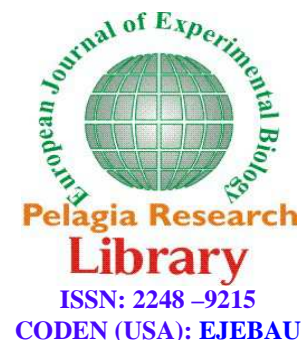




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European Journal of Experimental Biology, 2014, 4(5):143-148



Genotyping of Her1 SNP's in familial breast cancer by restriction fragment length polymorphism and sequencing

Ibtihal Riyadh Najeeb², Sudhakar Malla¹, Radhakrishnan Senthilkumar¹ and Selvam Arjunan^{1*}

¹Department of Biotechnology, Indian academy Degree College, Bangalore, India

²Department of Applied Genetics, Indian academy Degree College, Bangalore, India

ABSTRACT

Although the incidence of many types of cancer has declined globally over the last thirty years, the incidence of breast cancer globally has increased. Among women in worldwide, breast cancer remains one of the most common cancers. Genetic changes can occur at different levels and by different mechanisms. The gain or loss of an entire chromosome can occur through errors in mitosis. More common are mutations, which are changes in the nucleotide sequence of genomic DNA. Novel methods are being developed for the production of antibodies to specific antigens and thus helping in the process of development of protein based vaccines. HER1 gene was amplified from the tissue samples of the infected persons. In our present study 15 Indian families with hereditary breast cancer were studied for HER1 mutations using PCR RFLP method. Blood sample were collected and total genomic DNA was isolated using modified CTAB method. The isolated DNA was run on 1% agarose gel to verify the quality and the quantity was estimated by spectrophotometrically. PCR was done using the specific primer and the gene product was electrophorized in 2% agarose gel. The amplified product was subjected to restriction digestion using EcoRI and the PCR RFLP pattern was analyzed. The sample no 11 showed a single band, which is not digested with the restriction enzyme. So it confirmed the mutation in the recognition site of the enzyme. We eluted the PCR product along with two normal sample and send for sequencing. The sequencing result showed that there is a single mutation at the EcoRI restriction site. So RFLP can be used as to find out mutation or SNP in the gene responsible for the cancer development.

Key words: Breast Cancer, HER1, PCR-RFLP, SNP

INTRODUCTION

HER2 is a member of the epidermal growth factor receptor (EGFR/ERBB) family. Amplification or over expression of this gene has been shown to play an important role in the development and progression of breast cancer. In recent years the protein has become an important biomarker and target of therapy for approx. 30% of breast cancer patients [1, 2]. Both the epidermal growth factor receptor (EGFR/HER1) and HER2 are members of the ErbB family of the type I receptor tyrosine kinases [3], which also includes HER3 and HER4. These homologous receptors are comprised of an extracellular binding domain (ECD), a transmembrane domain, and an intracellular tyrosine kinase (TK) domain [5, 6, 7].

HER1 has several ligands including EGF, transforming growth factor α , amphiregulin, betacellulin, epiregulin and heparin binding-EGF [4, 8]. A HER2 ligand has not been identified, but over expressed HER2 is constitutively active [9]. In cells expressing both HER1 and HER2, binding of ligand to HER1 can induce HER1-HER1 homodimerization and HER1-HER2 heterodimerization. These active dimers transmit through signaling pathways including Ras/Raf/MEK/ERK and PI3K/Akt, which are important for tumor growth and metastasis [10, 11]. Recent studies have shown that HER1-HER1 homodimers and HER1-HER2 heterodimers also exist in inactive and non-ligand bound conformations also [12, 13]. These dimers are also thought to structurally rearrange to form actively signaling complexes [14].

HER2 over expression has been observed in several cancer types [17]. From 15 to 30% of human breast tumors display HER2 gene amplification or protein over expression, which is prognostic for poor outcome and predictive of a response to trastuzumab [16, 17]. HER1 over expression has also been observed in colorectal, gastric, breast, ovarian, non-small cell lung, and head and neck carcinomas as well as glioblastoma [18] and has been shown to contribute to cellular transformation and proliferation [19]. Potential cooperativity of HER1 and HER2 in mouse mammary tumorigenesis has been reported [20, 21].

