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p53 gene mutations among patients involved with breast cancer: types of detection

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Abstract

A significant transcription factor that is involved in the regulation of numerous cellular functions is the tumor suppressor p53. In disease, p53 weakens cell expansion in light of different boosts, including DNA harm, supplement hardship, hypoxia, and hyperproliferative signs, along these lines forestalling growth arrangement. It was detailed that the proficiency of Microarray and ABI 310 framework in distinguishing proof a wide range of p53 quality transformations. Microarray and ABI 310 analysis were used in this study to find p53 gene mutations in archived breast cancer tissues. Breast tissues from cancer patients who had been diagnosed with breast cancer were collected for this purpose and paraffin-embedded after being formalin-fixed. DNA was removed by the Microdissection technique and was cleaned with Microcon 50 channels (Millipore) prior to performing PCR. Twelve of the samples that were analyzed had ABI 310 system mutations in the p53 gene, the genomic DNA was acquired from micro-dissected tests without laser. The ABI 310 system identified p53 gene mutations in three of the nine ESCC specimens from patients who were examined by microarray. In laser-miniature analyzed examples changes were distinguished by ABI 310 framework. The extricated DNA obtained from laser miniature took apart examples was deficient for the evaluation of p53 quality changes with Microarray. It was resolved that Microarray was reliant upon how much tissues were utilized in DNA extraction. The resulting data of this study showed that selecting the appropriate method for extracting DNA from test samples in order to evaluate the p53 gene mutation is crucial. The ABI 310 system and Microarray were able to detect p53 gene mutations (for exons 5-8) with an efficiency of 99.6% and 27%, respectively. Consequently, involving new tissues for Microarray analysis is suggested. In conclusion, the application of Microarray to identify mutation for p53 gene, in breast cancer tissues, will be necessary for central hospitals, where fresh tissue samples are available easily.

Keywords: p53; DNA; Framework; Breast cancer

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Introduction

Breast cancer maintains its position as one of the most frequently detected types of malignant neoplasms, with only skin cancer surpassing it as the most common type of malignancy. It is

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the most frequent cause of related deaths. The five-year survival rate for patients who are diagnosed with localized breast cancer at an early stage is about 100 percent. Therefore, new prognostic markers and effective therapeutic approaches must be developed to lower the breast cancer-related morbidity and mortality rates. Detection of p53 gene mutations in breast cancer patients, as potential predictors of the onset of cancer or its progression and therapy efficacy, has sparked greater interest among researchers. The article reviews the detection of p53 gene mutations in breast cancer patients. The methods of in vitro mutagenesis and their modifications, including those for determining the status of p53 gene mutations in a cancer patient through the detection of ameristic or heteroplasid of the mutant alleles by radioactive or enzymatic molecular hybridization with differentiating probes, depend on the introduction of various genetic abnormalities into the nucleotide sequence of the p53 gene to confirm the tumor process development. Currently prevailing approaches suggest the systematic screen for pathogenic or likely pathogenic mutations, genetic variants of uncertain significance, and polymorphisms in the field of interest, and validation of identified variants. Recently, specific attention has been paid to the definition of larger deletions throughout the gene or microhomology around breakpoints of P53 intragenic deletions. It was published that several p53 mutations revealed by next-generation sequencing (such as homozygous deletions, indels, etc.) may not differ significantly from immunohistochemically detected TP53 mutations in terms of prognostic implications for breast cancer patients. In regular clinical service, routine p53 gene mutations by sequencing are important and will be focused on. The most frequent form of the lethal cancer in women is breast cancer (BC). Multifactorial etiology, including genetic alterations, contributes to BC development. The presence of such mutations prompts the cancerous transformation of breast cells. They also induce the absence of programmed cell death, which facilitates the proliferation of defective cells. Therefore, researchers have long focused on the mutations associated with anti-oncogene (tumor suppressor gene) p53, which is involved in the repression of cell division in response to damage and therefore in the initiation of programmed cell death (apoptosis). The p53 gene is indeed abnormally activated after genetic damage and regulates the cell cycle, the cycle ensuring the various stages of the cell's existence. This gene belongs to a family of three genes coding for proteins whose mechanisms of action are very close and which can compensate for each other in case of malfunction. The two other genes are p63 and p73. All the studies discussed in this report show that the p53 gene is associated with morphological and prognostic characteristics of the disease: grade, size, lymph node axillary and distal organ metastases and the success of treatment. Some authors have also evaluated the impact of this mutation on the response to chemotherapies. Indeed, the presence of excess p53 in a cell limits the effectiveness of chemotherapy based on cytotoxic drugs as these cells will migrate towards programmed cell death. At the end of the 2000s, the p53 gene and its protein were well studied for their potential application in the clinic in diagnosis, prognosis and treatment choices. Moreover, p53 could be a relevant biomarker of carcinogenesis in BRCA1 mutation carriers.

