SCREENING FOR 185delAG BRCA1 MUTATION IN BREAST AND/OR OVARIAN CANCER PATIENTS

Summary:
Breast cancer susceptibility gene, BRCA1, is a highly penetrant gene and mutations in BRCA1 contribute an estimated 56-85% lifetime risk of developing breast and/or ovarian cancer. A clinically important, frameshift, over-represented mutation is 185delAG in exon 2 of BRCA1 gene. We hypothesize that the prevalence of 185delAG BRCA1 mutation in breast and/or ovarian cancer patients in the south Indian population is similar to the Ashkenazi population, in which it occurs at a high frequency. To test this hypothesis, we propose to screen for 185delAG BRCA1 mutation in the familial as well as sporadic breast and/or ovarian cancer patients in the south Indian population. DNA will be tested for the presence of 185delAG BRCA1 mutation using the polymerase chain reaction – confirmation sensitive gel electrophoresis (PCR-CSGE) method. The variant bands will be subjected to DNA sequencing to confirm the mutation. This will enable us to get a better understanding about the prevalence of this mutation in the south Indian breast and/or ovarian cancer patients. Further, the results of this study will enable us to give life time risk assessment, clinical recommendations for carriers, genetic counseling and preventive therapy, identification of pre symptomatic mutation carriers and origin of mutation.

Objectives:
- To identify breast and/or ovarian cancer patients through various hospitals
- To screen for 185delAG BRCA1 mutation
- To offer carrier detection and genetic counseling

Background:
India is growing rapidly both in terms of industrialization and urbanization resulting in change of lifestyle factors. Factors such as increasing body weight due to high fat diet consumption, nuclear families due to urbanization and minimum physical activity are some of the important etiological factors. Possible contributions of these factors lead to a gradual increase in the incidence of various types of cancer in the country. Notably, breast cancer and ovarian cancer has become the most common cancer and remains as the principal cause of death from cancer among women in India (Yeole 2008). The incidence of breast cancer has surpassed cervical cancer and is now the most common malignancy among Indian women. The age-adjusted annual incidence rate (AAR) has increased from 19.9 to 26.6 per 100,000 women (1998 to 2002) among Indian women (Murthy et al, 2009a). On the other hand, an increase in the incidence of the ovarian cancer has also been noted through various Indian cancer registries. Within a short period spanning 2001-06, the age-standardized incidence rates (ASR) for ovarian cancer varied from 0.9 to 8.4 per 100,000 women amongst various registries (Murthy et al., 2009b).

Causative factors:
Breast as well as ovarian cancer occurs from both genetic and environmental factors whose effects accumulate as mutations in essential genes (Nathanson et al., 2001). The inherited or familial form of breast cancer and ovarian cancer is responsible for about 5-10% of the cases, the rest arising from a sporadic form (Claus et al., 1996).
BRCA1 gene (MIM - 113705) is associated with increased risk for breast and ovarian cancer. If there is a mutation in BRCA1, then the lifetime risk of breast cancer is estimated to be 55-85%, while the lifetime risk of ovarian cancer is 20-40% (http://www.ovariancancer.jhmi.edu/hereditary.cfm). BRCA1 was mapped to the long arm of chromosome 17 (17q12-21) using linkage analysis (Hall et al., 1990). The identified gene namely, BRCA1 spans 117 kb of genomic DNA, which codes for a 5.7 kb transcript composed of 24 exons. Twenty-two of these exons translate into a 220 kd protein comprised of 1,863 amino acids (Miki et al., 1994).

**Figure 1:** Schematic representation of BRCA1 gene. BRCA1 gene contains 24 exons, of which 22 are coding exons and 2 are non-coding (exons 1 and 4) exons. Exon 11, being the largest exon is split and shown as 4 parts for convenience.

