gel was simultaneously subjected to electrophoresis for Coomassie blue staining (Bio-Rad Laboratories, Hercules, CA).

Colony Formation Assay. Cells (2×10^5) were plated in six-well tissue culture plates 24 h prior to the transfection. The cells were transfected with 0.4 μg of either pCMV.CITE.IAP or pCMV.p16.CITE.IAP after *Not*I digestion and allowed to incubate for 24 h using the Effectene procedure. Selection for hygromycin-resistant colonies was started 24 h after transfection. Fourteen days after seeding, cells were stained with 0.05% crystal violet containing 50% methanol. Colonies of ~10 or more cells were counted.

Tumorigenicity Assay. Female athymic nude mice, 6–10 weeks of age, were used to assay tumor formation. For each cell populations, at least six sites (two sites/mouse) were inoculated s.c. with approximately 1×10^7 cells suspended in 0.2 ml of DMEM. The mice were monitored regularly for tumor formation, and palpable nodules were measured with calipers.

RESULTS

Evaluation of Subtraction Efficiency. The selectivity of the SSH method was evaluated using a manufacturer's instructions using a model system with a reconstituted artificial tester and revealed that the SSH was carried out with highly selectivity (data not shown). Subsequently, the subtraction efficiency was evaluated by G3PDH abundance as an indicator. Fig. 1*A* shows reduction of G3PDH abundance in subtracted population, whereas in the unsubtracted population, the corresponding bands appeared in earlier cycles. To see whether differentially expressed genes in subtracted sample have been enriched, cDNA Southern blot with subtracted and unsubtracted samples was carried out. The *collagen I-*α_2 and *H19* genes were known as differentially expressed genes in nontumorigenic and tumorigenic segregants, respectively (6). We used these fragments as probes, and the Southern blot revealed that SSH generated a significant enrichment of each differentially expressed genes in subtracted populations (Fig. 1*B*).

Differentially Expressed Genes. Each subtracted clone was used as a PCR template, and a set of two identical membranes consisting of 96 dots generated from the PCR products was prepared for dot blot hybridization. Fig. 2*A* compares the hybridization signals of two differential screening approaches (subtracted and unsubtracted) with duplicated membranes. The sensitivity of this procedure was increased by screening with subtracted probes and faint signals representing rarely transcribed differentially expressed genes could be distinguished. Positive clones were picked according to their hybridization signal intensity and used to inoculate new 96-well master plates for further analysis, including Northern and direct sequencing. In forward and reverse subtraction, 18 dots were designated as positive clones comprising categories *a* and *c* according to the criteria described in "Materials and Methods." To screen "false-negative," additional 20 dots that had been designated into category *b* were picked randomly. Finally, the Northern blot analysis revealed 9 fragments were positive in the forward subtracted clones, with redundancy (Fig. 2*B*). Hence, the total number of genes designated as differential expression was 9.4%, which is in close agreement with previous reports (12–14). The "true-positive" detection rate was significantly higher among the clones designated into categories *a* and *c* ($P < 0.05$;

