Analysis of the p53 gene alterations in mouse embryo fibroblast cell line Balb 3T12 and its derivative 312

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We have analysed the status of the p53 gene in the mouse embryo fibroblast cell line Balb 3T12 ($TD_{50} = 10^6$) and its transformed clonal derivative 312 ($TD_{50} = 10^4$) with an aim to determine whether there exists a correlation between increased tumorigenicity and clonal expansion of cells bearing a mutation in the p53 gene. While Southern hybridizations did not show any obvious changes in the p53 gene organization in 3T12 and 312 cells, sequencing the p53 cDNA revealed that 3T12 is mutated at the amino acid residue 233 (Tyr → Asp) whereas 312 is mutated at the residue 132 (Cys → Trp). Exploiting the altered RFLP pattern due to mutations, we identified that 3T12 contains p53 alleles that are different from the already identified mutant p53. On the basis of these observations, we conclude that 3T12 and 312 have evolved independently.

1. Introduction

Tumour suppressor gene p53 encodes a nucleophosphoprotein that regulates the growth and proliferation of normal cells (Jenkins and Struzbecher, 1988) and alterations such as mutations, deletions, and translocations in this gene are associated with transformation of normal cells. In addition, several viral proteins abrogate the function of p53 by forming complexes with it (Simmonds *et al* 1980; Symmonds *et al* 1991). Biochemically, p53 acts as a transcriptional activator or modulator (Raycroft *et al* 1990). In addition to the negative regulation of cell proliferation, p53 has been implicated in the control of differentiation (Shaulsky *et al* 1991), apoptosis (Younish-Rouach *et al* 1991), genomic stability (Livingstone *et al* 1992; Yin *et al* 1992) and cellular response to DNA damage (Kastan *et al* 1991; Kuerbitz *et al* 1992).

Mutations are the frequently observed alterations noticed in p53 (Hollstein *et al* 1991) and the cells containing mutated p53 have a growth advantage over other cells and expand clonally. Although mutation in one of the p53 alleles is sufficient to relieve the growth suppression activity of the wild-type p53 (Kress *et al* 1979), the loss of heterozygosity is also a frequent phenomenon with mutations in p53.

During the evolution of cell lines the tumorigenic potential increases gradually over passages. This increase in tumorigenic potential is due to the clonal expansion of the cells that possess growth advantage over the others. Neoplastic progression of the cell line HE correlates with the clonal expansion of the cells bearing p53 mutations (Le Rhun *et al* 1994). Similarly, the clonal cell line 312 (obtained from a tumour by injecting Balb 3T12 cells in Balb/c mice), has been shown to possess increased tumorigenicity compared to the parental cell line Balb 3T12 (Thathamangalam *et al* 1986). In this study, we have analysed whether the increased tumorigenicity of 3T12 correlates with the clonal expansion of mutant p53 bearing 3T12 cells. Our results indicate that 312 cell line has not evolved from 3T12 cells by clonal expansion but has evolved independently.

2. Materials and methods

2. *Cell culture*

Mouse embryo fibroblast cell lines 3T12 and 312 were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS,

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100 U/ml penicillin and 50 μg/ml streptomycin in a humidified environment containing 5% CO_2.

2.2 *DNA isolation*

Genomic DNA from the cell lines was isolated by following the procedure as described by Sambrook *et al* (1989).

2.3 *Construction of cDNA libraries*

cDNA libraries for the cell lines were constructed following the procedure described by Gubler and Hoffman (1983). About 5 μg of mRNA was reverse transcribed and cloned in λgt10 using *Eco*RI linkers. In order to identify the clones containing p53 cDNA, about $2{\cdot}5 \times 10^5$ pfu were screened with a full length p53 cDNA probe and the clones containing the largest inserts were subcloned into pGEM3 vector and used for sequencing.

2.4 *DNA sequencing*

Series of deletion clones were generated from the recombinant plasmid clones using Erase-a-base system (Promega). The inserts of the deletion clones were sequenced by the dideoxy chain termination method (Sanger *et al* 1977) using Gemseq K/RT system (Promega).

2.5 *Polymerase chain reaction*

PCR was performed using the phage DNA (100 ng) from cDNA libraries and genomic DNA (500 ng). Amplification was carried out for 40 cycles using the following conditions: DNA was denatured for 1 min at 94°C, the primers were annealed for 1 min at 60°C, and the annealed primers were extended at 72°C for 2 min. Subsequently, the DNA was incubated at 72°C for 5 min. Amplified PCR products were separated on a 1·5% agarose gel and the amplified fragment corresponding to the normal gene was eluted from the gel, restriction digested, cloned in the plasmid Bluescript (Stratagene) and sequenced. The primers used for amplification were:

P1 5′ GCTTCCACCTGGGCTTCCTGC 3′
[from nt 314 to 334; the ATG is +1 (Pennica *et al* 1984)].
P4 3′ GCCCTGGGAGAGACCGCCG 5′
(from nt 455 tp 471 – sequence of the opposite strand).
DP2 3′ CGGCACAGGCGCGGTACCGGTAG 5′
(from nt 455 to 471 – sequence of the opposite strand).
DP1 5′ TGCTGGCTCGCTCTGCCACTGCG 3′
(intron 4 specific primer).
P3 5′ CCGGCTCTGAGTATACCACC 3′
(nt 665 to 684).

