DISCUSSION
DISCUSSION

Breast cancer is a multifactorial disease. An accumulation of genetic alterations is responsible for the transformation of normal cells into cancer cells. Several hereditary (germline) and acquired (somatic) genetic alterations are known to induce genomic instability, resulting in a dis-balance between cell proliferation and cell death, and ultimately in growth development and progression (Klijn et al., 2002). Hereditary genetic alterations (germline mutations) are responsible for hereditary breast cancers and acquired (somatic mutations) are responsible for sporadic breast cancers. The dominant transmission of a trait implicates its acquisition in 50% of the offspring. More than 25 years ago, the observation that the penetrance for cancer mutation in the germline is incomplete prompted Alfred G. Knudson to suggest that something else had to take place at the genotypic level for the development of cancer (Knudson, 1971). He hypothesized that cancer results from the occurrence of a second mutation in a somatic target cell, so that the only difference between hereditary and non-hereditary tumours is the timing of the first mutation (prezygotic or postzygotic). The second mutations can either affect the remaining normal allele of the same gene (recessive trait) or one copy of a different gene (dominant trait).

Anecdotal evidence from individual families in which breast cancer occurs very frequently, and large epidemiological studies have shown that some women have a familial predisposition to breast cancer (Mant and Vessey, 1991). The anecdotal evidence includes the pedigree of Broca's family (Broca, 1866). He was a famous French surgeon (1824 –1880), and in his family tree (which comprised over five generations) 10 out of 24 women died of breast cancer. Systematic epidemiological studies performed in the second half of the 20th century have shown that, in women with a family history of breast cancer, the risk of breast cancer is increased two to three fold ('familial breast cancer'). Such studies have also shown that there are families in which breast cancer risk is inherited in an autosomal-dominant fashion ('hereditary breast cancer'). Since the first demonstration of evidence for dominant genetic susceptibility to breast
cancer (Williams and Anderson, 1984), more recent genetic epidemiologic-based estimates suggest that approximately 5-10% of all breast cancer cases are associated with hereditary predisposition conferred by an autosomal dominant genetic determinant of relatively high penetrance (Newman et al., 1988; Colditz et al., 1993; Slattery and Kerber, 1993; Claus et al., 1996). This type of genetic predisposition is characterized by a non-sex-linked (autosomal) single gene mutation inherited from one or the other parent (dominant) and causes a relatively high probability (high penetrance) that the associated phenotype (breast cancer) will develop at some point over a lifetime. The term *hereditary cancer* is most commonly used in association with this type of genetic predisposition, and in recent years, it has been shown that germline mutations in the *BRCA1* and *BRCA2* genes account for a large proportion of cases of hereditary breast cancer.

Epidemiological studies indicate that *BRCA1* mutation carriers have a lifetime risk of breast cancer that is on the order of 60–80% (Ford et al., 1994; 1998; Antoniou et al., 2003). The lifetime breast cancer risk for *BRCA2* mutation carriers approaches that of *BRCA1* carriers: however, disease onset has been documented to be at a later age (Ford et al., 1998; Antoniou et al., 2003). In other words, women with an altered *BRCA1* or *BRCA2* gene are 3 to 7 times more likely to develop breast cancer than women without alterations in those genes, with very high relative risks for early disease onset (before age 40) of about 30-fold. Carriers of *BRCA1* and *BRCA2* mutation(s) are also at increased risk for other cancers – in particular, both genes increase the risk of ovarian cancer, while *BRCA2* confers greatly increased risks of male breast cancer also. Additional, but more modest, risks are found for uterine, cervical, early onset prostate and pancreatic cancers for *BRCA1* (Thompson et al., 2002b) and prostatic, pancreatic, gallbladder, bile duct, stomach cancers and melanoma for *BRCA2* (BCLC, 1999).

The spectrum of *BRCA1* and *BRCA2* mutations has been characterized in different populations worldwide, with significant variation of the relative
contribution of these genes to hereditary cancer between populations and with examples of population specific founder mutations (BRCA1: 185delAG, 5382insC, BRCA2:6174delT in Jews, BRCA2: 999del5 in the Icelandic population) (Szabo and King, 1997). The contribution of BRCA1/BRCA2 mutations pertaining to early onset familial breast/breast and ovarian cancer from North India has been accumulating (Valarmathi et al., 2003; 2004; Saxena et al., 2002; 2006). Previous study from Kerala population analyzed the frequencies and patterns of germline BRCA1 gene mutations among 29 hereditary breast/ovarian cancer families and identified the proportion of BRCA1 gene mutations as 38% (11/29) (Vinodkumar et al., 2007). While the number of patients in the study is small, the results suggest that the frequencies of BRCA1 germline mutations in Kerala (South India) is similar to that seen in the US and Europe. However, the contribution of mutations in BRCA2 gene to breast cancer patients in the South Indian population remains relatively unexplored. In fact there is paucity of BRCA2 mutation reports on breast/ovarian cancer patients from South Indian population. Hence there is the need for screening a larger number of samples to investigate the role of BRCA2 gene mutations in the high-risk group of familial as well as early onset cases, in order to determine the prevalence of these mutations and to evaluate whether clinical testing for BRCA2 mutations would be useful in this population.