Several drugs that target HER1 and HER2 receptors have been utilized in both preclinical and clinical models of breast and other cancers. Treatment with the humanized monoclonal HER2 antibody trastuzumab is now standard of care for individuals with HER2-positive invasive breast cancer in metastasis [14, 20]. However, fewer than 50% of patients with metastatic HER2-positive breast tumors show initial benefit from trastuzumab treatment, and many of those eventually develop resistance [21]. Thus, exclusive measurement of total HER2 receptor level may not provide sufficient information for prediction of drug response. Human epidermal growth factor receptor HER2 over expression is present in approximately 20–30% of breast cancer tumors. HER2 over expression is associated with a more aggressive disease, higher recurrence rate, and shortened survival [21].

HER2 is part of the epidermal growth factor (EGF) family, along with 3 other receptors: epidermal growth factor receptor (HER1, erbB1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4). The HER2 gene is located on the long arm of chromosome 17 and encodes a 185-kDa transmembrane protein [21]. The HER2 receptor extracellular domain has no identifiable ligand, unlike the other EGF family receptors. It is present in an active conformation and can undergo ligand-independent dimerization with other EGF receptors [13]. The most active and tumor promoting combination is thought to be the HER2/HER3 dimer [15]. HER2 is co-localized, and, most of the time, co-amplified with the gene GRB7, which is a proto-oncogene associated with breast, testicular germ cell, gastric, and esophageal tumours. HER2 proteins have been shown to form clusters in cell membranes that may play a role in tumorigenesis [16, 17].

The present study is aimed to isolate the DNA from tissue samples infected by breast cancer. The study mostly concentrated on isolating the HER1 and HER2 gene and to fingerprint the DNA to trace out the mutations by PCR-RFLP method. This method of study might be used for vaccine production against the HER1 & HER2 antigens and also to find out the mutation effects of the substitution. And also to identify the SNPs in the HER1 and HER2 regions by PCR-RFLP and to sequence the SNP.

MATERIALS AND METHODS

Sample collection: Samples were obtained from families at high risk for breast cancer, with three or more individuals with early-onset breast cancer. DNA was extracted either directly from peripheral blood lymphocytes. Blood was collected in EDTA collection tubes and stored at 4°C till use. Approximately 3mL venous blood were collected from each patient, and added to the Falcon Screw Cap Sterile Tubes (50mL) containing 50 μ L of 0.5M EDTA.

DNA extraction: The DNA extraction was carried out by the Phenol-Chloroform- Isoamyl Alcohol (PCI) method. Briefly to 3mL of the EDTA treated blood samples 45mL of RBC lysis buffer pH 7.3 (10X buffer: 89.9gms of NH₄Cl, 10gms of KHCO₃, and 2mL of 0.5M EDTA(pH:8)) was added and incubated at room temperature for 1hour. The contents are then mixed thoroughly and centrifuged at 1000rpm for 30 minutes, and the supernatant carefully removed and discarded without disrupting the visible white pellet. The white pellet was resuspended in 1mL of RBC lysis buffer and the mixture was then centrifuged at 10,000 rpm for 15 minutes. The supernatant obtained was removed and discarded without disrupting the visible white pellet. The white pellet was thoroughly washed in 1mL

of PBS (phosphate buffered saline). The contents are then centrifuged at 10,000 rpm for 10 minutes, and the supernatant was removed and discarded without disrupting the visible white pellet. To the pellet, 5 μ L of Proteinase-K (10mg/mL), 500 μ L of DNA extraction buffer(100 mM Tris HCl, 100mM EDTA, 1.4 M NaCl, 1% CTAB), 50 μ L of β -mercapto ethanol, 25 μ L of 1M DTT(Dithiothreitol) were added and mixed thoroughly.

The contents are then incubated the above mixture at 37°C for 30minutes in incubator, followed by 66°C for 30-60 minutes in water bath. To the above mixture equal amounts of Phenol/Chloroform/Isoamylalcohol (25:24:1) are added and centrifuged at 10,000 rpm for 15 minutes. The aqueous layer was slowly aspirated and to this equal amount of Chloroform/Isoamyl alcohol (24:1) was added and mixed gently. The mixture was centrifuged at 10,000 rpm for 10 minutes and the aqueous layer separated into a fresh microfuge tube and an equal amount of Isopropanol was added for precipitating the DNA. The DNA was pelleted down by centrifuging at 10,000 rpm for 15 minutes. The pellet obtained was washed with 70% ethanol and dissolved in 30 μ L of sterile Milli-Q water and stored at -20°C until further use. The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). The DNA samples obtained were run on 1% agarose gel to quantify them. Primers were designed to either introduce or destroy a restriction site in either the wild-type or the mutant allele. The HER1/2 specific primers were designed using Primer3 Plus software.