![GenBank ID: U14680; Source: BIC database](image)

**Functional role of BRCA1 protein:**

BRCA1 plays an important role in:

- maintaining chromosomal stability
- DNA double strand break repair
- transcriptional coactivation
- cell cycle regulation (Venkitaraman, 2001)

**Mutations in BRCA1 gene:**

Mutations in major genes such as BRCA1 have strong effects which can cause early onset of cancer and the development of multiple tumors. For example, most women who carry a highly penetrant mutation in BRCA1 develop breast or ovarian cancer. A single copy of the mutation in BRCA1 i.e., a heterozygous allele is enough to develop a cancerous phenotype and they are inherited as dominant alleles (Frank 2004).

Mutations in the BRCA1 gene have been identified in a large number of families with multiple cases of early-onset breast and/or ovarian cancer (Narod et al., 1995). Most germ line mutations are predicted to result in protein truncation caused by frame shift/nonsense or splice site alterations; these mutations are spread along the length of the coding region (lau et al., 2001). Most of these mutations have been reported in the Breast Cancer Information Core (BIC) database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). However, as many of these variants are polymorphic, they do not exhibit a phenotypic effect. Among Ashkenazi Jews, two predominant mutations in BRCA1 (185delAG and 5382insC) account for the majority of germ line mutations in high-risk breast and/or ovarian cancer families (Abeliovich et al., 1997). However, there have been very limited studies on the prevalence of BRCA1 mutations in Indian breast cancer patients (Valarmathi et al., 2004; Kumar et al., 2002).
185delAG – a founder mutation:

A founder mutation can be defined as a mutation that appears in the DNA of one or more individuals who are founders of a distinct population. Founder mutations can get passed down to other generations. A common, ancient BRCA1 mutation, 185delAG have been identified predominantly among Ashkenazi Jews and is called as “Ashkenazi mutation”. It is also found in many other populations around the world. The 185delAG mutation is carried by 1% of Jewish Ashkenazim population due to a founder effect [Roa et al., 1996]. The BRCA1 protein is 1863 amino-acid long and possesses two well conserved structural domains: a zing finger of the RING type at its N-terminus, and a BRCT (BRCA1 C-terminus) domain at its C-terminus. This mutation is located at the 5’ end of the gene and predicted to cause truncation at the beginning of the zinc-binding region of the RING of the putative polypeptide (Simard et al., 1994). Ruffner et al., 2001 have proposed that mutations within the BRCA1 RING domain predispose to cancer by inactivating BRCA1 ubiquitin protein ligase activity. There is no report on the specific mechanism of 185delAG mutation, although it may be noted that it occurs in an area of multiple adenosines, which is prone to DNA slippage. In India, 185delAG has been reported in almost all populations studied.

From the BIC database, we understand that there are 1981 185delAG BRCA1 mutations out of 2131 mutations reported in exon 2.

**Figure 2:** The tumor types in the proband as reported in the BIC database for the presence of 185delAG mutation is summarized in the form of a pie chart

![Pie Chart](image_url)
International status:

Thousands of carriers of 185delAG, which introduces a protein termination codon very early in the coding sequence at amino acid 39, have been identified worldwide. Although the BRCA1 185delAG mutation is commonly referred to as the ‘Ashkenazi mutation’, it is not restricted to this particular ethnic subgroup (refer figure 3). Rather, it is reported in many other Jewish as well as non-Jewish ethnic subgroups (Table 1), with a unique haplotype for Jews which is different from the haplotypes of non-Jewish groups (Bar-sade et al., 1998).

It is well established that BRCA1 mutation carriers face a high lifetime risk of breast cancer and additionally, they also face a high risk of second primary cancer in the contralateral breast (Graeser et al., 2009). It is interesting to observe that the risk of developing contralateral breast cancer in BRCA mutation carriers reduces with the age of diagnosis and increases with the number of breast cancer affected first-degree relatives (Metcalfe et al., 2011). About 19.4% of bilateral breast cancer patients are reported to have 185delAG BRCA1 mutation (Skasko et al., 2009). Recently, there are reports about the response to therapy in BRCA1 mutation carriers. Krieger et al., 2011 observed that BRCA1 mutation carriers were less sensitive to taxane chemotherapy compared to non-BRCA1 mutation carriers.