Identification of susceptibility to cancer requires compiling a family history that will provide sufficient information to assess the significance of familial or hereditary factors. According to Kubba et al. (1993), establishment of a familial cancer registry is most helpful for cancer risk determination, surveillance and management programmes, identification of new cancer prone genotypes and etiological family studies. Hence, at the Regional Cancer Centre, Thiruvananthapuram, several cancer families have been identified through family history analysis in a face-to-face interview. This was found to be an easily acceptable and very productive method for the recognition of familial cancer aggregations and the identification of hereditary cancer associations. For all
patients with a positive family history, a detailed pedigree or family tree was constructed. Constructing a pedigree is an integral part of assessing an individual's genetic risk. This involves gathering a detailed cancer history (all anatomic sites, age of cancer onset, pattern of multiple primary cancers, including bilaterality of paired organs, and when applicable, exposures to carcinogenic agents) on all of the proband's first degree relatives (the patient's siblings, progeny and parents) and selected second degree relatives (both sets of grand parents, aunts and uncles). The second degree relatives may be highly informative in that being older, they will more likely have passed through the cancer risk age and thereby have expressed the pertinent hereditary cancer phenotype.

Pedigree analysis enabled the categorization of the 102 hereditary breast cancer patients into two groups: one group belonging to HBC families (hereditary breast cancer only families) and other to HBOC families (hereditary breast/ovarian cancer families). ie. Out of the 96 hereditary breast cancer families identified 84.3% (81/96) were breast cancer only families and 15.6% (15/96) were breast/breast-ovarian cancer families. The mean age of the HBOC patients was 42.88 ± 10.75 years. Even though the age of the HBOC patients ranged from 22-79 years, majority of the HBOC patients were below 50 years of age showing an early age of onset of the disease (Figure 18). Regarding the menstrual status, 72.4% of the patients were pre-menopausal (Figure 19d). Interestingly, hereditary breast/ovarian cancer patients showed a higher frequency of bilateral disease (9%) than those with sporadic breast cancer (4.44%) (Table 9). These characteristics are in support of salient features of hereditary cancers reported in the literature. A possible explanation for the earlier age of disease onset in BRCA2 mutation carriers could be high circulatory estrogen levels in younger women compared to elderly women. According to one hypothesis, the total number of ovulatory cycles and thus exposure to higher estrogen level is a significant factor contributing to the risk of breast cancer (Henderson et al., 1985). Estrogen exposure is hypothesized to increase the susceptibility to breast tissue
to carcinogenesis through continued cell division and proliferation resulting from multiple ovulatory cycles, principally between menarche and first birth, thus allowing for a concomitant increase in the accumulation of random genetic errors (Preston-Martin et al., 1991; De, 1992b). Increased estrogenic exposures increase the rate of proliferation, hence magnifying the effect.

Pearson $\chi^2$ analysis revealed no significant differences in the distribution of socio-demographic characteristics among HBOC cases and controls. Likewise the various clinico-pathologic characteristics analyzed such as tumour size, node involvement, metastasis, disease stage, laterality, histologic grade, estrogen receptor and progesterone receptor status showed no significant variation among HBOC and sporadic breast cancer cases in Pearson $\chi^2$ analysis.