PCR Amplification: The HER1 gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of HER1 gene. The forward primer for HER1, 5' CAC CTC CAA GGT GTA TGA AG 3' and the reverse primer, 5'- CTC TAG GAT TCT CTG AGC ATG G 3', were purchased from Eurofins, Bangalore. These primers correspond to the gene HER1 and thus the final PCR product was 470bp. The PCR mixture consisted of 10x reaction buffer with MgCl₂ (1.5mM), 2 μ L of dNTP mix (2.5mM), 2 μ L each of forward and reverse primers (10picomoles/ μ L each primer), 0.3 μ L of Taq DNA polymerase (5U/ μ L), and 50ng/ μ L of template DNA in a total volume of 20 μ L.

The PCR was performed with the following cycling profile: initial denaturation at 94°C for 5min, followed by 30 cycles of 40s denaturation at 94°C, annealing at 55°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 5min. The PCR products amplified were then qualitatively analyzed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose. The PCR assay was performed using 1 μ L of the DNA template in a total reaction volume of 20 μ L. The reactions were performed in a Thermo cycler (G-Strom, UK). Thirty amplification cycles were performed. After amplification, PCR products included 100 bp ladder was loaded in 1.0% Agarose gel electrophoresis. After a post staining in 0.50 mg/ml of Ethidium bromide solution, the results of gel electrophoresis were documented by using a gel documentation system.

SNP Detection by Restriction fragment length Polymorphism: To determine the SNP, RFLP analysis was performed separately by selected Restriction endonucleases enzyme *EcoRI* (Merck, India). The PCR products were mixed with PCR-RFLP reaction mixture (10X Assay Buffer, 100X BSA, and Restriction enzyme). The contents were mixed properly and incubated at 37°C for 4hrs. The PCR products obtained were digested with restriction enzymes and resolved on 2% agarose gel to check for the banding pattern. After Restriction digestion, the PCR products included 100bp ladder was loaded in 2.0% Agarose gel and Electrophoresis was performed.

Direct Sequencing of PCR Products for SNP detection: The samples showing aberrant heteroduplex pattern were reamplified from genomic DNA. The amplicons were purified using Bioline PCR purification kit (Bioline, UK) and subjected to cycle sequencing using ABI Protocol using direct incorporation of radiolabel was followed as per manufacturer's instructions. Cycling conditions were 30 cycles of 95°C for 30s, 56°C for 30s and 70°C for 1min. The sequencing primers were the same as those used to amplify the template.

RESULTS AND DISCUSSION

DNA isolation and quantification

The total genomic DNA was isolated by modified CTAB method from the infected blood samples. A total of seven blood sample was collected from the Govt .Hospital, Bangalore. After RBC lysis, the obtained pellet was used for the total genomic DNA isolation. The isolated DNA was electrophorized in 1% Agarose gel as shown in the figure 1.

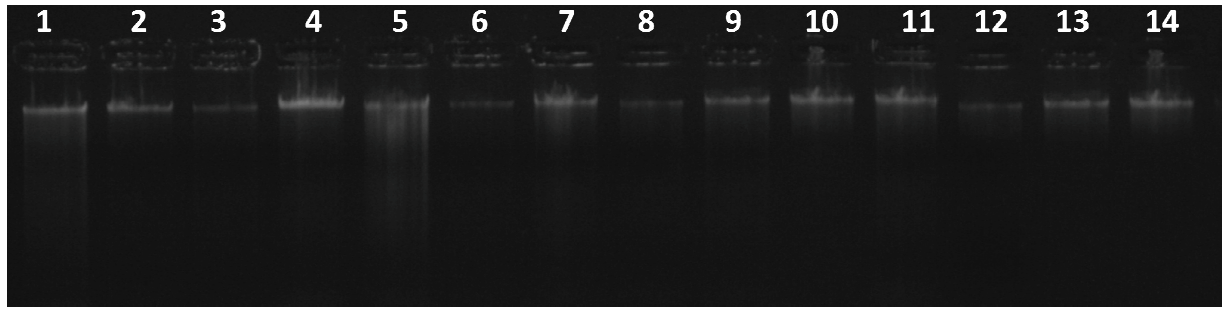


Figure 1: Genomic DNA isolated from patient blood samples (Lane 1-14 - Genomic DNA)

PCR amplification:

The predicted primers were validated initially *in silico* and subsequently in wet lab. The primers could yield an amplicon of the expected size of ~ 500 bp. The PCR product was electrophoresed and visualized by 1% agarose gel. (Fig) HER1 gene (chr 17q21) is a tumor suppressor gene that encodes tumor suppressor protein which acts as a negative regulator for tumor growth.