Table 1: Prevalence of 185delAG BRCA1 mutation in different ethnic groups around the world

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Type of cancer</th>
<th>Reported %</th>
<th>Haplotype information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spanish</td>
<td>Breast and Ovarian cancer</td>
<td>Case report</td>
<td>Ashkenazi haplotype</td>
<td>Ah Mew et al., 2002</td>
</tr>
<tr>
<td>Malay</td>
<td>Breast or ovarian cancer</td>
<td>0.53</td>
<td>Not available</td>
<td>Thirthagiri et al., 2008</td>
</tr>
</tbody>
</table>
Spanish Breast/ovarian cancer 1.85 Ashkenazi haplotype Diez et al., 1999
Chile breast/ovarian cancer 3.17 Not available Jara et al., 2004
Slovak Breast or ovarian cancer 5 Not available Cierniková et al., 2006
Iran Breast cancer 0.5 Not available Mehdipour et al., 2006
Jews and Pakistan Ovarian cancer 0.97 Not available Risch et al., 2001
Jews Ovarian cancer 19.4 Not available Muto et al., 1996
Ashkenazi Jews Pancreatic cancer 6.16 Not available Stadler et al., 2011

National status:
The 185delAG, a deleterious, frame shift mutation was first reported in a family residing in Trivandrum, in the state of Kerala in India, which is not far from the small towns with settlement of Jewish people (Kumar et al., 2002). It was later reported by several Indian studies (Table 2). Dinesh et al (2006) have reported that there is a BRCA1 involvement in the pathogenesis of sporadic breast cancer in Chennai, Tamil Nadu through his immunohistochemical studies on BRCA1 protein expression in tissue sections. Eachkoti et al., 2007 reported the presence of BRCA1 intronic mutations in sporadic breast cancer patients in an ethnic population of Kashmir.

Table 2: Prevalence of 185delAG BRCA1 mutation in Indian sub groups

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Type of cancer</th>
<th>Reported %</th>
<th>Haplotype information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>South India</td>
<td>breast cancer</td>
<td>7.14</td>
<td>Not available</td>
<td>Kumar et al., 2002</td>
</tr>
<tr>
<td>North India</td>
<td>breast/ovarian cancer</td>
<td>2.35</td>
<td>Not available</td>
<td>Valarmathi et al 2004</td>
</tr>
<tr>
<td>South India</td>
<td>breast/ovarian cancer</td>
<td>54.5</td>
<td>Not available</td>
<td>Vinodkumar et al 2007</td>
</tr>
<tr>
<td>South India</td>
<td>Breast and/or ovarian</td>
<td>16.4</td>
<td>Not available</td>
<td>Vaidyanathan et al, 2009</td>
</tr>
<tr>
<td>North India</td>
<td>Breast cancer</td>
<td>0.49</td>
<td>Not available</td>
<td>Saxena et al 2006</td>
</tr>
<tr>
<td>South India</td>
<td>Breast cancer</td>
<td>2</td>
<td>Not available</td>
<td>Soumittra et al 2009</td>
</tr>
<tr>
<td>South India</td>
<td>Breast cancer</td>
<td>Case report</td>
<td>Different from Ashkenazi Jews</td>
<td>Kadalmani et al 2007</td>
</tr>
</tbody>
</table>
Importance of the study:

Accurate knowledge of the proportion of cases carrying 185delAG BRCA1 mutation is important in order to offer genetic screening and counseling to women with breast cancer and/or ovarian cancer or family histories of any one of the types of cancer. The relative risk of developing breast cancer by age 42 was estimated to be 31 (CI 11-77) for 185delAG BRCA1 mutation (Neuhausen et al., 1996). Mutations with strong effects cause the early onset of cancer and tend to aggregate in families. Most women who carry a highly penetrant mutation such as 185delAG in BRCA1 develop breast or ovarian cancer. It is still not known about the prevalence of 185delAG BRCA1 mutation in Indian patients with breast and/or ovarian cancer at large. Therefore, the present study is undertaken to screen for such a frequently reported mutation, the 185delAG BRCA1 mutation in hereditary as well as sporadic breast and/or ovarian cancer patients.