Mutation analysis of the $BRCA2$ gene was carried out in a total of 264 study subjects, comprising of 102 hereditary breast/ovarian cancer (HBOC) patients, 72 early age onset (≤45 years) sporadic breast cancer patients and 90 age matched control individuals. For mutation analysis, in the present study, a comparatively recent technique called Conformation Sensitive Gel Electrophoresis (CSGE) was employed. CSGE is a technique which detects single base mismatches in DNA heteroduplex. Many single nucleotide changes as well as deletions were identifiable with CSGE technique suggesting that this is very sensitive and fast pre-screening method. This system differs from other heteroduplex based methods in that mildly denaturing solvents are used to amplify the tendency of mismatches to produce conformational changes. The increased tendency towards altered conformations results in differential migration of homo and heteroduplexes in the gel matrix. In CSGE, a different crosslinker, Bis Acryloyl Piperazine (BAP) is used instead of BIS Acrylamide (Ganguly et al., 1993; Williams et al., 1995). For the present $BRCA2$ gene mutation analysis using CSGE, 10% polyacrylamide gel was used. Introduction of BAP instead of BIS acrylamide increases the pour size of the gel to those of conventional 5% polyacrylamide gel and the resilience of a 10% polyacrylamide gel. CSGE is relatively inexpensive, reasonably high throughput, technically simple to perform,
and has been applied successfully for numerous genetic disorders – including BRCA1 and BRCA2 screening in many studies. It is estimated that 100% of all sequence variants can be detected with this method (Ganguly et al., 1997).

**BRCA2 GENE MUTATION ANALYSIS**

**BRCA2 gene mutations in HBOC Patients**

The entire coding regions and intron-exon boundaries of BRCA2 gene in 102 breast/ovarian cancer patients from 96 different breast ovarian cancer families were analyzed by means of CSGE, with heteroduplexes subsequently being identified through direct sequence analysis. A total of 19 distinct germline alterations including 12 distinct mutations were identified in 31.37% patients (Tables 10, 11 & 12). The results of the present study were comparable with the study from a South American population (Jara et al., 2006). In their study 70 breast/ovarian cancer patients from 64 high-risk breast and/or ovarian cancer families from Chile (South America) were screened for germline mutations in the BRCA2 gene using conformation-sensitive gel electrophoresis and detected a total of 15 alterations in 21.42% patients. Another study by Maillet et al. (2006) identified a total of 68 BRCA2 sequence alterations in a cohort of 350 Swiss breast/ovarian cancer families, accounting for 19.4% of the patient population. Analysis of BRCA2 in the 75 patients from 40 families with multiple cases of breast and ovarian cancer from Cyprus revealed the presence of BRCA2 sequence variants in 49% of the patient population including 3 truncating mutations, 14 missense mutations, 8 polymorphisms, and 12 intronic variants (Hadjisavvas et al., 2004). Thus, the prevalence of BRCA2 mutations varies among different population groups.

**BRCA2 Frame Shift Mutations:** By definition, Frame Shift mutation (FS) is a mutation in a DNA chain that occurs when the number of nucleotides inserted or deleted is not a multiple of three, so that every codon beyond the point of insertion or deletion is read incorrectly during translation. The two pathogenic frame shift BRCA2 mutations detected in the present study were c.4642delAA
and c.4926insGACC. These mutations are readily classified as cancer predisposing because they uniformly truncate \textit{BRCA2} prior to the COOH-terminal nuclear localization signals (Spain et al., 1999), resulting in exclusion of the mutant proteins from the nucleus and inactivation of all associated nuclear functions. The c.4642delAA is a frame shift deletion mutation which changes the reading frame of mRNA and causes a premature termination codon at position 1480. The c.4926insGACC is a frame shift insertion mutation which results in the ceasing of the translation of the \textit{BRCA2} protein at codon 1575, leading to the production of a truncated \textit{BRCA2} protein. Two of these deleterious mutations detected are novel and hence could be considered unique to the Kerala population. These two mutations were identified in 1.96% [2/102] of breast/breast ovarian cancer patients analyzed.

\textit{BRCA2 nonsense mutation:} Apart from the deleterious frame shift mutations, the present study identified one nonsense mutation 10204A>T [K3326X] also in 2/102 (1.96%) patients. The very same nonsense mutation was identified previously with a frequency of 0.02% in a US control population, and was not associated with an increase of susceptibility to breast cancer and ovarian cancer (Mazoyer et al., 1996). The authors of this study concluded that this mutation is a polymorphic nonsense variant, which does not increase cancer risk. Subsequently Krainer et al. (1997) described this mutation in a single case in a cohort of 73 early-onset breast cancers and did not find it in an age- and sex-matched control group of 130 individuals. The K3326X polymorphism has since been identified in patients with a variety of cancers either alone or in combination with obvious pathogenic mutations (Haraldsson et al., 1998; Claes et al., 2003). However, evidence that the K3326X polymorphism may be functionally deleterious came from Howlett and coworkers who, in demonstrating that biallelic inactivation of \textit{BRCA2} was a cause of Fanconi's anemia identified a Fanconi anemia individual with the K3326X variant as well as the 3033delAAAC frame shift mutation on exon 11, suggesting that the variant may cause Fanconi's anemia in the compound heterozygote state (Howlett et al., 2002). An alternative
The explanation is that the K3326X polymorphism may occur in linkage disequilibrium with other disease susceptibility alleles outside of the BRCA2 coding sequence. However, none of the genes adjacent to the BRCA2 locus are suspected to be tumour suppressor genes. In a previous analysis of BRCA2 mutations among 29 patients with familial pancreatic cancer, Murphy et al. (2002) identified three patients with the K3326X variant. More recently, Martin et al. (2005) reported that the BRCA2 K3326X polymorphism was significantly more prevalent in individuals with familial pancreatic cancer: 8/144 (5.6%) vs 3/250 controls (1.2%) (OR = 4.84; 95% CI = 1.27–18.55, P<0.01) thereby suggesting a deleterious role for this mutation.

