STUDY OF MUTATIONS IN p53 TUMOUR SUPPRESSOR GENE IN HUMAN SPORADIC BREAST CANCERS

Naina Makwane and Alpana Saxena*

Department of Laboratory Medicine, Chacha Nehru Bal Chikitsalaya, Geeta Colony, New Delhi- 110031, India

*Department of Biochemistry, Maulana Azad Medical College, New Delhi- 110001

ABSTRACT

Using Polymerase Chain Reaction followed by Single Strand Conformation Polymorphism analysis, we studied thirty seven cases of primary human breast cancer for the presence of mutations in exons 5 and 7 of p53 tumour suppressor gene. Only one of the thirty seven tumours tested (2.7%) showed the electrophoretic mobility shift indicative of a mutation in exon 5 of the p53 gene, while no such mobility shift was noted in exon 7. Our finding is in contrast to the findings in previous studies, wherein the mutation frequency has been reported to be 13-30% by direct DNA analysis. This may be related to the ethnicity and local population prevalence rather than technique.

KEY WORDS

Polymerase Chain Reaction, Breast Cancer, p53 Tumour Suppressor gene, DNA analysis.

INTRODUCTION

Carcinoma of the breast, like other malignancies, is a genetic disease with multiple genetic events leading to the malignant phenotype. A number of oncogenes (neu/ HER-2/ C-erbB-2, int-2, myc) and tumour suppressor genes (p53, Rb) have been implicated in the pathogenesis of breast carcinoma, amongst which p53 tumour suppressor gene is one of the most commonly mutated gene (1).

The p53 is a 20 Kb gene located on the short arm of chromosome 17 at 17p13.1 locus. It contains 11 exons, first of which is non-coding and some 10 Kb away from the rest (2). Comparison of the amino acid sequence of p53 gene products from different species has revealed 5 (I-V) highly conserved domains during evolution (2), which are critical for P53 protein function. The p53 plays a critical role in cell cycle regulation, cellular response to DNA damage and induction

of apoptosis. It operates at the G1/S check point and therefore in cells lacking functional p53, replication of damaged DNA with insufficient repair leads to genomic instability. p53 also directly stimulates DNA repair by activating transcription of GADD 45 and binding to ERCC3. If DNA damage is too extensive to be repaired, p53 causes damaged cells to self destruct by triggering apoptosis (3).

Inactivation of p53 gene is one of the most common somatic genetic alterations in most human malignancies including cancers of the breast (3). Most mutations are missense and clustered between 130-290 amino acid residues. Most p53 mutations are localized to the highly conserved domains II, III, IV and V which correspond to amino acid residues 117-142 (exon 4-5), 171-181 (exon 5), 234-258 (exon 6) and 270-286 (exon 8) respectively. Thus the core highly conserved region i.e. exons 5-8 is the target of the majority of p53 mutations in human malignancies (4). The p53 gene is mutated in 25-40% of breast carcinoma cases. There is higher than expected incidence of G to T transversions and Guanosine mutations in the non-transcribed strands are a preferred target. This suggests an etiological role of exogenous chemical carcinogens in sporadic breast carcinoma (5). Mutations in p53 are often but not always accompanied by allele loss at 17p13 (5). Studies have directly correlated p53 mutations with breast cancer prognosis and it has recently been demonstrated that p53 gene mutations (exons 4-10) are the single most

Address for Correspondence :

Dr. Naina Makwane

Dept of Lab Medicine

Chacha Nehru Bal Chikitsalaya

Geeta Colony, New Delhi-110031

Mobile: 09873327508

E-mail: nainamakwane@gmail.com

predictive indicator for recurrence and death in breast cancer. Direct detection of p53 mutations has substantially greater prognostic value than immunohistochemical detection (6, 7, 8).

In the present study, our aim was to detect mutations in exons 5 and 7 of p53 tumour suppressor gene in human sporadic breast cancers by Polymerase Chain Reaction amplification of the target DNA followed by Single Strand Conformation Polymorphism analysis and study their role as a prognostic indicator. There does not appear to be a breast cancer specific clustering of p53 mutations or "hot spots", but most have been identified in the core region. Exons 5 and 7 were therefore particularly chosen for the study.