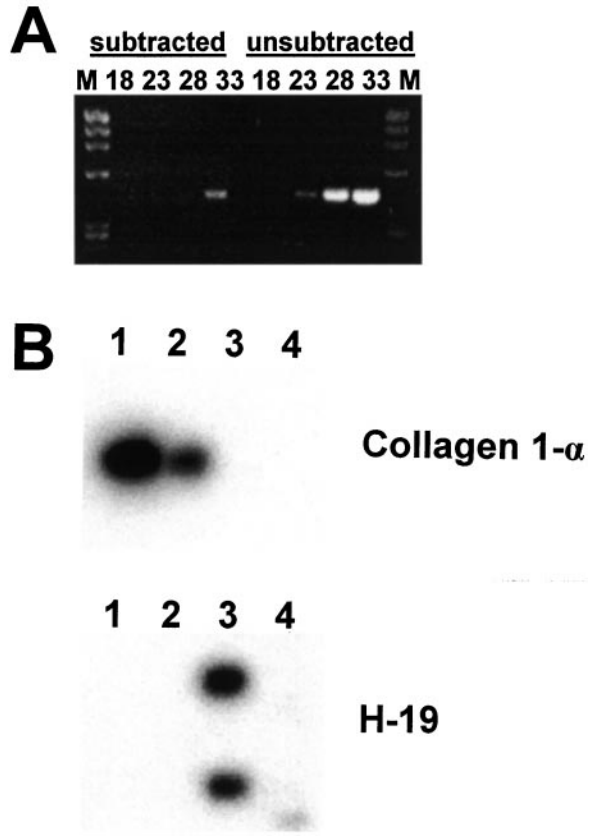

Fig. 1. Evaluation of subtraction efficiency. *A*, an example of *G3PDH* reduction in a successfully subtracted reaction by PCR analysis is shown on the *left*. PCR was performed on subtracted and unsubtracted secondary PCR product with *G3PDH* primers. A decrease in *G3PDH* abundance in the subtracted sample can be seen. *Lane M*, size marker. The numbers on the *top* correspond with the number of PCR cycles of this analysis. *B*, as positive controls for the enrichment of differentially expressed genes (9), *collagen I-*α_2 and *H19* is expressed in CGL1 and CGL3, respectively. After secondary PCR amplification, Southern blot hybridization was performed with these probes. The abundance of these up-regulated cDNAs is increased. *Lane 1*, forward subtracted cDNA; *Lane 2*, forward unsubtracted cDNA; *Lane 3*, reverse subtracted cDNA; *Lane 4*, reverse unsubtracted cDNA. CGL1 and CGL3 were used as tester and driver, respectively, in *Lanes 1* and *2*. CGL3 and CGL1 were used as tester and driver, respectively, in *Lanes 3* and *4*.

2 × 2 contingency tables). Therefore, this dot blot screening proved to be useful for this experimental system. The same screening was also carried out in the reverse subtraction. The differentially expressed genes of both directions were shown in Table 1. We identified seven genes that have not been identified by other methods, including the previous DD analysis as differentially expressed genes in tumorigenic and nontumorigenic hybrid cells (6). It has been reported that nearly one-half of subtraction library clones are nonredundant (7). It is getting harder to isolate unique genes because the frequency of a particular cDNA approaches 1/total colonies. Although we failed to detect either *collagen-I*α_2 or *H19* from the clones we analyzed, the genes found as differentially expressed in DD, it may be because of the low probability of detecting a particular gene in the library. However, it should be noted that using known differentially expressed genes is still useful for confirming whether the subtraction has been successful.

Establishment of Stable Transfectant. An expression vector pCMV.p16.IAP and control vector pCMV.CITE.IAP bearing a hygromycin resistance gene were transfected to the CGL3 cells. As shown in Fig. 3*A*, both CGL1 and CGL3 expressed the p16 protein before transfection, although the level in CGL1 was higher than that in CGL3. To obtain a high level of expression of exogenous p16 protein, the human CMV promoter was used to drive the expression of the *p16* gene (12). A high level of exogenous p16 protein expression was achieved in clone 19, whereas CGL3 transfected with vector alone showed the same level of endogenous p16 (Fig. 3*A*). The successful transfectants were prepared for subsequent tumorigenicity assay.

Suppressed Clonogenicity in p16-Transfected CGL3. Northern blotting of B-8 (forward) clone exhibited a differential expression between CGL1 and CGL3, and this difference was attributable to a p16 partial cDNA. This p16 cDNA fragment was 303 bp containing the exon 3 sequence, which enabled us to distinguish if from $p19^{ARF}$, the splicing variant of p16 (16). Genetic evidence has revealed that inactivation of p16 plays a critical role in the regulatory programs disturbed during cellular transformation (10). The *p16* gene is frequently mutated in familial melanomas, and a high frequency of its inactivation in broad range of human malignancies has been observed (10, 17). This evidence led us to examine whether the *p16* gene has tumor-suppressive function toward the tumorigenic phenotype. To address whether the *p16* gene is involved in the tumorigenic phenotypic change in the HeLa × fibroblast hybrid cell system *in vitro*, we performed colony formation assays in CGL3 cells. Transfection of CGL3 cells with p16 resulted in substantially reduced clonogenicity (47 ± 11%) in culture compared with the vector-transfected CGL3 (Fig. 3*B*). The magnitude of this suppression was comparable with previous studies (18, 19).