This premature stop codon results in loss of the final 93 amino acids of the BRCA2 protein. The C-terminus of BRCA2 is thought to be functionally important. For example, BRCA2 protein from a patient with Fanconi anemia with C-terminal deletion of BRCA2 (nts 3226–3418) was unable to co-localize with FANCD2 (Wang et al., 2004). Similarly, mouse cells with truncated BRCA2 lacking the last 188 amino acids are hypersensitive to radiation (Morimatsu et al., 1998). These data support the notion that the K3326X polymorphism is deleterious, which was further strengthened by the absence of this variant in the 90 analyzed control subjects from Kerala population.

**BRCA2 Missense mutations**: Nine distinct BRCA2 missense mutations (MS) were identified in 15/102 (14.7%) breast/ovarian cancer patients.

A sequence variant in exon 18 (c.8415G>T) resulting in a substitution of Lysine to Asparagine at codon 2729 (K2729N) was identified in two patients in the present study. This same type of missense alteration has been reported earlier in one young breast cancer case and 6 patients belonging to 5 families (Zhi et al., 2002). Further, this substitution is non conservative and occurs in an amino acid residue located in the highly conserved BRCA2 COOH domain. In the present study, the two breast cancer patients identified to harbour this mutation belonged to the same family (BF54) (Figure 15). None of the control samples in the present study
harboured this mutation. These observations are suggestive of disease association which was further supported by the absence of this variant in the previously studied Indian control samples (Saxena et al., 2002, 2006; Valamathi et al., 2004).

Of particular interest is the BRCA2 missense mutations, 4486G>T and 3257G>A, which appears to be a recurrent mutation in Kerala population because it was detected in four unrelated HBOC families and also in four sporadic patients. The 4486G>T resulted in the substitution of an acidic polar amino acid (aspartic acid) to neutral polar tyrosine. The very same mutation was reported more than 190 times in the BIC database. Although Hadjisavvas et al. (2004) reported the frequency of this missense mutation in the breast/ovarian cancer families and in the control population from Cyprus as 2.5% and 2% respectively, the present study failed to identify the same in the 90 control subjects from Kerala. Two other novel missense variants obtained in the present study 5206C>T and 3578T>C, resulted in the substitution of two neutral non polar amino acids (Proline and Isoleucine) to two other neutral polar amino acids (Serine and Threonine) respectively.

The missense variant c.5332C>T (p.P1702S) obtained in this study was a rare alteration, reported only once at the BIC database and the ethnicity has not been specified. This amino acid change P1702S is located in protein region following BRC5 repeat. The c.4501G>A missense change in exon 11 which results in the substitution of Asparagine for Aspartic acid at codon 1425 (D1425N) may affect the conformation of the protein, since an acidic residue is replaced by a polar uncharged residue. Similarly, the c.5299A>C missense change in the same exon which results in the substitution of lysine for glutamine at codon 1691 (K1691Q), may also affect the conformation of the protein, since a polar uncharged residue replaces a residue that is basic. However, the missense variant c.5007A>C, with a conservative glutamic acid to aspartic acid substitution (E1593D) obtained in the present study has previously been reported in two breast cancer patients from North India (Saxena et al., 2002). One additional report of this missense
alteration in a Pakistani patient is available in the BIC database. Although this missense substitution occurs in a residue that is invariant between humans and other mammalian species, it has been previously reported in 1 Pakistani individual from among 9 Indian and Pakistani population controls studied by Wagner et al. (1999), suggesting it is likely to be a population-specific neutral polymorphism. However the fact that this mutation was found only in one breast cancer patient (age: 68 years) (Table 11), coupled with its absence in the control subjects in the present study suggest the possibility of local variation in ethnic-specific alteration of the \textit{BRCA2} gene (Khoo et al., 2002). But, further studies of this variant are warranted in establishing whether it is disease associated.

