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Yeast functional assay of the p53 gene status in human cell lines maintained in our laboratory*

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Abstract

We used a yeast functional assay (functional analysis of separated alleles in yeast: FASAY) to determine the p53 gene status of human cell lines maintained in our laboratory. This assay enables the researcher to score wild-type p53 expression on the basis of the ability of expressed p53 to transactivate the reporter gene HIS 3 via the p53-responsive GAL 1 promoter in Saccharomyces cerevisiae. The cell lines examined were ten hepatoma, two hepatoblastoma, three in vitro immortalized fibroblast, two osteosarcoma, a chondrosarcoma, an ovarian teratocarcinoma and a colon cancer cell line. Out of 20 cell lines, 11 cell lines had mutations in both alleles of the p53 gene, and another 8 cell lines had no mutation in the p53 gene. Thus, 55% of the cell lines examined had mutations in the p53. Interestingly, PA-1 cells had both the normal and the mutant p53 alleles, showing that FASAY is a useful method for detecting the wild-type and mutated p53 genes simultaneously. As for the three liver cell lines harboring HBsAg, there was no relationship between their p53 gene status and the presence of HBsAg. Two cell lines were normal for p53 status, while the other had a mutation of the p53 gene.

KEYWORDS: p53 mutation, FASAY, cultured human cells

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Yeast Functional Assay of the p53 Gene Status in Human Cell Lines Maintained in Our Laboratory

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We used a yeast functional assay (functional analysis of separated alleles in yeast: FASAY) to determine the p53 gene status of human cell lines maintained in our laboratory. This assay enables the researcher to score wild-type p53 expression on the basis of the ability of expressed p53 to transactivate the reporter gene *HIS 3* via the p53-responsive *GAL 1* promoter in *Saccharomyces cerevisiae.* The cell lines examined were ten hepatoma, two hepatoblastoma, three *in vitro* immortalized fibroblast, two osteosarcoma, a chondrosarcoma, an ovarian teratocarcinoma and a colon cancer cell line. Out of 20 cell lines, 11 cell lines had mutations in both alleles of the p53 gene, and another 8 cell lines had no mutation in the p53 gene. Thus, 55% of the cell lines examined had mutations in the p53. Interestingly, PA-1 cells had both the normal and the mutant p53 alleles, showing that FASAY is a useful method for detecting the wild-type and mutated p53 genes simultaneously. As for the three liver cell lines harboring HBsAg, there was no relationship between their p53 gene status and the presence of HBsAg. Two cell lines were normal for p53 status, while the other had a mutation of the p53 gene.

Key words: p53 mutation, FASAY, cultured human cells

We have established many human cell lines from hepatoma, hepatoblastoma, colon cancer, chondrosarcoma and osteosarcoma cells. In addition, we have been able to immortalize normal human fibroblast cells by treatment with 4-nitroquinoline1-oxide or ^{60}Co gamma rays. Details of these cell lines are shown in Table 1. These cell lines are now deposited in the Japanese Cancer Research Resources Bank (JCRB) and available to the scientific community. For the convenience of the users of these cell lines, further study is needed to fully characterize these cell lines. Thus, examination of the p53 gene status in these cell lines will be of value, because more than 50 % of human malignant tumors have mutations in the p53 gene (for reviews, 1 and 2). Recent studies indicate that the p53 protein is involved in gene transcription, DNA synthesis and repair, senescence, genome plasticity, and in programmed cell death (for reviews, 1 and 2).

To detect mutations in the p53 gene, there are several methods, such as single strand conformation polymorphism (SSCP) analysis (3), sequencing amplified p53 genomic fragments and cDNA of the reverse transcribed (RT) mRNA by polymerase chain reaction (PCR). However, it is time consuming to analyze all the coding regions of the p53 gene by these methods. Furthermore, frequent appearance of artificial bands makes it difficult to determine whether or not the abnormal bands are bona fide mutations of the p53 gene. The status of the p53 gene can also be detected by assaying the transcriptional activity of the p53 protein expressed in *Saccharomyces cerevisiae.* This functional analysis requires only a few steps, can be readily automated, and enables one to identify the wild-type and the mutated p53 gene separately (4). By this method, we tested the p53 gene status of 20 human cell lines, 12 of which were established in our laboratory. Herein, we show that 55 % of these cell lines have mutations in the p53 gene.