MATERIALS AND METHODS

Collection of samples: Tumour tissue specimens from thirty seven cases of Carcinoma breast were collected from the surgical operation theatre of Lok Nayak Hospital, New Delhi. All the patients of Carcinoma breast underwent modified radical mastectomies and axillary lymph node dissection. p53 studies were done on fresh tissue specimens. A small piece of tumour tissue 0.5-1 cm was dissected free of the adherent normal tissue. The adjacent normal breast tissue and blood of the same patient were taken as the control samples. The tissues were frozen at -70°C until analysis.

DNA extraction from tissue: High molecular weight cellular DNA from breast tissue (cancerous/normal) was extracted by grinding with sea sand and 4ml 1XTE (1 M Tris, 0.5M EDTA pH 8), 2ml lysis buffer (3% SDS in 2 XTE) and digested with proteinase K (100µg/ml, Boehringer Mannheim) followed by routine phenol chloroform isolation (9) and precipitation overnight in 2.5 volume ethanol with 1/10 volume of 3M Sodium Acetate. Vacuum dried DNA pellet was dissolved in TE (Tris-EDTA) and DNA concentration was measured either by mini gel electrophoresis or by spectrophotometric method.

DNA extraction from blood: 5-10ml of peripheral venous blood was collected into heparinised blood vials (15ml; Corning, USA). To this were added 3 volumes of blood lysis

buffer (155mM NH₄Cl, 10mM KHCO₃, 0.1 mM EDTA pH 8.0), kept on ice for 15 mins and centrifuged at 3000 rpm for 10 mins at 4°C. The lymphocyte cell pellet was suspended in 5ml SE solution (75mM NaCl, 20 mM EDTA pH 8.0) to which was added 1ml 10% SDS and Proteinase K. (100µg/ml) and incubated overnight at 37°C. Standard phenol chloroform extraction and precipitation with alcohol was used to obtain genomic DNA as for tissue biopsies.

PCR Amplification: Amplification of exon 5 and exon 7 of p53 gene was carried out by standard PCR reaction mix containing 10 mM of Tris pH 8.3, 1.5 mM MgCl₂, 5mM KCl, 20 pmol of each primer, 200µM of each dNTPs (dATP, dCTP, dGTP, dTTP), 2 units of Taq DNA polymerase (Bangalore Genei, India) and 100ng of tumour DNA per 100µL reaction mix was used (20µL from the prepared reaction mix were then used for each sample). The reaction conditions were optimized. Primers were initially denatured at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 secs. In the last cycle the extension at 72°C was allowed for 5 min. Then 10-15µL of PCR product were electrophoresed on 3% Nusiev Agarose gel along with øx174 Hae III digested DNA molecular weight marker and photographed under a UV transilluminator. The primer sequence of p53 exon 5 and exon 7 used were:

Single Strand Conformation Polymorphism: Analysis of single strand conformational polymorphism was performed by radiolabelling the PCR products following regular amplification of 30 cycles for an additional 10 cycles where dCTP was replaced with [α -p32]dCTP {specific activity, 4000 Ci/mmol; BARC, Mumbai, India} in the PCR mix. Thus for a typical PCR mix of 100µL meant for 10 samples (10µL each), 1µL of [α -p32]dCTP was added, with other reaction components being the same. 1µL of radiolabelled PCR product was diluted with 9 volumes (10 times) of denaturing solution (95% Formamide, 20mM EDTA pH-8, .05% Xylene cyanol and .05% Bromophenol blue), heat denatured for 5 mins at 95°C and then chilled on ice for 5 mins. 3µL of this diluted product was then subjected to non-denaturing gel electrophoresis in a 6% polyacrylamide gel with 10% glycerol. The gel was then run in

Table 1: Oligonucleotide primer sequences used for the amplification of Exon 5 and 7 of p53 gene

Primer	Nucleotide position	Amplimer size	Primer sequences
p53 Exon 5	13055-13074 13219-13238	184 bp	US- 5'-TAC TCC CCT GCC CTC AAC AA-3' DS- 5'-CAT CGC TAT CTG AGC AGC GC-3'
p53 Exon 7	13992-14012 14096- 14116	125 bp	US- 5'-TCT CCT AGG TTG GCT CTG ACT-3' DS- 5'-TCC TGA CCT GGA GTC TTC CAG-3'

0.5 XTBE (0.045M Tris borate, 0.001M EDTA pH 8.0) for 12 hours at 200V in a Base Ace TM sequencing gel apparatus (Stratagene GmbH, Germany) at 17 +/- 1°C. The gel was then dried and exposed to X-ray film with intensifying screen at 70°C for 48 hours. Alterations in electrophoretic mobility in single strand DNA bands were analyzed by comparison with normal controls.