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Methods

Tissue Collection and Dissection for DNA Preparation 12 Formalin-fixed, paraffin-embedded breast cancer from cancer patients diagnosed with breast cancer were collected for analysis. No patient had been given chemotherapy or radiotherapy before the operation. Serial sections of 10-µm thickness were prepared for DNA extraction. Tissue areas with high neoplastic cellularity (>50%) were dissected from dewaxed slides, and the materials were digested by proteinase K in SDS containing buffer for 3-5 days at 50°C to release DNA suitable for PCR.

DNA extraction

Three breast diagnosed with breast were used for laser microbeam micro-dissection to extract DNA. These samples were selected from those who had p53 gene mutations in exon 5-8 using ABI 310 system. Serial sections of 3-µm thickness were prepared for this purpose. Tissue areas with high (>70%) neoplastic cellularity were selected from dewaxed, eosin-stained slides under visual control.

These areas were isolated from the surrounding tissue by the focused nitrogen laser beam with a microdissection apparatus (PALM Laser MicroBeam System; P.A.L.M, Wolfrathausen, Germany). The materials were harvested with the increased energy of the laser and the micro-dissected area was catapulted by a single laser shot as described by other [9]. The detached tissue samples were then collected in a microfuge cap coated with the PCR oil and mounted above the object slide. The cap containing samples was placed on a microfuge tube filled with 100-µL SDS containing buffer. DNA was isolated using digestion of Proteinase K, Phenol/Chloroform extraction and ethanol precipitation.

Uniplex PCR and DNA sequencing

Genomic DNA was mixed with PCR buffer containing 1.5 mM MgCl2, 10 mM Tris-HCl and 500 mM KCl with pH=8.3, 0.2 mM of each dNTP, 3 ng/µL of each primer and 2.5 Unit Taq polymerase (from Roche company) in a final volume of 50 µL. Exons 5-8 of the p53 gene were amplified by a single 40-Cycle PCR using primers. Cycling conditions were as follows: primer annealing at 57°C for 1 min, polymerization at 72°C for 30 s and strand separation at 95°C for 1 min in a Thermocycler Model PTC-150-16 & 25 from MJ Research Co. PCR products were purified with Microcon 100 filters (Millipore) and sequenced directly by BigDyeTM fluorescent dye dideoxy sequencing and microcapillary electrophoresis with an ABI 310 Genetic Analyzer according to the supplier's Instructions (Applied Biosystems International).

Multiplex PCR and p53 GeneChip Array

For this purpose, only 9 samples were selected from those had p53 gene mutations in exon 5-8 using ABI 310 system, (nine samples out of 12 had p53 gene mutations). Multiplex polymerase chain reaction (PCR) was performed by a single 35 cycle using 20 primer pairs

covering exon 2 to 11 of the p53 gene. Genomic DNA was mixed with 10 mM MgCl2, 200 mM of each dNTP, 5 µL of mixed 20 primers and 2 Unit/µL of Ampli Taq Gold in a final volume of 50 µL.