Although truncating mutations may be assumed to cause disease, pathogenicity involving missense mutations is more equivocal because of insufficient information concerning both protein function and genetic variation. The significance of missense mutations and their associations with breast cancer are largely unknown because there is currently no suitable functional assay to evaluate their effect on tumourigenesis. For this reason, most of the detected missense mutations are recorded in the BIC database as unclassified variants (BIC, 2003).

It has been postulated that some missense mutation may be pathogenic, depending upon the nature of the amino acid substitution and its effects on protein structure and function and may play a role in cancer pathogenesis (Fleming et al., 2003). More over, missense mutations in conserved protein motifs are more likely to be deleterious. Missense amino acid changes in the p53 binding domain or the trans-activation domain of \textit{BRCA1} adjacent to a BRCT repeat have been shown to be pathogenic (Monteiro et al., 1997; Zhang et al., 1998). The role of \textit{BRCA2} unclassified variant mutation in breast carcinoma pathogenesis has not yet been reported. Unclassified variant mutations in \textit{BRCA2} gene might also be critically involved in \textit{BRCA2} function and consequently associated with a high penetrance and predisposition to cancer.
This variant might also be a modifier of BRCA2 or a related gene function with similar impact on cancer occurrence as protein truncating mutation.

Interestingly, 53% (10/19) BRCA2 sequence variants identified were novel, all (except intronic variants) localized in exon 11. Seven BRCA2 germline alterations described in this study is located in the short BRC repeat sequences in exon 11. This could be of functional relevance as they are located in the RAD51-binding domains and might therefore interfere with the BRCA2-RAD51 interaction (Katagiri et al., 1998). Using in vitro studies, it has been shown that BRCA2 binds directly to RAD51 through 6 of the 8 BRC repeats (BRC 1, 2, 3, 4, 7, and 8 motifs) (Wong et al., 1997) that are highly conserved between species (human, monkey, rat and mouse) (Bignell et al., 1997). Therefore, changes in the BRC motifs might interfere with the binding of RAD51 and lead to a loss of function (Chen et al., 1998). Thus, inactivating mutations in the RAD51 binding sites may be responsible for breast and ovarian cancer predisposition in these families. More over, 80% (15/19) of the mutation carriers in the present study had missense mutations suggesting an important role for these variants in breast cancer predisposition. Above all, none of these missense alterations were identified in the control subjects analyzed in the present study as well as in control subjects from North Indian populations (Saxena et al., 2002; 2006; Valarmathy et al., 2004). Screening a large number of appropriate controls might be a useful adjunct to other methods for evaluation of unclassified variants (Chenevix-Trench et al., 2006). Considering these aspects it is reasonable to suspect that missense mutations might have predisposed this group to cancer occurrence.

Thus the present study identified a total of twelve distinct germline BRCA2 gene mutations (two frame shifts, one nonsense and nine missense) in 18.6% (19/102) of hereditary breast/ovarian cancer patients in Kerala. In many populations, germline BRCA2 mutations are thought to account for 15 -20 % hereditary breast cancer and nearly all of large families with both early onset breast and ovarian cancer (Ford et al., 1998; Nathanson and Weber, 2001). A recent study by
Miramar et al. (2008) reported a comparable BRCA2 mutation frequency in Spanish population in which 15% of high-risk breast and/or ovarian cancer patients tested positive for BRCA2 deleterious mutations. In line with this study, various studies on several other populations had reported similar mutation frequencies for BRCA2 gene (Dutch 12% by Peelen et al., 2000; Germany 12% by Hamann et al., 2002; US 27% by Schubert et al., 1997b). However, Ozdag et al. (2000) identified BRCA2 frame shift mutations in 33% of hereditary breast cancer cases, a frequency that is rather high and similar to the Icelandic population. On the contrary much lower BRCA2 mutation frequencies have been reported from various other populations (Hispanic families 6.3% by Vogel et al., 2007; Portuguese 7% by Peixoto et al., 2006). However, contradictory results among these studies might probably be because of differences in the characteristics of the patient population analyzed, and criteria used for the selection of these families. Variation in the sensitivity of the screening techniques might also have accounted for the differences in the reported results. Additional variability in the results of population based studies might also be attributed to the variable contribution of founder mutations in different ethnic populations (Thorlacius et al., 1998; Loman et al., 2001 and Shih et al., 2002).