RESULTS

In the present study, thirty seven cases of human sporadic breast cancers have been analyzed for mutations in exons 5 and 7 of p53 tumour suppressor gene by PCR-SSCP analysis. Some important observations are presented below. The results of PCR-SSCP have been depicted from Figures 1 to 5. Mutation was found in only one of the thirty seven cases, in exon 5- clinical details of which have been listed. No mutation was seen in exon 7.

Characteristics of Patient population (n=37)

Table 2: Age distribution of cases of breast carcinoma at diagnosis

Age range	Number of cases	Percentage (%)
26-35 yrs	5	13.5
36-45 yrs	17	45.9
46-55 yrs	9	24.3
56-65 yrs	4	10.8
66-75 yrs	2	5.4

Table 3: Menstrual status

Menstrual status	Number of cases	Percentage (%)
Pre-menopausal	25	67
Post-menopausal	12	33

Table 4: Clinical stage

Clinical stage	No. of cases	Percentage (%)
Stage I	6	16.2
Stage II	12	32.4
Stage III	19	51.4
Stage IV	0	0

Table 5: Tumor size

Tumor size	No. of cases	Percentage (%)
<2 cm	1	2.7
2-5 cm	21	56.8
>5 cm	15	40.5

Table 6: Lymph node status

Lymphnode status	No. of cases	Percentage (%)
Positive	26	70.3
Negative	11	29.7

Table 7: Histological grade (Bloom and Richardson)

Histological grade	No. of cases	Percentage (%)
Grade I	18	48.6
Grade II	11	29.7
Grade III	8	21.6

Particulars of the case positive for p53 mutation on exon 7

Age: 36yrs; Menstrual status: Premenopausal; Parity: G₂P₂A₀; Family history: No significant history; Lymphnode status: Multiple ipsilateral axillary nodes palpable; Tumour size: 4-5 cms; TNM stage: T₂N₂M₀; Histopathological staging: Grade II

For quantitation of genomic DNA by gel electrophoresis, 2µL of extracted DNA solution was electrophoresed on 1% Ethidium Bromide stained mini gel, along with 1µg of λ DNA marker digested with HIND III. Presence of good quality high molecular weight DNA was evident from the presence of a single intact band without smearing or degradation. Comparison of band intensities of DNA from breast cancer specimens with that of standard 1µg HIND III digested λ DNA molecular weight marker showed almost similar concentrations of DNA in different samples.

The amplification of exon 5 and exon 7 of p53 tumour suppressor gene was done in 25µL reaction volume using appropriate p53 exon primers and the amplified product was checked by electrophoresis on 3% ethidium bromide stained agarose gel. Thus for p53 exons 5 and 7, the amplimers obtained in different breast cancer tissue samples and normal control samples were of sizes 184 and 125 bp respectively. DNA from the tumour as well as control samples was utilized for PCR which showed a good amplification of the exons (see Fig 1 and 2).

SSCP was performed by radiolabelling the PCR products for an additional 10 cycles where dCTP was replaced with [α -p32] dCTP in the reaction mix. Loading of the denatured labeled product on non-denaturing polyacrylamide gel followed by its autoradiography revealed presence of several bands on the autoradiograph. The mutation of exon 5 of the

Fig 1: PCR amplification of exon 5 of p53 tumour suppressor gene showing amplimers of exon 5

Fig 2: PCR amplification of exon 7 of p53 tumour suppressor gene showing amplimers of exon 7

Fig 3: PCR SSCP analysis of exon 7 of p53 tumor suppressor gene showing no band shift or extra band, indicating the absence of mutation

p53 gene was seen as presence of an extra band in SSCP in addition to the other bands which were homogenously present in other tumour as well as in normal control samples.

The electrophoretic mobility shift in the tumour sample of exon 5 was analyzed in comparison to the normal controls.

