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Presence of p53 Mutations in 3Y1-B Clone 1-6: A Rat Cell Line Widely Used as a Normal Immortalized Fibroblast

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The 3Y1 cell line, established from a rat whole embryo, is widely used as a normal immortalized fibroblast. We analyzed p53 mutations in four clonal lines derived from the 3Y1 cell line; 3Y1-B clone 1-6, 3Y1-C and two clonal lines (3Y1 cl-3 and 3Y1 cl-6) which had been transformed by the human papilloma virus E6 gene. Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis and DNA sequencing showed that three clonal lines had a double mutation at codons 130 and 136 on the same allele and that the other clonal line, 3Y1 cl-3, had no mutations. 3Y1-B clone 1-6, which has been registered as the standard clonal line at the Japanese Cancer Research Resources Bank, demonstrated weak bands of the wild type allele, suggesting the existence of heterogeneous cell types in this "clonal" line. PCR-SSCP analysis of 25 subclones obtained by limiting dilution of 3Y1-B clone 1-6 cells revealed a mixture of two types of cells; 12 subclones showed only the bands of mutated allele, and 13 subclones showed both bands of the wild and mutated p53 alleles. These findings should be taken into consideration when using this cell line as a normal immortalized cell line.

Key words: 3Y1 — p53 — Mutation

3Y1 is a rat cell line established by Kimura *et al.* from a Fischer 344 rat embryo according to the transfer schedule for murine 3T3 cells.[1] The 3Y1 cell line shows a very low saturation density, requires a high concentration of serum, and is supposed to be a homogeneous population. Its karyotype is normal diploid and relatively stable.[2] Based on these characteristics, the 3Y1 cell line has been used as an immortalized cell system for studies of the mechanisms underlying cell transformation and of cellular signal transductions.[3,4]

Transfection of a mutant p53 tumor suppressor gene can immortalize primary rat embryo fibroblasts.[5] Further, p53 alteration is a common event in spontaneous immortalization of primary murine embryo fibroblasts.[6] For these reasons, we analyzed p53 mutations in the 3Y1 cell line.

Four clonal lines were used for this study. 3Y1-B clone 1-6, which is widely used as the standard clonal line of 3Y1, was obtained from JCRB.[5] A second clonal line, 3Y1-C, which had been frozen-stored before establishment of 3Y1-B clone 1-6, was obtained from Dr. G. Kimura, Kyushu University, and analyzed at more than ten passages. The two other clonal lines used, 3Y1 cl-3 and 3Y1 cl-6, had also been derived (by K.S.) from 3Y1 before establishment of 3Y1-B clone 1-6, by transfecting the HPV E6 gene (details will be described elsewhere). The cells were all cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air.

Total RNA was purified from log-phase cultures of 2 or 3 10-cm dishes by the acid guanidinium thiocyanate/phenol/chloroform extraction method.[7] Genomic DNA was prepared from confluent 10-cm dish cultures using Nonidet P-40 (Sigma, St. Louis, MO) and Tween 20 (Bio-Rad, Richmond, CA).[8]

cDNA was synthesized from total RNA by RNase H^- reverse transcriptase (SuperScript, GIBCO BRL, Gaithersburg, MD). Four regions (Y1, Y2, Z1 and Z2 in Fig. 1) of the p53 gene, containing all 4 mutational hot spots observed in human cancers, were amplified from the synthesized cDNA by PCR using primers end-labeled with [^{32}P]γ-ATP (Table I). PCR consisted of thirty cycles of the reaction at 94°C, 55°C, and 72°C for 0.5, 1.0, and 1.0 min, respectively. The PCR products were run on acrylamide gels after heat denaturation at 95°C. The Y1 region, where a mobility shift was found, was asymmetrically amplified using 50 and 5 pmol of up-

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[5] Abbreviations: JCRB, Japanese Cancer Research Resources Bank; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; HPV, human papilloma virus.

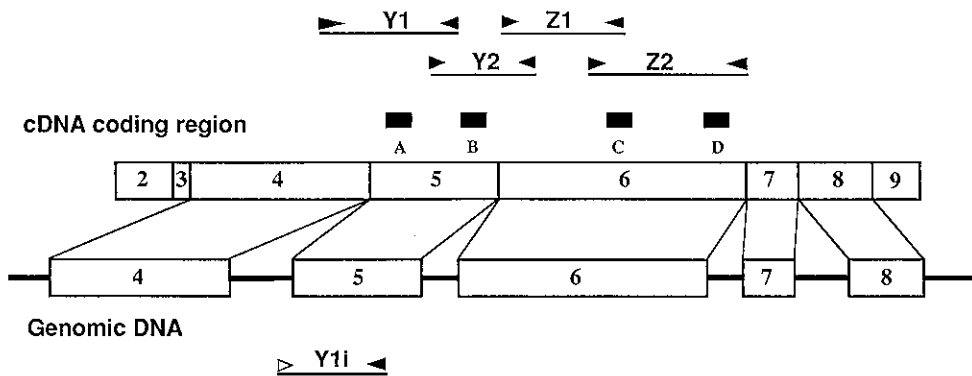

Fig. 1. Primer design for the analysis of rat p53 cDNA and genomic DNA. Solid boxes show four mutational hot spots (A–D) reported in human tumors. Filled arrowheads show primers from exon sequences. An open arrowhead shows a primer from an intron sequence. There is no intron in the rat p53 gene at the region corresponding to the boundary of exons 6 and 7 in human and mouse.