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Materials and Methods

Cells and cultures. Cells used are summarized in Table 1. Hepatoma cell lines were maintained in Dulbecco's modified medium and the other cell lines in Eagle's minimum essential medium. Each defined medium was supplemented with heat inactivated fetal bovine serum at a concentration of 10%. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured with 0.2% trypsin (1:250, Difco, Detroit, MI, USA) plus 0.02% EDTA solution in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline when the cells became confluent in culture dishes.

RT-PCR. Reverse transcribed cDNA from cellular RNA extracted by the acidic guanidine isothiocyanate method was treated with RNaseA, purified by phenol/chloroform extraction, and precipitated with ethanol in the presence of glycogen as a carrier. The cDNA from 2 μg RNA was amplified by 30 cycles of polymerase chain reaction (PCR). We used the thermostable polymerase enzyme *Pfu* (Stratagene, La Jolla, CA, USA) which possesses a proofreading characteristic which reduces mutations caused by polymerase error (5).

FASAY method. FASAY was carried out as described previously (4). Briefly, we used a yeast strain ySS 5 containing the reporter plasmid (pSS 1) encoding the *HIS* 3 gene which was expressed under a p53-responsive promoter and a *TRP* 1 selectable marker. As the *HIS* 3 gene is located downstream of a p53 responsive promoter, it can be expressed only when the normal p53 protein binds to the promoter. A p53 expression vector pSS 16 containing a *LEU* 2 selectable marker was digested with the restriction enzymes *Hind* III and *Stu* I, to form a gap between the codons 66 and 348 of the p53 coding region. PCR amplified p53 cDNA containing all the coding sequence was co-transformed with the p53 expression gap vector. Homologous recombination of the p53 cDNA and the gap vector resulted in constitutive expression of the *LEU* 2 selectable gene and allowed yeasts to grow on a plate lacking the amino acids leucine and tryptophan (leu-, trp-). The plate was incubated at 30°C for 2 days. Yeast colonies grown on the leu- and trp- plate were transferred to a plate lacking histidine, leucine and tryptophan (his-, leu-, trp-) and grown at 37°C for 2 more days. Since the *HIS* 3 gene is expressed in a wild-type p53-dependent manner, the colonies transformed with the wild type p53 were large, whereas the colonies with the mutant p53 were small.

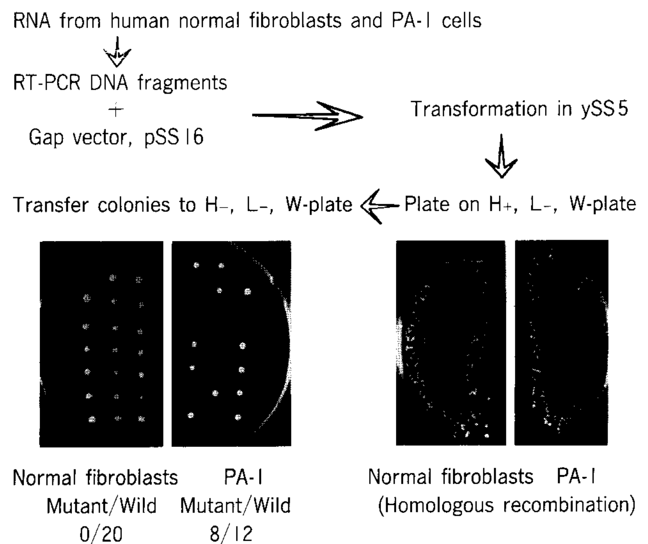

Fig. 1 Schematic diagram of functional amalysis of separated alleles in yeast (FASAY) result.