primer annealing at 60°C for 30 s, polymerization at 72°C for 45 s and strand separation at 95°C for 30 s with a final extension of 10 min at 72°C in a Master Cycler from Eppendorf Co. The multiple PCR product was analyzed in 4% agarose gel in a standard electrophoretic apparatus (Horizon 58 Life Technologies Co.). The protocol was continued when bands presented on the gel (Fig. 1). The samples were rejected with missing bands or bands of decreased intensity on the gel. 45 µL of the whole multiple PCR product fragmented with 0.25 Unit of DNase I and 2.5- µL of 1 Unit/µL Alkaline Phosphatase at 25°C for 15 min and at 95°C for 10 min. fragmented samples labeled with 1 mM of Fluorescin-N6-ddATP, 1.4 µL of 25 Unit/µL Terminal transferase and 5 mM of CoCl2 at 37°C for 45 min and at 95°C for 5 min. 250 µL of 12X SSPE+10% Triton X-100 (containing 1.8 M NaCl, 0.12 M NaH2PO4, 0.012 M EDTA and 100 ml Triton X-100), 50 µL of 20 mg/ml Acetylated BSA, 10 µL of 100 nM Control Oligonucleotide F1 (5′-FluorescinCTGAACGGTAGCATCTTGAC-3′) and 90 µL distilled water were added to each fluorescent labeled fragmented DNA sample. Finally, these samples were washed over the Chip and allowed to bind to complementary oligonucleotide probes on a p53 GeneChip Array at 45°C for 30 min in a DNA-Chip Machine (Affymetrix, Inc., Santa Clara, CA). Hybridized probe arrays were then read using the GeneArray Scanner. All sequences were compared with the control human placental DNA (Fig. 2 a,b).

Results

Analysis of micro-dissected samples (without Laser) by ABI 310 System

p53 gene mutations were identified in 9 out of 12 analyzed samples by ABI 310 system. Detected mutations were as follows:

1. A missense mutation at codon 248 (CGG→TGG) in sample GC-F14R,
2. A missense mutation at codon 152 (CGG→CTG) in sample GC-F17R,
3. A missense mutation at codon 152 (CGG→CTG) in sample GC-F18R,
4. A missense mutation at codon 213 (CGA→TGA) in sample GC-F15R,
5. A missense mutation at codon 248 (CGG→TGG) in sample GC-F27R,
6. Tandem mutation at codon 242 (TGC→TTT) in sample GC-F59R;
7. Missense mutations at codon 258 (GAA→AAA) and at codon 282 (CGG→TGG)in sample GC-F14R;
8. A missense mutation at codon 248 (CGG→TGG) in sample GC-F37R;
9. A missense mutation at codon 273 (CGT→CAT) in sample GC-F39R

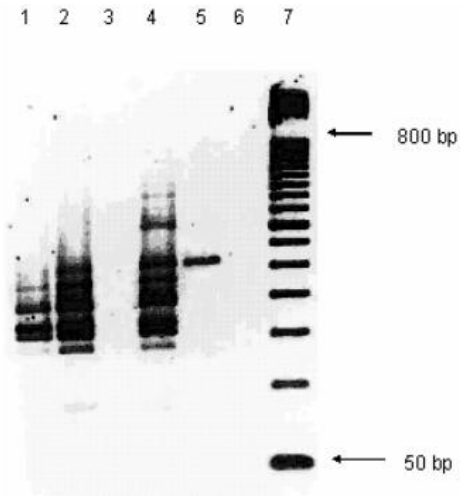

Figure 1.

Gel electrophoresis of PCR product from multiplex and uniplex PCR method. The genomic DNA flanking exons 2-11 was amplified using set of primers. Lanes 1, 2 products of multiplex PCR for samples GCF17R and GC-F18R, respectively. The sample GC-F17R was rejected because of missing bands. The sample GC-F18R was identified p53 mutation with score 23 by Microarray analysis.

Analysis of Laser-micro-dissected samples by ABI 310 System

For two samples, (samples 8 and 9 or GC-F37R and GC-F39R), DNA was extracted from esophageal neoplastic cells by laser microdissection. By using laser in DNA extraction, cancer cells have been separated from other cells and in this way mutation in codon 248 (CGG→TGG) was shown clearly and without the presence of the normal tissue peak.