Figure 4: An overview of the project. The flow chart shows that breast and/or ovarian cancer patients will be identified and registered. Blood will be used to isolate DNA, which will then be analysed for the presence of 185delAG mutation using appropriate primers.

If we screen only the breast cancer patients with family history of cancer, we will be underestimating the prevalence of 185delAG BRCA1 mutation. This is because of the fact that family history alone is a poor predictor of BRCA1 mutation status at least in Asian families (Kang et al., 2010). Many studies have reported the presence of 185delAG BRCA1 mutation in sporadic breast and/or ovarian cancer patients (Soumittra et al., 2009; Dinesh et al 2006). Risch et al 2001 have reported the presence of 185delAG BRCA1 mutation in women with no family history of cancer. A high BRCA1/BRCA2 mutation ratio (8.8%) among sporadic breast cancer patients was reported by Leeneer et al., (2011). Therefore, it is necessary to screen both the familial and sporadic breast and/or ovarian cancer patients along with controls to exactly understand the prevalence of this particular mutation in the local population.
There are a number of potential benefits that can be inferred from the results of this project.

1. Identification of carriers
2. Preventive therapy for carriers (preventive mammography and surgical intervention)
3. Pre-clinical diagnosis becomes possible
4. Genetic counseling can be offered to the affected family
5. Origin of mutation through haplotype analysis

Here, I would like to make it clear that our previous project (funded by DST; File No. SR/FTP/LSA-104/2002) aimed at screening for complete BRCA1 gene (all the 22 coding exons) mutations in hereditary breast cancer patients of Tamil Nadu. But the present project aims at screening for only one particular mutation in exon 2, which is 185delAG BRCA1 mutation, a highly frequent mutation reported in all the populations studied. In addition, the present study aims at both familial and sporadic breast and/or ovarian cancer unlike the previous study which aimed at only hereditary breast cancer patients. To my knowledge, there are no studies conducted on the prevalence of 185delAG BRCA1 mutation in Indian breast and/or ovarian cancer patients, though there are reports on complete BRCA1 screening.
Preliminary work:

We have published our work on:


**Figure: 5A**

*5A - Pedigree of a family with 185delAG mutation in the BRCA1 gene. The proband (III-3) was diagnosed with breast cancer at the age of 53 years. Individual III-4 developed breast cancer at the age of 47 years. Numbers immediately above the symbols are individual identification numbers. Numbers below symbols indicate the current age for the individuals in the pedigree. The symbol * indicates the individuals who did not participate in the study (Source: Kadalmani et al 2007).*
**5B** - An ethidium bromide–stained CSGE gel of exon 2 (258 bp) in individuals of generation IV. Lane M is a 100bp DNA ladder while lane 10 is a control sample from an unaffected, unrelated person. Lanes 1-9 represents the carriers of 185delAG mutation (lanes 4, 7, and 9) and noncarriers of the mutation (lanes 1, 2, 3, 5, 6 and 8) of generation IV (Source: Kadalmani et al 2007).