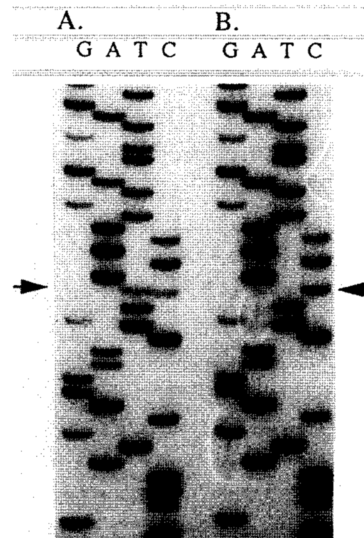

Fig. 2 Sequence analysis of p53 mutation in PA-1 cells. The mutated point is indicated by the arrows. **A**: direct sequence from RT-PCR product shows both the wild-type and the mutated bands. **B**: sequence of p53 in plasmids forming a small yeast colony shows only the mutated band.

Sequence analysis. RT-PCR fragments were purified by gel filtration (Chroma Spin 30, Clontech Lab., Inc., Palo Alto, CA, USA). The purified PCR fragments were directly sequenced with an AmpliTaq cycle

sequencing kit (Perkin-Elmer Setus, Branchburg, NJ, USA). Yeasts were digested with zymolyase (Seikagaku Co., Tokyo, Japan), and the plasmid DNA was extracted by alkaline lysis and transfected into *E. coli*, DH5. Mutations of p53 were identified by sequencing of the plasmids from each colony.

Results and Discussion

Fig. 1 shows one of the experimental assays. The yeast strain ySS5 was transformed with the p53 expression gap vector pSS16 and with PCR amplified p53 cDNAs from normal human fibroblasts and from PA-1 cells. The yeast colonies containing the cDNA from the normal human fibroblasts were large, indicating that the p53 gene status was wild-type. On the other hand, out of 20 colonies produced by the yeasts transfected with cDNA from PA-1, 12 colonies were large and 8 colonies were small, indicating that one allele of the p53 gene was wild and the other was mutant. In fact, the presence of a p53 gene mutation in an allele of PA-1 cells was confirmed by sequencing the RT-PCR product and the plasmid DNA extracted from the small yeast colonies (Fig. 2). The mutation was located at one of the hot spots in exon 7 at the amino acid codon 239, where asparagine was replaced by aspartic acid.

In a previous study, mutated p53 was not detected in PA-1 (6). This may be due to having cloned and sequenced only the wild-type allele of the genomic DNA. On the other hand, we found out that the wild-type and the mutated p53 were always expressed at a ratio of 1:1 in PA-1 cells at different passage levels. These results

Table I P53 gene status of human cell lines maintained in our laboratory

Cell lines	Origin[a]	Wild	Mutant	p53 status[b]	Remarks[c]	Ref
Liver						
Hep3B	HCC	19	1	W	AFP, ALB, HBsAg	11
HepG 2	HB	17	3	W	AFP, ALP, N-ras 61 mutation	11
HuH-6	HB	20	0	W	AFP, ALB	12
JHH-1	HCC	19	1	W		13
JHH-7	HCC	20	0	W	AFP, ALB, HBsAg	14
HLE	HCC	0	20	M[d]	AFP, p53 codon 249 mutation, Rb-	15
HuH-7	HCC	0	20	M[d]	AFP, ALB, mp53 codon 220 mutation	16
JHH-2	HCC	0	20	M	AFP	13
JHH-4	HCC	0	20	M	AFP, ALB	13
JHH-5	HCC	0	20	M	AFP	17
JHH-6	HCC	0	20	M		17
PLC/PRF/5	HCC	0	20	M[d]	AFP, HBsAg, p53 codon 249 mutation	18
Fibroblasts						
KMST-6		0	20	M	p53 codon 179	19
OUMS-24F		0	20	M	p53 codon 248	20
SUSM-1		0	20	M	p53 codon 179	21
Bone						
HuO9	Osteosarcoma	19	1	W	Rb-, c-myc amplification	22
HuO9-3M	Osteosarcoma	19	1	W	Rb-	22
Others						
OUMS-23	Colon carcinoma	17	3	W		23
OUMS-27	Condrosarcoma	0	20	M		In preparation
PA-1	Ovarian	12	8	W/M	p53 codon 239 mutation in one allele	24