3. Results

312 is a fibroblastic cell line established as a clonal derivative of tumours obtained by injecting 3T12 in Balb/c mice (Thathamangalam *et al* 1986). Though both 3T12 and 312 cell lines showed transformed phenotype, 312 has higher tumorigenic potential ($TD_{50} = 10^4$ cells) than 3T12 ($TD_{50} = 10^6$ cells, Aaronson and Todaro 1988). In 3T12 cells, p53 has been shown to be mutated at the amino acid residue 233 (Tyr → Asp). It has generally been assumed that a decrease in the genetic stability of the genome is involved in the accumulation of a multitude of genetic changes leading to the generation of a neoplastic cell. Since the p53 gene which is involved in the control of genomic stability is mutated in 3T12, therefore more mutational events might have accumulated in 312 which is reflected by its tumorigenic potential. In order to analyse this possibility, we first investigated the status of the p53 gene in 312 cells.

3. *p53 is altered in 312*

The structure of the p53 gene in 312 cells was analysed by Southern blotting. Genomic DNA (20 μg) of 312 and normal mouse spleen was digested with *Eco*RI and hybridized with a radiolabelled full length p53 probe. As expected, two bands of 16 and 3·3 kb representing the normal and the pseudogene were obtained (figure 1). The

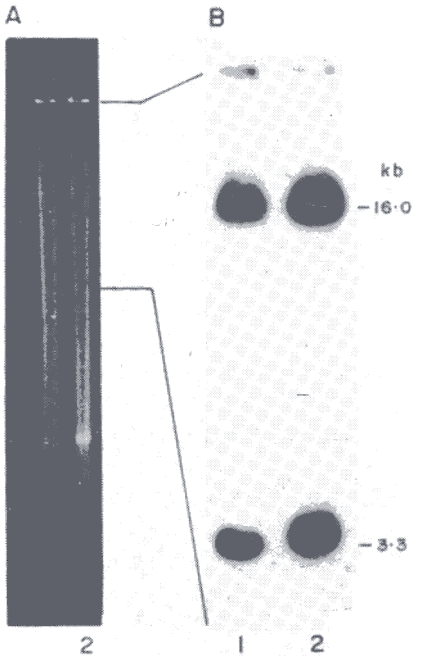

Figure 1. Southern blot analysis of 312 (lane 1) and normal mouse (lane 2). The *Eco*RI digested chromosomal DNA (**A**) was hybridized to a radiolabelled p53 probe (**B**). The 16 and 3·3 kb bands correspond to the functional and the pseudogene respectively.

pattern and intensity of the bands in 312 was similar to that of the normal mouse indicating absence of any gross changes in the structure of the p53 gene. Therefore, to determine whether there was any change in the primary structure of p53, a cDNA library was constructed. A representative clone containing the full length p53 cDNA was identified by screening the 312 cDNA library with a p53 probe and its nucleotide sequence was determined by following the dideoxy method. Surprisingly, sequence comparison with the wild-type p53 revealed that 312 cells contained a p53 allele mutated at codon 132 (Cys → Trp; figure 2) and not at codon 233 (Tyr → Asp), as observed in 3T12 (figure 3A).

3.2 *PCR analysis of p53 sequences*

The sequenced cDNA represented only one p53 allele and to determine the p53 alleles expressed in both 3T12 and 312 cell lines, the p53 sequences encompassing the conserved regions II to IV (from nucleotide 314 to 838) were amplified using the DNA isolated from the cDNA libraries using the primers P1 and P4 (figure 3B). Both 3T12 and 312 are hypo-tetraploids, thus they would contain either two or more than two p53 alleles. Furthermore, 3T12 is not a clonal cell line and due to heterogeneity, there is a possibility of existence of several p53 alleles. Since the p53 alleles which are expressed at very low levels cannot be detected by direct sequencing, the PCR products were first cloned in the plasmid pBS-SK and used for sequencing. The results obtained by sequencing 20 recombinant clones from 3T12 and 312 confirmed our previous results i.e., p53 is mutated at codon 132 in 312 and 233 in 3T12 and no other p53 allelic sequence was noticed in these cell lines. The p53 alleles that were amplifed from the cDNA library are represented based on their expression, thus the alleles that are expressed at very low levels might have not been detected by this method.