Tumorigenicity in Nude Mice. To determine whether p16 can suppress tumorigenicity of CGL3 *in vivo*, female athymic nude mice, 6 weeks of age, were given s.c. injections of the HeLa × fibroblast hybrids including the nontumorigenic CGL1, tumorigenic CGL3, vector-transfected CGL3, and p16-transfected CGL3. Each mouse received two injections of 1×10^7 cells of the above four cell types. There was no significant difference of tumorigenicity among CGL3 and the transfectants (Fig. 3*C*). The nontumorigenic CGL1 cell showed no tumors up to 5 weeks from the inoculation date.

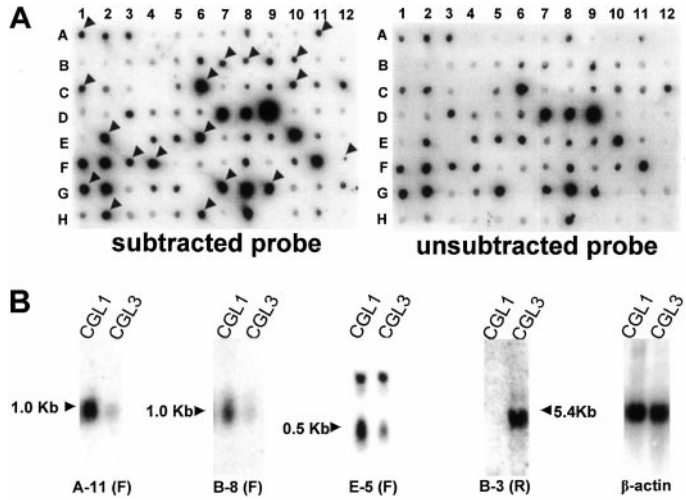

Fig. 2. Differential screening for the forward subtracted library. *A*, 96 randomly selected colonies were amplified from the TA cloning vector using adaptor-specific primers. After duplication of these membranes derived from forward subtracted (CGL1-CGL3) products, each of them was hybridized with two different radiolabeled probes; *left*, forward subtracted probes; *right*, forward unsubtracted probes. *Arrows*, the positive clones of this screening. The same procedure was performed for the reverse subtracted cDNAs. *B*, clones with differential signal in the screening was used for each probe. The probes detected differentially expressed transcripts with Northern blotting. Each lane contains 1.0 μg of poly(A)$^+$ or 25 μg of total RNA from CGL1 and CGL3. *Arrowheads*, four representative SSH fragments differentially expressed in either CGL1 or CGL3. The *letter* and *number* at the *bottom* of each picture is the clone ID. *F*, forward subtracted probes; *R*, reverse subtracted probes. β-actin probe confirmed that lanes were loaded equally.

Table 1 *Genes identified differentially expressed by SSH*

Clone number	Fragment size	Description	mRNA size	Unigene
		A. Forward subtracted clones (expressed in CGL1)		
A-11, E-6, G-7	600	Ribosomal protein L7a	1.0	Hs.99858
B-7, D-3	300	Ribosomal protein L18	0.7	Hs.75458
B-8	420	Cyclin-dependent kinase inhibitor 2A	1.0	Hs.1174
E-5	400	S100 calcium-binding protein A6	0.5[a]	Hs.275243
F-3	250	Breakpoint cluster region protein uterine leiomyoma, 1; barrier to autointegration factor	0.8[a]	Hs.268763
G-1	650	Eukaryotic translation elongation factor 1γ	1.9	Hs.2186
		B. Reverse subtracted clones (expressed in CGL3)		
B-3, H-6	720	Carbamyl-phosphate synthetase 1	5.4[a]	Hs.50966
F-1	400	Carbamyl-phosphate synthetase 1	5.4[a]	Hs.50966

[a] The probe hybridized with one or more additional transcripts that were not differentially expressed.