**BRCA2 Silent Mutations and Intronic Variants:** All the silent/intronic variants obtained in the present study (except the novel ones) had been originally reported as a polymorphism in many populations (Wagner et al., 1999; Hadjisavvas et al., 2004). However, the present study failed to detect these variants in any control samples. In evaluating the sequence variants found in the control populations, those variants occurring with a frequency of >1% in both global and specific populations may be classified reliably as polymorphisms. Even where only a single occurrence of a sequence variant is detected in the control individuals, this may be considered a strong indication of polymorphism (Wagner et al., 1999). Additional research into control populations is needed to clarify this issue.
The silent mutation L1132L was detected in four HBOC patients (F01, F09, F15, and F16). In F15, the protein truncating mutation c.4642delAA was identified. In F16, the missense mutation c.4486G>T was identified. Moreover, out of the 21 HBOC patients reported with silent/intronic variants, 43% (9/21) were found to harbour another frameshift/missense/nonsense mutations. It seems unlikely, therefore, that these variants are of clinical relevance. However, two examples of silent mutations inducing exon skipping in the 'fibrillin-1 gene' and the 'calpain gene' have been reported (Liu et al., 1997b; Richard and Beckmann, 1995). These findings imply that all sequence variants specific to HBC/HBOC might, at the very least, be of potential, functional relevance. Thus, similar to missense alterations the functional relevance of intronic variants - except of intronic splice site mutations - also often remains enigmatic. Evaluating the effect of these variants on BRCA2 mRNA processing could support its functional significance.

Out of the 19 distinct BRCA2 sequence variants identified in the present study, 13 (68.42%) of the alterations were confined to exon 11, which, to date, is reported with a great proportion of mutations identified in most of the studies. ie. Most BRCA2 related families carry mutations located in exon 11 of the gene. Peixoto et al. (2006) reported 71.4% (5/7) of BRCA2 mutations in exon 11. Although there is no obvious explanation for this observation, it may be efficient to analyze exon 11 in the initial stage of the screening protocol, which will be helpful in reducing the time and cost of mutational analysis, in Kerala population.

It has been suggested that BRCA2 mutations are clustered in site specific breast cancer families (Ford et al., 1998; Ikeda et al., 2001; Ellisen and Haber 1998). This has been interpreted as evidence for an increase in ovarian cancer risk associated with BRCA1 but not BRCA2 mutations. Some studies have postulated a BRCA2 central region (nucleotides 3035–6629 in exon 11), the ovarian cancer cluster region (OCCR) which is a target of mutations associated with a higher risk of developing ovarian cancer (Gayther et al., 1997; Thompson and Easton 2001; Levy-Lahad and Friedman, 2007). Compared to other BRCA2 mutations, OCCR mutations are associated with higher ovarian cancer and lower
breast cancer risk (Thompson and Easton, 2001). However, this genotype-phenotype correlation doesn't explain results of the present study because in the present study, all the 13 identified BRCA2 alterations in exon 11 (19 families) were located within the OCCR but only 4 families had cases of ovarian cancer whereas the remaining 15 families were site specific breast cancer families. Other investigators also failed to demonstrate an increased incidence of ovarian cancer in the BRCA2 OCCR (Frank et al., 1998; Ikeda et al., 2001; de la Hoya et al., 2002; Claes et al., 2004). The results of the present study are also in agreement with an earlier report from North India (Valarmathi et al., 2004), suggesting that there exists a difference in susceptibility of BRCA2 mutation carriers to ovarian cancer between Indians and other populations. Furthermore, specific mutations could have unique risk profiles, and their relative prevalence in different studies could affect final risk estimates. In this context, one should note the locations of the common Ashkenazi Jewish and Icelandic mutations, which constitute a substantial fraction of carriers in many studies. In BRCA2, the common Jewish mutation, 6174delT, is within the OCCR, whereas the common Icelandic mutation, 999del5, is outside the OCCR. It is possible that some mutations outside the OCCR are also associated with an increase in ovarian cancer risk. Alternatively, other genetic or environmental factors present in our population may increase the ovarian cancer risk associated with a BRCA2 mutation, but these associations require much larger sets of families to be discernible.

Male breast cancer risk in BRCA2 mutation carriers has been reported to be 6% by 70 years age (Easton et al., 1997), which is approximately 80-100 times higher than in general population (Thompson and Easton 2001). Several studies suggest a correlation between male breast cancer and BRCA2 mutations (Thorlacius et al., 1996; Friedman et al. 1997; Frank et al., 1998; Ellisen and Haber, 1998; Syrjääläkoski et al., 2004). Linkage studies indicate that more than 80% of families with male breast cancer are related to BRCA2 mutations (Ford et al., 1998; Thompson and Easton 2001). In the present study, out of the 4 male
breast cancer patients included, one patient (F94) was found to harbour the nonsense mutation K3326X. The very same patient belonging to the male breast cancer family MBF88 was also found to harbour a silent mutation in exon 14 and a novel intronic variant. Another male breast cancer patient, F95, belonging to the family MBF89 was found to harbour the silent mutation S2414S in exon 14 (Tables 11 &12). It is presumed that some other genes (other than BRCA2) may be involved in the pathogenesis of male breast cancer in the remaining families.