Fig 4: PCR SSCP analysis of exon 5 of p53 tumor suppressor gene in breast cancer showing presence of and additional band (shifted band shown by arrow) indicative of a mutation

DISCUSSION

Most p53 gene mutations found in human cancers produce missense proteins with altered or absent transcriptional regulation activities and are associated with high concentration of P53 protein detectable by immunohistochemistry. The loss of p53 function eliminates growth arrest in response to certain DNA damaging agents and enhances the frequency of gene amplification, suggesting a role for p53 in the control of cell cycle check point and in the maintenance of integrity of the genome (10).

The p53 gene, according to most studies, is mutated in 25-40% of sporadic breast carcinoma cases. Although there does not appear to be a breast cancer specific clustering of p53 mutations, or "hot spots", within the p53 gene, most have been identified within the highly conserved core region, which is exons 5 to 8. Exon 5 and 7 were therefore particularly chosen for the present study. Although 20% of mutations are outside of exons 5-8, these mutations are predominantly of the null type, resulting in truncated proteins or products of abnormal splicing.

There is a higher than expected incidence of G to T transversions and guanine mutations in the transcribed strand are a preferred target. This suggests an etiological role of exogenous chemical carcinogens in sporadic breast carcinoma (6). The world wide incidence of breast cancer varies by at least four fold and exposure to environmental mutagens has been hypothesized to underlie this variation. In support of this hypothesis, cross cultural studies of Japanese women have shown that native populations have a very low risk of breast cancer, and that this risk rises dramatically within one generation of moving to a high risk area, for example the USA (10). In the present study, a mutation could be detected in only one of the thirty seven cases in exon 5; it is therefore difficult to comment upon the etiology from analysis of the present study.

Most of the studies on p53 mutations in breast carcinoma have used immunohistochemical detection of P53 overexpression, which is only an indirect assay and gives an approximate estimate of mutation frequency. Wild type p53 is generally not detected by this technique because of its short half life i.e. about 6-20 min (12). It is generally accepted that p53 gene mutations lead to an overexpression/ stabilization or increase in the half life of the protein. Alternatively, p53 may form complexes with E6/E7 (protein coding regions in the genomic organization of HPV) during HPV infection and this p53- E6/E7 complex formation may lead to stabilization of P53 protein till such time that it is degraded through the Ubiquitin pathway (5). It is also not unlikely to find some residual stable P53 during the process of complex formation and degradation. This may be the reason for the observation of overexpression of p53 messenger RNA transcripts detected by immunohistochemistry which are not necessarily due to mutation.

PCR-SSCP and sequencing correlate with each other in 96.5% cases (20). Although sequencing is the most unambiguous method, it is technically cumbersome and expensive. Therefore DNA analysis by PCR-SSCP was particularly

chosen for the study, since there is 100% correlation between band shift in SSCP and mutation of the p53 gene.

More recently, certain studies have directly correlated p53 mutations with breast cancer prognosis. It has been demonstrated that p53 gene mutations (exons 4-10) were the single most predictive indicators for recurrence and death in breast cancer. Exons 5-8 were screened for p53 mutations and it was observed that mutations in only exons 5 and 6 had a significant correlation with high S phase index (21).

A study by Andersen et al (8) also showed that the highest frequency of p53 mutations in breast carcinoma is in exon 7 per base pair, which were screened by constant denaturant gel electrophoresis and demonstrated a correlation of p53 mutations with adverse prognostic factors and decreased survival. Thorlacius et al (22) analysed only exons 5, 7 and 8 for p53 mutations and showed an association with low estrogen content and high mortality rates. A study by Gentile et al (23) has suggested that missense mutations in the zinc binding domain of the P53 protein contribute substantially to tumour aggressiveness and adverse prognosis.

In contrast to the findings of previous studies which have shown p53 gene mutation as an important prognostic factor in patients with breast carcinoma, no such prognostic role of p53 mutation could be assessed from the present study since mutation was found in only one of the thirty seven cases.

To summarize, thirty seven cases of carcinoma breast were analyzed by PCR-SSCP for mutations in exon 5 and 7 of p53 tumour suppressor gene in the present study. Mutation was found in only one of the thirty seven cases, i.e. in 2.7% of the cases. This is in contrast to the findings of the previous studies where the mutation frequency has been reported to be 13-30% by molecular analysis. This suggests that the occurrence of p53 mutations relatively low in Indian women with breast cancer as there are only few other reported studies about the mutation frequency in p53 gene in the Indian population.