DISCUSSION

For a better understanding of cellular behavior, the identification of genes differentially expressed in a certain type of cell has been a major goal in recent biological research. The EST project and Cancer Genome Anatomy Project are two major EST sequencing efforts to identify all of the expressed human genes. However, the rate of novel gene identification through the EST project had declined dramatically from 10.6% of EST sequences in 1996 to only 2.7% of EST sequences collected in 1998, despite the fact that many expressed genes still were unidentified (20). This indicates that the remaining unidentified genes are becoming progressively harder to find as EST sequencing continues, because they are of progressively lower abundance or are more cell type restricted (21). In a given cell, most unique transcripts at fewer than five copies/cell are expressed at low levels, with <25% of the mRNA mass of the cell comprising 95% of total transcripts, and nearly one-half of the unique transcripts have not been identified (9). It is therefore important to note that even such a small abundant genes may have the potential to include many of the most biological significance.

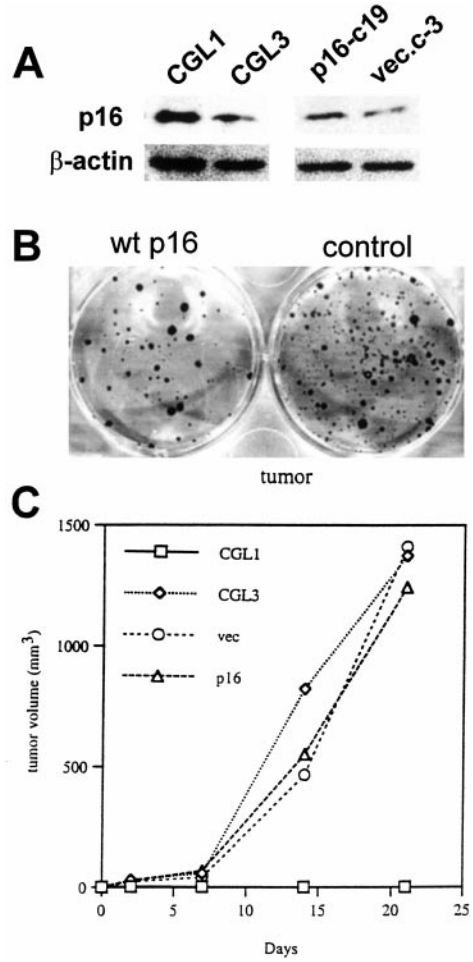

Fig. 3. Functional assay of wild-type p16 in the tumorigenic CGL3. *A*, Western blot analysis of p16 in the nontumorigenic CGL1, tumorigenic CGL3, wild-type p16 transfected CGL3 (clone 19), and control vector-transfected CGL3 (clone 3). A higher level of p16 expression was observed in p16-c19 than the endogenous p16 level in CGL3. *β*-Actin expression was used for an internal control. *B*, colony formation assay. Suppressed clonogenicity in wild-type p16-transfected CGL3 was observed. *C*, tumorigenicity of the nontumorigenic CGL1, tumorigenic CGL3, p16-transfected, and vector-transfected CGL3. In each case, 1×10^7 cells were inoculated s. c., and tumor masses were measured periodically over a 25-day period. There was no significant difference in CGL3 and its transfectants. No tumor was observed in the nontumorigenic CGL1.

There are some commonly used PCR-based methods that have been shown to be useful methods to isolate genes differentially expressed between two different types of cells. RDA, a process of subtraction coupled to amplification, was initially developed by Lisitsyn *et al.* (22) for genomic DNA to isolate differences between complex genomes. Subsequently, RDA protocols were adapted to the examination of differential gene expression between two mRNA populations (23). Diatchenko *et al.* (8) developed a new PCR-based cDNA subtraction method, SSH, which is essentially similar to RDA but is based on the suppression PCR effect. The SSH has overcome the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes sequence abundance during the course of subtraction by standard hybridization kinetics. It has also been reported that the SSH method can detect differently expressed mRNAs with low prevalence (7, 14).