Analysis of Micro-dissected Samples (without Laser) by Microarray

Microarray could detect mutations in 3 of 9 ESCC specimens in which p53 gene mutations were identified by ABI 310 system. p53 gene mutations were detected in three micro-dissected samples by Microarray and the scores of mutations was recorded 16-29. The score was calculated automatically by the Affymetrix Inc. software. Microarray detected a missense mutation for p53 gene at codon 248 (CGG→TGG) in sample GCF14R with score 16, a missense mutation at codon 152 (CGG→CTG) in sample GC-F18R with score 23, a missense mutation for p53 gene at codon 248 (CGG→TGG) in sample GC-F27R with score 29 (Fig. 2b). Other mutations out of exons 5-8 was not found in these specimens. The tandem mutation (in sample 6 or GC-F59R, TGC→TTT), could not be detected by Microarray.

In Figure 2a and in the top row the normal nucleotide sequence of the codon 247 (AAC) is recorded. The score was zero for this codon. In Figure 4b and in the top row the normal and mutant nucleotide sequence of the codon 248 (CGG) is recorded. For this sample, score was 29. The antisense strand was converted automatically to sense strand. Figure 2b records the mutation in codon 248 (CGG→TGG) with a score of 29. Three samples (GC-F55, GC-F88, GC-

F34) had no mutations using ABI 310 system, therefore they were not selected for Microarray and ABI system analysis. The results from assessing p53 gene mutations with Microarray were compared with the results of nucleotide sequence determination by ABI system.

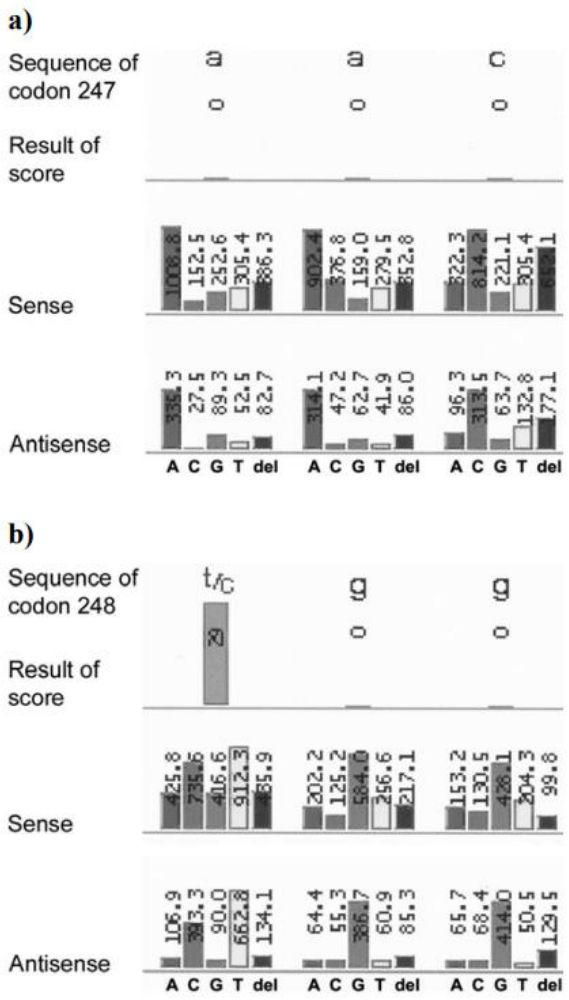

Figure 2.

a. The normal nucleotide sequence of the codon 247 (AAC) is showed. The second row is the result of the comparison of the nucleotide sequence of the control sample and the test sample; the same row also presents no score. The third and fourth rows present the nucleotide sequence in sense and antisense strands related to the test sample. b. Mutation analysis by a p53-Chip showing base substitution mutation at codon 248 (CGG→TGG) in tumor GC-F27R with score 29. For each nucleotide 5 different columns were shown to identify different nucleotides and del for 1-bp deletion.