Table I. Primers Used for Genomic- and cDNA-PCR-SSCP

Region	Upstream	Downstream
Y1i	5′-GATTCTTTCTCCTCTCCTAC-3′	5′-TGTAGATGGCCATGGCACGG-3′
Y1	5′-GGCAACTATGGCTTCCACCT-3′	5′-TGTAGATGGCCATGGCACGG-3′
Y2	5′-CCTCCACCTGGTACCCGTGT-3′	5′-ACCATACGGATTTCCTTCCA-3′
Z1	5′-TCCCCACACTCTTATCCGGG-3′	5′-CAGGAGCTGTTGCACATGTA-3′
Z2	5′-TATACCACTATCCACTACAAG-3′	5′-CAATGCTCTTGGCTTTTTGC-3′

Y1 region primers were also used for PCR-direct sequencing.

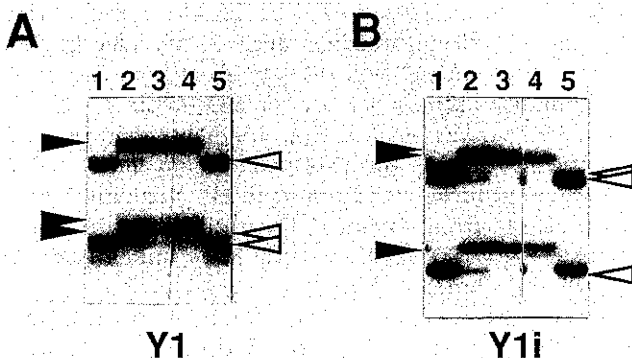

Fig. 2. Detection of p53 mutations in the 3Y1 clonal lines. Lane 1: F344 rat normal liver DNA as a control. Lane 2: 3Y1-B clone 1-6 obtained from JCRB. Lane 3: 3Y1-C. Lanes 4, 5: HPV E6 transformants 3Y1 cl-6 and 3Y1 cl-3. 3Y1 cl-6 and 3Y1-C showed only mutated bands (closed arrowheads). 3Y1-B clone 1-6 showed both mutated and wild type bands (open arrowheads). 3Y1 cl-3 showed only wild type bands. A. cDNA-PCR SSCP analysis. The ratio of mutant and wild type alleles can be easily determined from the upper band. B. Genomic PCR-SSCP analysis. The ratio of mutant and wild type alleles can be easily determined from the lower band.

stream and downstream primers, respectively, and the product was directly sequenced using the end-labeled downstream primer.[9] Further, the presence of a mutation in the Y1 region was confirmed in genomic DNA by PCR-SSCP, using primers within intron 4 and exon 5 (Fig. 1).

By cDNA-PCR-SSCP analysis, 3Y1-B clone 1-6 was found to have both mutated and wild type bands of the Y1 region (Fig. 2A). The 3Y1-C clonal line showed only mutated bands with the same mobility shift as that of 3Y1-B clone 1-6. The two HPV E6 transformants showed only wild type bands (3Y1 cl-3) or mutated bands (3Y1 cl-6) in the Y1 region. The mutated bands of all these three clonal lines demonstrated the same mobility. No mobility shift in any other region was observed in any of the four clonal lines. The same pattern of mutant and normal alleles was confirmed among the four clonal lines by genomic PCR-SSCP analysis (Fig. 2B).

Direct sequencing revealed that the 3Y1-B clone 1-6 had a double mutation at the second letter of codon 130 and at the first letter of codon 136 (Fig. 3). These mutations altered the encoded amino acids from lysine