a : HCC: hepatoma, HB: hepatoblastoma.
b : A few mutant colonies detected in the wild-type cell lines may be due to the mutation during PCR amplification cycles. Although fidelity of Pfu polymerase is 6-fold higher than that of Taq polymerase, 4.9% mutation is expected in 30 cycles of PCR amplification using Pfu enzyme as described in the instruction manual. Based on this information and the present technical errors, we judged the p53 status as follows: When there are more than four mutant colonies among the total of 20 colonies, the p53 status is mutant.
M: mutant, W: wild-type.
c : AFP: α-fetoprotein, ALB: albumin, ALP: alkaline phosphatase.
d : Mutations of these cell lines also have been reported by others (7).

indicate that the mutated and the wild-type p53 gene are present in separate alleles in a cell. On the other hand, there is another possibility that half of the population of the cells has the mutated p53 gene and the other half has the wild-type p53. However, this possibility seems unlikely since this cell line has been cultured for more than 10 years, and the population of the cells ought to be homogeneous. In fact, the karyotypes of this cell line have been kept stable in the diploid region since the early stages of culture.

Table 1 shows the p53 gene status of the human cell lines maintained in our laboratory. Out of 20 cell lines, 11 lines had mutations in the p53 gene. Among these 11 lines, other investigators (7) have already reported mutations in three cell lines, HLE, HuH-7 and PLC/PRF/5. We were also able to confirm these results in the present study. This percentage of the p53 mutation correlates fairly well with that of the clinical data. In fact, Levine *et al.* reported that the p53 gene was mutated in 60 % of human tumors (2). It is believed that cells in culture are prone to genetically changeability. However, our present data do not sustain this belief. All the present cell lines examined have been maintained in culture for a long period of time, at least 2 or more years.

Among the 20 human cell lines examined, 12 lines were of hepatic origin, hepatocarcinoma and hepatoblastoma, and the mutation frequency of the p53 gene in these hepatic lines was about 58 %. These data are consistent with those reported by Hsu *et al.* (8). They showed that the mutation frequency of the p53 gene in poorly and well differentiated hepatomas was 76.9 % and 27.3 %, respectively. The hepatoma cell lines in our laboratory may have been derived from poorly differentiated hepatomas.

Three hepatocarcinoma cell lines (Hep 3B, JHH-7, PLC/PRF/5) harbored HBsAg. Hep 3B and JHH-7 were normal for p53 status, whereas PLC/PRF/5 showed the mutation of the p53. Thus, it seems that there is no relationship between p53 gene status and the presence of HBsAg. A similar conclusion was reported by Hsu *et al.* (7).

In this study, we were able to confirm the usefulness of FASAY as developed by Ishioka *et al.* (4) for detecting mutations of the p53 gene. The virtue of this method is that it requires only a few steps to determine the p53 gene status in cells. Furthermore, this method can simultaneously detect the wild-type and mutated type of p53 in a cell, as shown in the case of the PA-1 cells (Fig. 1). In addition, this method is simple, rapid and accurate as compared with such previous methods as SSCP analysis and sequence analysis of PCR-amplified fragments.

Gene therapy using retrovirus or adenovirus vector expressing the human wild-type p53 and E1b mutant adenovirus which can selectively kill p53 deficient tumor cells is now in the clinical phase (9, 10). Thus, detection of mutated p53 genes in biopsy samples at an early stage of cancer is of value since it allows the oncologist to predict the effectiveness of the gene therapy. The FASAY method is useful for this detection.

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