SNP analysis RFLP method

In this study, 15 Indian families with hereditary breast cancer were studied for HER1 mutations using PCR RFLP method as shown in the figure 2.

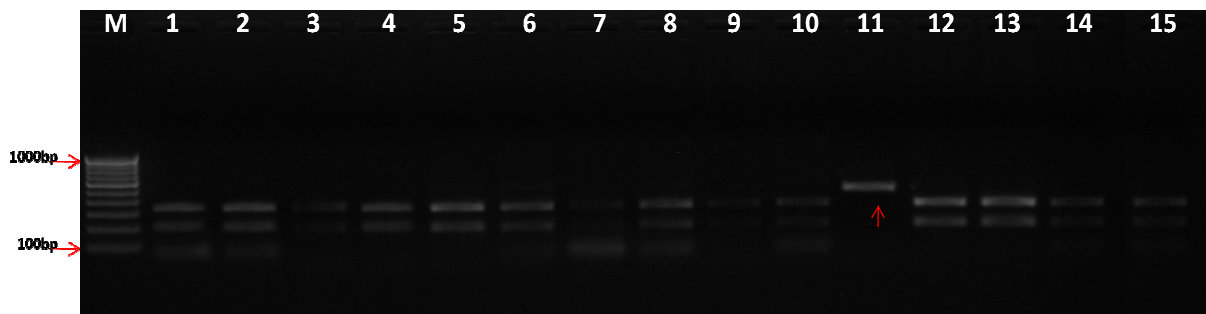


Figure 2: SNP detection by using RFLP analysis of HER1 gene using *EcoRI* restriction enzyme. M: Marker 1kb

The amplified product was subjected to restriction digestion using *EcoRI* and the PCR RFLP pattern was analyzed. The sample no 11 showed a single band, which is not digested with the restriction enzyme. So it confirmed the mutation in the recognition site of the enzyme. We eluted the PCR product along with two normal sample and send for sequencing. The sequencing result showed that there is a single mutation at the *EcoRI* restriction site.

SNP analysis by direct PCR product sequencing: The PCR products, after restriction digestion which showed single band, were sequenced by cycle sequencing to identify the nature of mutation. Appropriate control PCR products from normal persons were also sequenced. We were able to identify the mutations in the sample 9 as shown in the sequence data. This mutation leads to the inhibition of digestion of the *EcoRI* restriction enzyme which forms an uncut single band in the RFLP pattern.

Normal sample (EGFR/HER1)

CCGGAGTCCCGAGCTAGCCCCGGCGGCCGCGCCGCCAGACCCGGACGACAGGCCACCTCGTCGGCG
 TCCGCCCCGAGTCCCCGCCTCGCCGCCAACGCCACAACCACCGCGCACGGCCCCCTGACTCCGTCCAGT
 ATTGATCGGGAGAGCCGGAGCGAGCTCTTCGGGGAGCAGCGATGCGACCCTCCGGGACGGCCGGGGC
 AGCGCTCCTGGCGCTGCTGGCTGCGCTCTGCCCGGCGAGTCGGGCTCTGGAGGAAAAGAAAGTTTGCC
 AAGGCACGAGTAACAAGCTCACGCAGTTGGGCACCTTTGAAGATCATTTTCTCAGCCTCCAGAGGATG
 TTCAATAACTGTGAGGTGGTCTTGGGAATTTGGAAATTACCTATGTGCAGAGGAATTATGATCTTTCC
 TTCTTAAAGACCATCCAGGAGGTGGCTGGTTATGTCCTCATTGCCCTCAACACAGTGGAGC**GAATTC**CT
 TTGGAAAACCTGCAGATCATCAGAGGAAATATGTACTACGAAAATTCCTATGCCTTAGCAGTCTTATC
 TAACTATGATGCAAATAAAACCGGACTGAAGGAGCTGCCCATGAGAAATTTACAGGAAATCCTGCAT
 GCGCGCTGCGGTTTCAGCAACAACCCTGCCCTGTGCAACGTGGAGAGCATCCAGTGGCGGGACATAGT
 CAGCAGTGACTTTCTCAGCAACATGTCGATGGACTTCCAGAACCACCTGGGCAGCTGCCAAAAGTGTG
 ATCCAAGCTGTCCAATGGGAGC