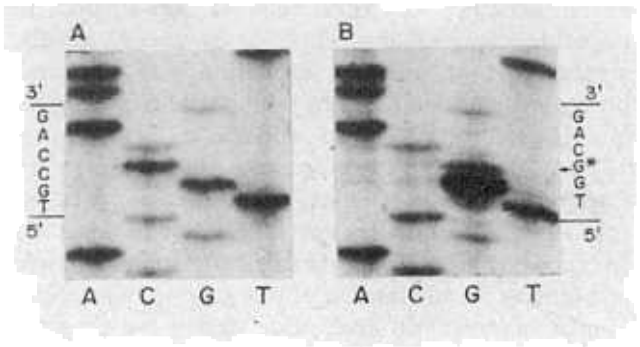

Figure 2. The autoradiograph of the p53 nucleotide sequence from wild type p53 (**A**) and 312 (**B**) at codon 132. Mutated nucleotide is marked by an asterisk.

3.3 *PCR-RFLP analysis*

While the mutation at codon 132 has generated a new *Bbv*I restriction site in 312 cells, the mutation at codon 233 has abolished the pre-existing *Rsa*I site in 3T12 cells. Therefore, to determine the presence of other p53 alleles (both expressing and non-expressing), the genomic p53 sequences from nucleotide 664 to 839 including the 320 bp intron 7 were amplified from both 3T12 and 312 using the primers P3 and P4. These primers amplify both the normal and pseudogenes. The PCR product corresponding to the normal gene (495 bp) was isolated on an agarose gel, eluted and digested with *Rsa*I, run on a denaturing polyacrylamide gel, Southern blotted and hybridized with the p53 probe (figure 4). The presence of a faint signal at 460 bp region in 3T12 suggested the presence of other p53 alleles in 3T12.

To determine whether 312-p53 allele is one of the p53 alleles present in 3T12, the p53 sequences (240 nucleotides; 130 from intron 4 and the nucleotides 360 to 477 from exon 5) were amplified using the primers DP1 (intron 4 specific primer) and DP2 and digested with *Bbv*I. The *Bbv*I digested products were separated on 8% denaturing polyacrylamide gel, Southern blotted and hybridized with a p53 probe (figure 5). The absence of the signal at the 180 and 60 bp regions suggested that no 312-p53 allele is present in 3T12.

4. Discussion

Availability of cell lines Balb 3T12 (parent) and its

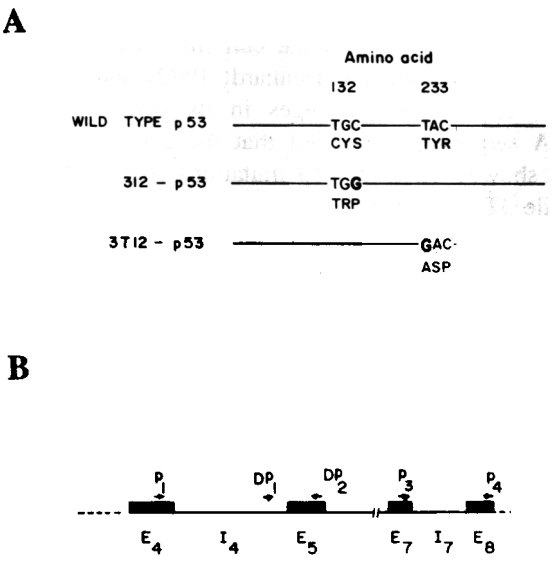

Figure 3. (**A**) Sequence comparison between wild type p53, 312-p53, and 3T12-p53. The nucleotide sequence and the three letter amino acid code of the codons 132 and 233 are shown. The mutated nucleotide is shown in bold. (**B**) Strategy used to amplify the p53 sequences. Arrows represent the orientation of the primers. E, exon; I, intron; P/DP, primers.

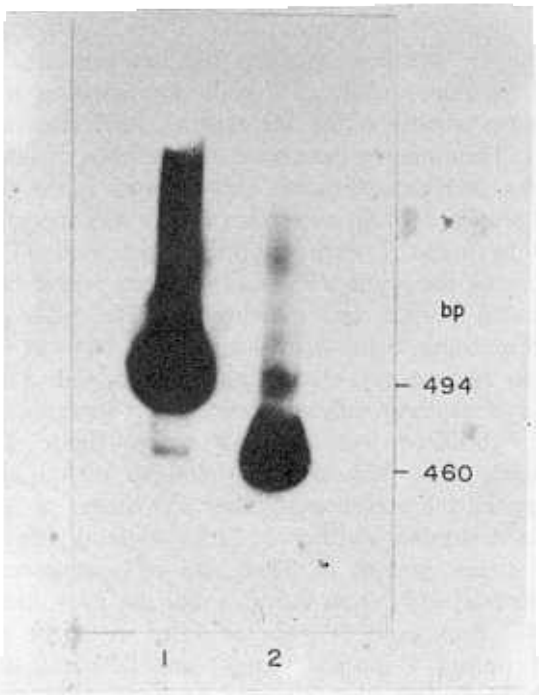

Figure 4. PCR-*Rsa*I digestion of PCR amplified (using primers P3 and P4) p53 products of normal gene from 3T12 (lane 1) and 312 (lane 2). The size of the PCR product is shown on the right.