**BRCA2 Gene Mutations in Sporadic Breast Cancer Patients**

Although most breast cancers are diagnosed in postmenopausal women, approximately one third are diagnosed in the premenopausal years (Greenlee et al., 2000). Breast cancer in young women has been shown to be clinically more aggressive (Armes et al., 1999; Vicini et al., 2000). While young breast cancer has no specific age cut-off, a reasonable upper limit is 45 years of age, so most affected individuals are in the premenopausal years (Hafty et al., 2006). It is likely that the underlying molecular and genetic basis of breast cancer in younger women is distinct from that of breast cancer diagnosed later in life. Inherited cancer susceptibility genes may also act in sporadic cases (Moolgavkar and Knudson, 1981; Weinberg, 1991). Hence the present study analyzed BRCA2 gene mutations in 72 early age onset sporadic breast cancer patients and detected only two missense and one silent mutation in five patients (Table 13), all of which are reported in HBOC cases also. No truncation mutations were detected in the early age onset sporadic breast cancer cases. The BRCA2 mutation data for sporadic breast cancer patients from the present study is in agreement with the observations of Ozdag and colleagues (2000) who also failed to identify any mutations in the 27 analyzed early onset breast cancer cases. Similar were the results of yet another study from Greece (Armakolas et al., 2002). However, this has not been the case in the study of Neuhausen et al. (1996) that reported an 8% incidence of a recurrent BRCA2 6174delT mutation in patients with early onset sporadic breast cancer. In another study, 7.5% of the sporadic patients diagnosed with breast cancer at young age were found to
harbour BRCA1/2 mutations (Claes et al., 2004). Although the number of sporadic breast cancer patients in the present study is rather small and not all coding exons of BRCA2 were screened in them, the observations indicate the lesser involvement of BRCA2 germline mutation in the development of sporadic breast cancer in Kerala. It is also possible that mutations in genes other than BRCA2 may be associated with a high risk of breast cancer in these young women (Dite et al., 2003). Also, the absence of somatic mutations of BRCA2 in the majority of sporadic breast cancer favors the hypothesis of a different carcinogenic mechanism in hereditary cases.

**BRCA2 GENE POLYMORPHISMS**

A polymorphism is defined as a non-disease-causing change and/or a change found in at least 1% of the alleles in the population (HGVS, 2004). Thus far, a number of genetic polymorphisms of various genes have been studied for their association with breast cancer risk; these genes include mostly genes for steroid hormone biosynthesis and metabolism enzymes (CYP17, CYP19, COMT, and so forth) and carcinogen metabolism enzymes (GSTP1, CYP1A1, and so forth), and other genes (ER-α, PR, TP53 and so forth) (Dunning et al., 1999). Recently, genetic polymorphisms of BRCA2 have been attracting a considerable attention because deleterious germ-line mutations in BRCA2 confer a greatly increased risk of breast cancer (Easton et al., 1993a). Until now, several BRCA2 polymorphisms (a-26g, N/H298, N/H372, T/M1915, R/C 2034) have been studied for their association with breast cancer risk, and only one polymorphism (N/H372) has been shown to be significantly associated with breast cancer risk (Healey et al., 2000; Spurdle et al., 2002). However, some other studies had failed to show such significant association with regard to the N/H372 polymorphism (Goode et al., 2002; Ishitobi et al., 2003; Cox et al., 2005).

In the present study the BRCA2 polymorphic variants N289H and S455S were identified by CSGE. Here, the homozygous variants of these polymorphisms were not detected in CSGE as this technique could only distinguish between
homozygotes and heterozygotes. However, despite the absence of the homozygous variants in the present study, the \textit{BRCA2} heterozygous variants were found to be significantly associated with sporadic breast cancer susceptibility risk ($P=0.02$) (Table 15). The present study results were comparable to another study from Japan (Ishitobi \textit{et al.}, 2003). This study investigated the association of \textit{BRCA2} polymorphism at codon 784 (Met/Val) with breast cancer risk using the PCR-SSCP method and reported the complete absence of homozygous variant (V/V 784) either in cases or controls. Moreover, \textit{BRCA2} MN784 polymorphism (heterozygous variant) was significantly associated with breast cancer risk compared to M/M784 homozygotes (Ishitobi \textit{et al.}, 2003).