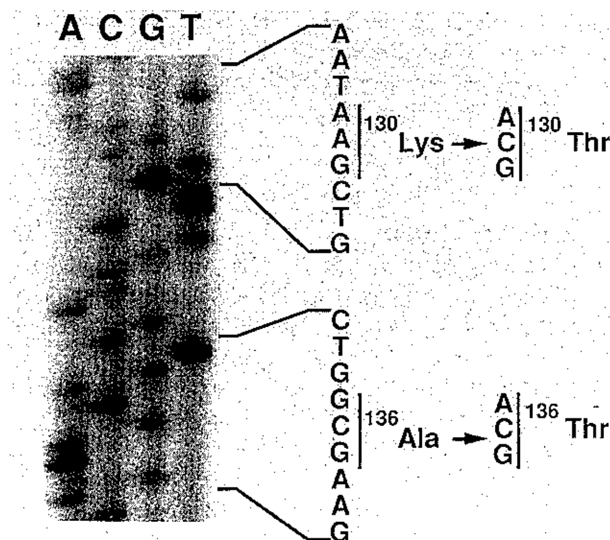

Fig. 3. Nucleotide sequence of p53 in the 3Y1-B clone 1-6 cell line. Direct sequencing of the Y1 region revealed an A to C transversion at the second letter of codon 130 and a G to A transition at the first letter of codon 136, which would result in substitution of threonine for lysine and of threonine for alanine. These two mutations were confirmed to be on the same allele by subcloning PCR products and sequencing them.

Table II. Cell Populations from 3Y1-B Clone 1-6 with Wild Type and Mutant Alleles

Genotype	Number of subclones	Rate (%)
$^{130}K^{136}A/^{130}K^{136}A$	0	
$^{130}K^{136}A/^{130}T^{136}T$	13	52
$^{130}T^{136}T/-$ or $^{130}T^{136}T/^{130}T^{136}T$	12	48

3Y1-B clone 1-6 cells were subcloned by limiting dilution and 25 subclones were tested for presence of wild type and mutated alleles in the Y1i region by genomic PCR-SSCP. $^{130}K^{136}A/^{130}K^{136}A$, $^{130}K^{136}A/^{130}T^{136}T$ and $^{130}T^{136}T/-$ or $^{130}T^{136}T/^{130}T^{136}T$ represent subclones which contained the bands of wild type allele only, wild type and mutated alleles and mutated allele only, respectively.

to threonine and alanine to threonine, respectively. To analyze whether the observed two point-mutations were on the same allele, PCR products were subcloned into plasmids and sequenced as described.[10] The results confirmed that the two mutations were on the same allele (data not shown).

The signal of the wild type allele ($^{130}K^{136}A$) in the Y1 region was much weaker than that of the mutated allele ($^{130}T^{136}T$) in 3Y1-B clone 1-6, indicating that this clonal line is composed of different types of cells. 3Y1-B clone 1-6 cells were thus subcloned by limiting dilution, and 25 resulting subclones were investigated for p53 mutation by the genomic DNA-PCR-SSCP method. 3Y1-B clone 1-6 was shown to be a mixture of two types of cells, one type $^{130}K^{136}A/^{130}T^{136}T$ and the other $^{130}T^{136}T/^{130}T^{136}T$ or $^{130}T^{136}T/-$ (Table II). The population sizes of the detected two types of subclones were almost equal. There was no subclone which contained only $^{130}K^{136}A$. Although selection against this type of 3Y1 subclone cannot be ruled out, the population should be very small, judging from the weak band intensity of $^{130}K^{136}A$ in the SSCP analysis (Fig. 2B, lane 2).

The presence of the same mutation in three clonal lines (3Y1-B clone 1-6, 3Y1-C and 3Y1 cl-6) indicates that the mutation was generated when the 3Y1 cell line was established or that it was generated in a cell in quite early passages before these three clonal lines were established. In the former case, 3Y1 cl-3 (HPV E6 transformant), which had only $^{130}K^{136}A$ (wild type), is speculated to have originated from a cell heterozygous for $^{130}K^{136}A$ and $^{130}T^{136}T$ and generated through a gene conversion or loss of the mutant allele. It is speculated that transformation with E6 allowed the survival of cells without p53 mutation and that p53 mutation is essential for immortalization of the 3Y1 cell line. In the latter case, 3Y1 cl-3 is speculated to have originated from a cell homozygous for $^{130}K^{136}A$ which still existed as one of plural heterogeneous cell types in early passages of the 3Y1 cell line. p53 mutation is speculated to have been an independent event from immortalization of the 3Y1 cell line.

As described above, the present 3Y1-B clone 1-6 is a mixture of two cell types. The most plausible explanation is that the 3Y1-B clone 1-6 had $^{130}K^{136}A/^{130}T^{136}T$ when isolated and that a subclone spontaneously lost $^{130}K^{136}A$ (wild type) during the passages. The subclone without $^{130}K^{136}A$ is expected to have some growth advantage over the heterozygous subclone because the subclone without $^{130}K^{136}A$ has increased up to almost the same population as the heterozygous subclone after a number of passages.

The p53 mutation observed in the 3Y1-B clone 1-6 might have contributed to the susceptibility to transformation by genes which cooperate with mutant p53, such as E7 of human papilloma virus.[11] Attention must be paid to insidious changes of cell population when using this clonal line of 3Y1.

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