clonal transformed derivative 312 with higher tumorigenic potential has led us to ask whether the mutational events in 312 are responsible for the increased tumorigenicity. Since it is known that the tumour suppressor p53 is involved in the stability of the genome, we first analysed the p53 alleles in both the cell lines. Southern blotting of 3T12 (Rittling and Denhardt 1992) and 312 did not show any obvious changes in its structure. However, DNA sequencing revealed that the cell lines 3T12 and 312 showed different p53 mutations (figures 2 and 3A). While 3T12 showed a mutation at the amino acid residue 233 (Tyr –> Asp), 312 showed a mutation at the amino acid residue 132 (Cys → Trp). Furthermore, sequencing the PCR amplified p53 cDNA and Southern blotting of restriction-digested PCR-amplified genomic DNA showed that only one mutant p53 allele is predominant in 3T12 and 312 cell lines. The sensitive PCR-RFLP digestions, however, showed that the p53 allele other than the already detected mutated p53 is present at extremely low copies in 3T12. Although no differences in the pattern and intensity of bands were noticed in Southern blotting between the 312 and normal mouse, densitometric scanning of PCR-amplified genomic p53 showed four-fold less amplification for the normal gene as compared to the pseudogene (data not shown) suggesting that the functional p53 alleles are present in hemizygous state in both 3T12 and 312.

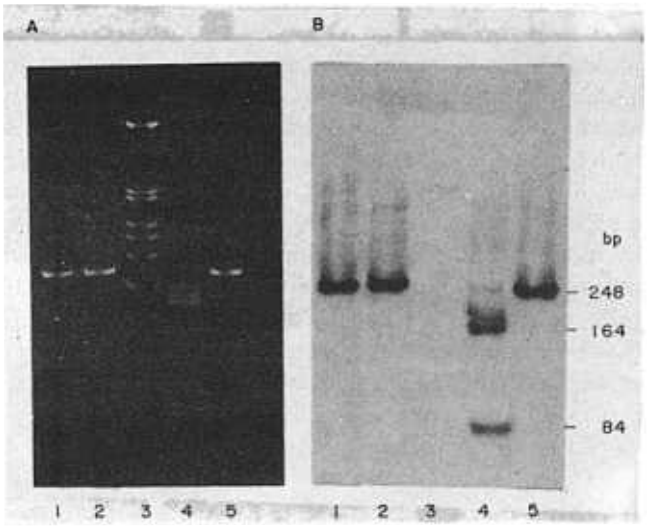

Figure 5. PCR-*Bbv*I digestion of PCR amplified (using primers DP1 and DP2) p53 products from 3T12 (lanes 1 and 2), 312 (lanes 4 and 5) are shown. Lanes 1 and 4 are *Bbv*I digested, 2 and 5 are undigested PCR products. Lane 3, pBR322 digested with *Hin*fI (molecular weight marker). The size of the products is shown on the right.

4.1 *Has 312 evolved from 3T12?*

Since 312 is a cloned derivative of a tumour obtained by injecting 3T12 cells in mice, it is expected that both the cell lines should have the same p53 sequence. However, the experimental results indicate that they have evolved from two different cell types as evidenced by the presence of two different p53 mutant alleles. This can be explained only if it is assumed that at the time of transplantation, 3T12 was a heterogenous population containing both 3T12 and 312 cells. This would suggest that during maintenance of 3T12 in culture, 312 cells were either lost or evolved later so that they existed at an undetectable number. Lack of differences in the cell doubling time (data not shown) between 3T12 and 312 cells suggests that this may not be a reason for the selective growth advantage of 3T12 cells in culture.

The existence of 3T12 cells as an almost clonal population suggests that 3T12 has expanded clonally. A similar kind of a clonal expansion was observed in benzopyrene treated rat embryo lung epithelial cells where a single cell with a mutation in the p53 gene at codon 130 (AAG > AGG), expanded to a subpopulation which finally became homozygous for the mutation (Le Rhun *et al* 1994). These studies indicated a pattern of directional selection for mutant p53 towards neoplastic development and reflects the importance of this gene in tumorigenesis. Similar observations were made during the progression of leukemia *in vitro* (Wada *et al* 1994).

In conclusion, these experimental results indicate that 3T12 and 312 cells may have evolved independently.

Therefore, these cell lines cannot be used in further understanding the accumulation of mutational events that are evolved in the neoplastic progression.

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