**5C** - Sequence electropherogram of cloned exon 2 of the BRCA1 gene. The upper panel shows the presence of 2 copies of AG (boxed) and is from a healthy normal individual, while the lower panel that shows deletion of one copy of AG (boxed) is from the proband (Source: Kadalmani et al 2007).
5D - Haplotype analysis among the selected members of the family (see figure 1). The STR markers that were used (indicated on the left) and the alleles observed are given below the symbols for the individuals. The haplotype linked to the mutation is shown in the box (Source: Kadalmani et al 2007)

The results of the above study clearly showed us that 185delAG mutation in BRCA1 does play an important role in causing breast and/or ovarian cancer in patients from south India and the origin of this mutation through haplotype analysis demonstrates that it is independent and different from Ashkenazi Jews haplotype.
**Detailed Methodology:**

Patients having a clinically documented history of breast and/or ovarian cancer will be included in the study. We have contacts with few of the cancer hospitals and oncology division of government hospitals, who will be referring their patients for this study. A detailed family history will be collected from the patients and pedigrees will be drawn for each familial case. Healthy individuals matched for age and sex without any family history of any type of cancer will be included as controls.

We will be collecting blood samples (about 2 ml) from 250 sporadic cancer (breast and/or ovarian), 250 familial cancer (breast and/or ovarian) patients along with 500 healthy, age and sex matched controls. We have obtained clearance to conduct this study from our institutional biosafety and ethical committee (IBEC-8). Informed written consent will be obtained from each individual who will be part of the study. Confidentiality will be maintained by appropriate coding.

A standard questionnaire will be used to record relevant information from patients registered for the study. Data on patient's personal and clinical history will be recorded in the questionnaire. These include patient's age, sex, age at menarche, age at childbirth, parity, age at menopause, menopausal status and type, family history of cancer, type of cancer, stage of cancer and mode of treatment. A comprehensive questionnaire and a consent form have been designed, standardized by our pilot study.

**Blood collection and DNA isolation:**

We will collect 2 ml of blood using EDTA vacutainer tubes. DNA will be isolated using QIAamp DNA minikit (Qiagen, USA, cat no. 51304). The amount of DNA will be quantitated using UV-VIS spectrophotometer. The quality of the DNA will be checked using agarose gel electrophoresis. Storage of the DNA at -80°C will be done till the samples are analysed.

**Requirements:**

EDTA vacutainer tubes, tourniquet, disposable syringes, cotton, alcohol, DNA isolation kit, electrophoresis tank, buffers, loading dye, gel documentation system, -80°C freezer, and agarose

**Amplification of BRCA1 exon 2:**

The exon 2 of BRCA1 gene will be amplified using the following primer set.

Forward primer: 5'-GAAGTTGTCATTTTATAAACCTTT
Reverse primer: 5'-TGTCTTTTCTTCCCTTAGTATGT
PCR product size: 258bp

**Preparation of Reagents for PCR**

In a sterile 0.5 ml microfuge tube, the following reagents for PCR amplification will be mixed.

- 10X Amplification buffer: 5 µl
- 20 mM solution of four dNTPs (pH 8.0): 1 µl
20 µM forward primer 2.5 µl
20 µM reverse primer 2.5 µl
1-5 units/µl thermostable DNA polymerase 1-2 units
Sterile water 28-33 µl
Template DNA 5-10 µl
Total volume 50 µl

The reactions will be carried out in a thermal cycler, usually for 30-35 cycles through varying temperatures (denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 30 seconds) to amplify the exons of BRCA1 gene. We will optimize PCR conditions for different DNA templates.

Requirements
Automated thermal cycler, Micropipettes, Sterile eppendorf tubes and tips, Sterile deionised water, 100mM MgCl2, 10X amplification buffer, 100mM dNTPs, 50µM oligonucleotide primers 1 and 2, 25 µg/ml template DNA, 5 U/µl Taq DNA polymerase

Mutation detection by Confirmation Sensitive Gel Electrophoresis (CSGE)
CSGE is a mutation detection method that is based on heteroduplex formation of DNA (Grompe, 1993; White et al 1992). It is simple, effective for larger size fragments and does not involve radioactivity. The sensitivity of the technique can be appreciated from the studies conducted by Ganguly et al, 1993, where they detected 60 of 63 point mutations for PCR products ranging from 200-800 bp.