Discussion

Discussion An interesting point in nucleotide sequence determination with Microarray method in this research is the use of formalin-fixed, paraffin-embedded tissue; other researchers prefer to use fresh tissue in order to assess mutation in p53 gene with this method [10-15]. DNA

breaks into pieces during the processes of formalin fixation and paraffin embedding. The quality of extracted DNA from formalin-fixed, paraffin embedded samples is dependent on the quality of the starting materials including formalin buffer. Keeping tissues in formalin after an operation or biopsy for short period, is effective in preventing breakage of DNA [16-20]. Therefore, the extracted DNA was purified with Microcon 50 filters (Millipore) before performing PCR to remove the fragmented DNA. By this measure, we could detect only 3 point mutations for p53 gene by Microarray analysis (samples 1, 3, 5 in Table 1). The results indicate that the choice of method for extracting DNA from test samples to assess mutation in p53 is very important. In addition, the thickness of the tissue in each DNA extraction test has its own importance in achieving precise results (the optimal thickness of the tissue for laser microbeam microdissection is 3-µm) [21-25].

It was determined that Microarray is dependent on the amount of tissue in DNA extraction and the extracted DNA which obtained from laser-micro-dissected samples cannot be sufficient for the assessment of p53 mutation with Microarray. However, this amount of DNA is perfectly suitable for determining nucleotide sequence with micro-capillary method. It was reported that the efficiency of Microarray and ABI 310 system in the identification of all types of p53 gene mutations (for fresh tissues) are 95% and 91%, respectively [26]. The detection rate of point mutations by Microarray and ABI 310 system are 100% and 92%, respectively [27]. The efficiency of Microarray in the detection of p53 gene mutations was 30% for archived samples. Therefore, using fresh tissues to detect p53 gene mutations is recommended. b) Although the accuracy of Microarray in identifying mutations of p53 gene between exons 2 and 11 has been proven, a number of mutation types cannot be identified by this method. Concerning the structure and design of p53 Chip probes, in which only point mutations or one nucleotide base deletion is identifiable, tandem mutation or frameshifts (insertions or deletions other than single base deletions) are not identifiable with Microarray [28]. As it was explained in the results section, tandem mutation is not identifiable with Microarray. Tandem mutation is very rare in breast cancer [29-34].

Tandem mutation and complex deletion and insertion are not common in p53 gene for tumor tissues. It was found that p53 gene mutations are significant predictors of treatment response and for survival in patients [35-40]. In order to determine the success rate of cancer treatment methods in hospitals, detection of p53 gene mutations is necessary. Identification of mutations along exons 2 to 11 for p53 gene using Microarray happens in a shorter period (4/5 h) compared to the conventional DNA sequencing analysis. Therefore, the application of Microarray to identify mutation for the p53 gene, in tumor tissues, will be necessary for central hospitals, where fresh tissue samples are available easily.

Conclusion

Breast cancer is the second most common cancer in the world. The p53 gene mutations are one of the factors which expression in the genesis of a tumor can be significant for the individual

diagnosis as well as therapy. The scores can individualize the method frequency of detection, type of a used analysis. It can also show the intensity of reports in lesional progression. They represent the expression of the progress of changing the aspect of breast cancer and of related researches. In this paper, the current methods of detection and mutation of genes associated with breast cancer research were presented. For the future of effective therapy, continued shows the creation of new methods for studying p53 gene mutations in breast cancer.

The creation of new modern methods of research is of great importance for individual patient therapy. Creation of new methods of research p53 gene will contribute enormous benefits in personalized medicine. The identifying of p53 gene mutations is the basic step in further research of new modern protocol (procedures) for treatment of breast cancer. Mutations can be found performing molecular genetic, molecular cytogenetic tests, research of transcriptome as well as proteome. Genomic and proteome-based profiling method research are the most modern methods of p53 gene mutations. These methods are capable of detecting as a single mutation as well as multiple p53 gene mutations. In the last few decades, the most significant progress has been made in the detection of single and multiple gene mutations of p53.

Competing interests

The authors declare no conflict of interest.

Ethics Statement

All the animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The animal protocols were approved by the Ethics Committee of the Department of Public Health and Mortality Studies, Mumbai India.

Authors' contributions

All authors shared in the conception and design and interpretation of data, drafting of the manuscript and critical revision of the case study for intellectual content and final approval of the version to be published. All authors read and approved the final manuscript.

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