SNP sample (EGFR/HER1)

CCGGAGTCCCGAGCTAGCCCCGGCGGCCGCGCCGCCAGACCCGGACGACAGGCCACCTCGTCGGCG
 TCCGCCCCGAGTCCCCGCCTCGCCGCCAACGCCACAACCACCGCGCACGGCCCCCTGACTCCGTCCAGT
 ATTGATCGGGAGAGCCGGAGCGAGCTCTTCGGGGAGCAGCGATGCGACCCTCCGGGACGGCCGGGGC
 AGCGCTCCTGGCGCTGCTGGCTGCGCTCTGCCCGGCGAGTCGGGCTCTGGAGGAAAAGAAAGTTTGCC
 AAGGCACGAGTAACAAGCTCACGCAGTTGGGCACCTTTGAAGATCATTTTCTCAGCCTCCAGAGGATG
 TTCAATAACTGTGAGGTGGTCTTGGGAATTTGGAAATTACCTATGTGCAGAGGAATTATGATCTTTCC
 TTCTTAAAGACCATCCAGGAGGTGGCTGGTTATGTCCTCATTGCCCTCAACACAGTGGAGC**GAATTA**CT
 TTGGAAAACCTGCAGATCATCAGAGGAAATATGTACTACGAAAATTCCTATGCCTTAGCAGTCTTATC
 TAACTATGATGCAAATAAAACCGGACTGAAGGAGCTGCCCATGAGAAATTTACAGGAAATCCTGCAT
 GCGCGCTGCGGTTTCAGCAACAACCCTGCCCTGTGCAACGTGGAGAGCATCCAGTGGCGGGACATAGT
 CAGCAGTGACTTTCTCAGCAACATGTCGATGGACTTCCAGAACCACCTGGGCAGCTGCCAAAAGTGTG
 ATCCAAGCTGTCCAATGGGAGC

CONCLUSION

Breast cancer is one of the most common malignancies affecting women worldwide. In India, breast cancer is the second most common malignant condition among women. Genetic predisposition for familial early onset of breast cancer accounts for approximately 5-10% of all breast cancers. Mutations in two autosomal dominant genes, HER1 and HER2 have been linked to familial breast cancer. In the present study 15 Indian families with hereditary breast cancer were studied for HER1 mutations using PCR RFLP method. Blood sample were collected and total genomic DNA was isolated using modified CTAB method. The isolated DNA was run on 1% Agarose gel to verify the quality and the quantity was estimated by spectrophotometrically. PCR was done using the specific primer and the gene product was electrophorized in 2% Agarose gel.

The amplified product was subjected to restriction digestion using *EcoR1* and the PCR RFLP pattern was analyzed. The sample no 11 showed a single band, which is not digested with the restriction enzyme. So it confirmed the mutation in the recognition site of the enzyme. We eluted the PCR product along with two normal sample and send for sequencing. The sequencing result showed that there is a single mutation at the *EcoR1* restriction site. So RFLP can be used as to find out mutation or SNP in the gene responsible for the cancer development. Gene testing of HER1/2 is available as a routine clinical test for diagnosing hereditary breast/ovarian cancer (HBOC) in the US and other Western countries, while only a few reports have been published concerning the prevalence of HER1/2 mutations among Indian people. In conclusion, our results indicated that HER1 mutation has a role in breast cancer but a considerable proportion of the early breast cancer and familial breast cancer may be due to genes other than HER1 mutation.

Until recently, the study of EGFR function in breast cancer biology has been largely limited to HER-2 receptor [16]. By adding *HER-1* in MDA-MB 231 cancer cells I showed how the transfected cancer cells respond to anticancer drugs. Understanding how growth factor receptors and their downstream kinases are activated by HER-1 (and vice-versa) is a central goal for maximizing treatment opportunities in breast cancer. In addition to other EGFRs, it is predicted that modulating the activity of HER-1 is expected to provide novel prevention and treatment approaches for breast cancer patients.

Single nucleotide polymorphism (SNP) plays an important role in the study of complex genetic diseases, in pharmacogenetic analysis, in population genetics and evolutionary studies. Gene testing of HER1/2 is available as a routine clinical test for diagnosing hereditary breast/ovarian cancer (HBOC) in the US and other Western countries, while only a few reports have been published concerning the prevalence of HER1/2 mutations among Indian people.

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