A previous comparative study among electronic subtraction, SSH, and DD revealed the low amount of overlap between all three experiments, which suggests that many more differentially expressed mRNAs are yet to be identified, with low prevalence in particular (7). It is, likely, therefore, to find some other differentially expressed genes with different approaches, such as SSH and DD. On the other hand, in contrast to DD, SSH is basically designed for cDNA library construction. Most cDNA libraries currently used for the genome-wide gene identification are generated exclusively through oligo(dT) priming through reverse transcription (20). However, there is very rare chance of identifying low-abundance genes with direct sequence alone. Therefore, normalization (equalization) by subtraction is considered to be a suitable technique to reduce the high abundance copies and to increase the representation of the low-abundance copies to identify the genes expressed at low level. Recently, Wang *et al.* (20) reported that the random hybridization can occur anywhere along to the poly(dA) and poly(dT) sequences during the normalization/subtraction process because of presence of 3′ poly(dA/dT) sequences in

cDNA templates and therefore can result in loss of templates. We identified seven genes using SSH, none of which has been reported as differentially expressed and were not detected in previous DD analysis (6). In previous studies, investigators have reported that they did not detect the expected differentially expressed genes with SSH (13, 24). This subtraction process makes it possible to isolate different sets of differentially expressed genes; however, it should also be noted that templates may be lost during the subtraction process. These facts along with our results suggest that there may be different biases associated with each technique (13); thus, SSH and DD could possibly be useful complimentary techniques. Although neither SSH nor DD could detect *IAP*, the expression of which is most tightly linked to tumorigenic phenotype, recent study with oligonucleotide microarray has demonstrated that the *IAP* expression level is invariably correlated to the tumorigenic phenotype in this system (25).

It has been reported that wild-type p16 transfection resulted in significantly reduced clonogenicity in various types of cell lines (18, 19). Examination of cell growth of adenovirus-p16 infected human non-small cell lung carcinoma cell lines showed an inhibition of cell proliferation of the cells lacking wild-type p16 protein expression and not of the cells carrying the wild-type p16 (26). The tumorigenic CGL3 has a substantial level of the wild-type p16 protein expression, although this level is lower than that seen in the nontumorigenic CGL1 (Fig. 3*A*). In addition, PCR-microsatellite analysis did not show homozygous deletion on chromosome *9p21* (data not shown). These facts led us to examine whether a higher level of p16 in CGL3 causes a tumor-suppressive effect. Although the wild-type p16 transfection to CGL3 resulted in a clear growth inhibition, no tumor suppression was observed when the p16-transfected CGL3 was injected into nude mice. This might indicate that transfectants expressing a higher level of p16 were suppressed in growth, but the transfectants are still capable of proliferating *in vivo*. In previous observations, it was found that both nontumorigenic hybrids and their tumorigenic segregants were able to grow and form colonies, with the tumorigenic lines being the most efficient (26). The present study revealed that the level of p16 is different between CGL1 and CGL3, and p16 transfection resulted in growth suppression of CGL3 *in vitro*. Furthermore, we have examined Rb function in these hybrids, and no perturbation of Rb function was observed.[5] Therefore, it has been suggested that the p16 expression plays some roles in this system, at least.

We demonstrated that SSH can be a useful method for the identification of differentially expressed genes. However, SSH still requires labor and a time-consuming screening step to identify "true positive" among the subtracted clones. Recent technological advances, such as DNA microarrays, have enabled one to screen a large number of known genes, which is now capable of aligning an equivalent number of colonies obtained with an SSH for the screening. It has been reported that the combination of SSH and cDNA microarray can be applied successfully to identify differentially expressed genes including novel sequence (15). 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 with SSH will detect the rest of cell-specific or low-abundance unidentified genes, but *in vivo* studies are still necessary to elucidate the biological functions of differentially expressed genes.

ACKNOWLEDGMENTS

We thank Dr. Sun Chi for technical assistance and Drs. Luda Diatchenko and Yen Nguyen for critical comments on the manuscript.

[5] E. J. Stanbridge *et al*., unpublished data.

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