Principle
A heteroduplex is double-stranded DNA where the base complementarity between the two DNA strands is not perfect. When such DNA is denatured, the two strands are separated. On renaturation, complementary DNA strands reassociate and form a homoduplex. However, when there is a mutation in one of the strands, then heteroduplexes is also formed. The electrophoretic mobility of a heteroduplex in polyacrylamide gel is different from a homoduplex due to conformational changes, and these can be detected as an extra slow moving band. This concept of heteroduplex formation forms the basis of mutation screening by CSGE. Additionally, in the CSGE technique, mildly denaturing solvents such as polyacrylamide and bis-acryloyl piperazine (BAP) will be used to enhance the conformational change in heteroduplex DNA (refer figure 6). Primer sequences, PCR conditions and the heteroduplex detection protocols will be as per our previous protocol (Kadalmani et al., 2007). Sequencing of PCR amplicons showing heteroduplex band will also be done as per our previous method (Kadalmani et al., 2007).

Requirements:
Acrylamide, Bis-acryloyl piperazine, ethylene glycol, deionized formamide, TTE buffer, TEMED, Electrophoretic gel apparatus and high-voltage power pack
Figure 6: Detection of mutations by using CSGE. This method involves isolation of DNA from blood sample of a patient and a normal person (N). The coding regions of BRCA1 gene are amplified by polymerase chain reaction. After amplification, heteroduplexing was carried out (see text for details) and electrophoresed in a CSGE gel. If there is a single band in the electrophoregram, it indicates normal sample, while additional bands appear in the case of the patient. The sample showing the additional bands will be subjected to DNA sequencing.

Time schedule:

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<tr>
<th>Period of Study</th>
<th>Achievable targets</th>
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<tbody>
<tr>
<td>6 months</td>
<td>Contacts with cancer hospitals will be established and registration of relevant cases</td>
</tr>
<tr>
<td>12 months</td>
<td>Collection of blood samples from the patients and storage of samples</td>
</tr>
<tr>
<td>18 months</td>
<td>Isolation of DNA from the samples and checking the DNA for its quality and quantity</td>
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<tr>
<td>24 months</td>
<td>PCR-CSGE will be performed on DNA samples</td>
</tr>
<tr>
<td>30 months</td>
<td>Sequencing will be done to confirm the mutation wherever needed and analysis of results obtained</td>
</tr>
<tr>
<td>36 months</td>
<td>Summarizing the data and interpretation of results</td>
</tr>
</tbody>
</table>

Source: Lakhotia and Somasundaram, 2003
**Expected Outcome:**

There is a great public utility as an outcome of the study in terms of pre symptomatic counseling and pre clinical diagnosis for carriers. Since these DNA based tests are not routinely done across the counter, this project assumes great importance in terms of public as well as patient awareness about cancer. The outcome of this project will also have an impact on therapeutic strategies planned for the patient in the context of preventive mammography and surgical intervention.

**Future Plans:**

The present project will give us an exact proportion of cases with this particular 185delAG BRCA1 mutation in south Indian population. A larger sample size will be planned in future to throw more light on other contributing factors such as family history, age at diagnosis and ethnicity. If the prevalence of this mutation is found to be high, then developing a diagnostic kit for detecting 185delAG BRCA1 mutation can be planned. We will also plan to look for the presence of any biomarker (Heerma et al., 2011 and Honrado et al., 2006) in 185delAG BRCA1 mutation carriers, which will enable easy identification of susceptible individuals who might develop cancer in their lifetime.

**Budget:**

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<th>S.No</th>
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</tr>
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</table>

**References:**


White MB, Carvalho M, Derse D, O'Brien SJ, Dean M. Detecting single base substitutions as heteroduplex polymorphisms. Genomics, 1992 (12);301-306.