REFERENCES

1. Dhingra K, Hortobagyi GN. *Seminars in Oncology* 1996; 23 (4): 436-45.
2. Soussi T, de Fromental C, May PC. Structural aspects of P53 protein in relation to gene evolution. *Oncogene* 1990; 5: 945-52.
3. Shimamura A, Fisher DE. p53 in life and death. *Clin Cancer Res* 1996; 2: 435-40.

4. Nigro JM, Baker SJ. Mutations in p53 gene occur in diverse tumour types. *Nature* 1989; 342: 705-8.
5. Levine AJ, Momand J, Finley CA. The p53 tumour suppressor gene. *Nature* 1991; 351: 453-6.
6. Lemione NR. Molecular biology of breast cancer. *Ann Oncol* 1994; 5 (Supl 4): 5312-37.
7. Kovach JS, Hartmann A, Balszyk H. Mutation detection by highly sensitive methods indicate that p53 gene mutations in breast cancers can have important prognostic value. *Proc Natl Acad Sci USA* 1996; 93: 1093-96.
8. Andersen TI, Holm R, Nesland JM, Heimdal KR, Ottestad L, Borresen AL. Prognostic significance of TP53 alterations in breast carcinoma. *Br J Cancer* 1993; 69: 540-48.
9. Das BC, Sharma JK, Gopalakrishna V, Luthra UK. Analysis by Polymerase chain reaction of the physical state of the human papilloma virus type 16 DNA in cervical preneoplastic and neoplastic lesions. *J Gen Virology* 1992; 73: 2327-36.
10. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991; 253:49-53.
11. Eeles RA, Bartkova J. The role of the p53 in breast cancer development. *Cancer Surveys* 1994; 18: 57-75.
12. Helland A, Holm R. Genetic alterations of the p53 (TP53) gene, p53 protein expression and HPV infection in primary cervical carcinomas. *J Pathol* 1993; 171:105-14.
13. Mittal KR, Lin O. Cervical squamous dysplasias and carcinomas with immunodetectable p53 frequently contain HPV. *Gynaecol Oncol* 1995; 58: 289-94.
14. Varley JM, Brammer WJ. Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. *Oncogene* 1991; 6: 413-21.
15. ThorAD, Moore DH. Accumulation of p53 tumour suppressor gene protein: An independent marker of prognosis in breast cancer. *J Natl Cancer Inst* 1992; 84: 845-55.
16. Coles C, Condie A. p53 mutations in breast cancer. *Cancer Res* 1992; 52 (1): 5291-8.
17. Davidoff AM, Kerns BJM. Maintenance of p53 alterations throughout breast cancer progression. *Cancer Res* 1991; 51: 2605-10.
18. Cornalis RS, Vanvliet M. Evidence for a gene on 17p13.3, distal to TP53, as a target for allele loss in breast tumours without p53 mutations. *Cancer Res* 1994; 54(15): 4200-6.
19. Prosser J, Thompson AM. Evidence that p53 behaves as a tumour suppressor gene in sporadic breast tumours. *Oncogene* 1990; 5: 1573-9.
20. Chakravarty G, Redkar, Mitra I. A comparative study of detection of p53 mutations in human breast cancer by flow cytometry, Single Strand Conformation Polymorphism and Genomic sequencing. *Br J Cancer* 1996; 74: 1181-7.
21. Merlo GR, Bernardi A, Diella F, Venesio T, Cappa AP, Callahan R, Liscia DS. In primary human breast carcinomas, mutations in exons 5 and 6 of the p53 gene are associated with a high S-phase index. *Int J Cancer* 1993; 54: 531-35.
22. Thorlacius S, Borresen AL, Eyfjord JE. Somatic p53 mutations in human breast carcinomas in an Icelandic population- a prognostic factor. *Cancer Res* 1993; 53: 1637-41.
23. Gentile M, Bergman Jungstrom M, Olsen KE, Soderkvist P, Wingren S. p53 and survival in early onset breast cancer: analysis of gene mutations, loss of heterozygosity and protein accumulation. *Eur J Cancer* 1999; 35: 1202-7.