ASSOCIATION BETWEEN \textit{BRCA2} GENE MUTATION STATUS AND CLINICOPATHOLOGICAL CHARACTERISTICS

Women predisposed to hereditary or familial breast cancer form a heterogeneous group. It would be useful if the high-risk \textit{BRCA2} gene carriers could be identified and target the expensive and time-consuming genetic testing to individuals who most probably carry those mutations. Several models have therefore been developed for evaluating the probability of carrying a \textit{BRCA1} or \textit{BRCA2} mutation (Parmigiani \textit{et al.}, 1998; Gilpin \textit{et al.}, 2000; Vahteristo \textit{et al.}, 2001). In a previous study of Finnish breast cancer families, multivariate analysis suggested simple family history criteria for breast cancer onset under the age of 40 years and the presence of ovarian cancer to be most strongly associated with \textit{BRCA1/2} mutation status (Vahteristo \textit{et al.}, 2001). However, in addition to family history, it is important to find other markers that could help to identify mutation carriers.

Besides family history, clinicopathological parameters could also be useful in distinguishing patients and families likely to carry a \textit{BRCA2} germline mutation from mutation-negative families and breast cancer patients in general. However a more systematic analysis involving various clinical parameters with respect to the
BRCA2 gene mutation status from specific populations is required to derive clear criteria to distinguish between the mutation positive and negative families.

Breast cancer is characterized by a number of histopathological subtypes. Generally, the vast majority of invasive breast carcinomas fall into the category 'ductal, not otherwise specified'; of note is that the tumours in this category still show heterogeneous morphology. Ductal carcinomas not otherwise specified can be divided into subgroups with different grades of malignancy on the basis of degrees of differentiation, pleomorphism and mitotic activity (van de Vijver, 1999).

Invasive ductal carcinoma is the most common histological type in BRCA2 breast tumours (Honrado et al., 2006). There is no agreement about whether there is any special histological type with a higher frequency among BRCA2 mutation carriers. Marcus et al. (1996; 1997) reported a higher incidence of tumours belonging to a “tubular lobular group” including invasive lobular, tubular, and cribriform carcinomas in BRCA2 patients. Armes et al. (1998) found that BRCA2 tumours were more frequently pleomorphic lobular carcinomas. However, other series have not exhibited statistically significant differences in breast cancer histotypes between BRCA2 and sporadic breast carcinomas (Lakhani et al., 1998; Agnarsson et al., 1998). Interestingly, all the breast cancer patients in the present study were diagnosed with infiltrating Ductal Carcinoma (IDC) with different histological grades.

BRCA2 associated hereditary breast cancers have not consistently revealed a specific pattern of characteristics, which may reflect the heterogenous pathogenesis of BRCA2 induced breast cancer (Iau et al., 2001). Indeed, while recent investigations have suggested definite differences between BRCA1 and BRCA2 related breast cancers, BRCA2-related tumours may be more difficult to distinguish from sporadic cancers.

BRCA2 tumours are more frequently moderately or poorly differentiated carcinomas (grades 2 and 3) (Lakhani et al., 1998; Lynch et al., 1998; Agnarsson et al., 1998; Palacios et al., 2003; Eerola et al., 2005a). The Breast Cancer
Linkage Consortium (BCLC, 1997) observed that this difference in grade was significantly greater due to less tubule formation in BRCA2 tumours; there were no significant difference between BRCA2 tumours and sporadic cases in the extent of nuclear pleomorphism and the mitotic count. Besides this difference in tubule formation, Agnarsson et al. (1998) have reported more nuclear pleomorphism and higher mitotic rates in BRCA2 tumours than in sporadic tumours. Likewise, BRCA2 tumours have a higher proportion of continuous pushing margins that occupy >75% of the tumour than do sporadic tumours (11% versus 5%) (Lakhani et al., 1998).

The steroid receptor status of BRCA2 related tumour is of particular interest in the light of the potential for preventive strategies for carriers. In the National Surgical Adjuvant Breast and Bowel Project P-1 Study (Fisher et al., 1998), tamoxifen was found to decrease the incidence (or, more likely, delay the appearance) of oestrogen receptor (ER) positive tumours by 69% after a median follow-up of 55 months. 60–90% of BRCA2 carcinomas have been reported to be ER positive and 40–80% PR positive, which is a similar proportion as seen in sporadic breast tumours (Armes et al., 1999; Lakhani et al., 2002; Palacios et al., 2003; Robson et al., 2004; Eerola et al., 2005a; Oldenburg et al., 2006). In BRCA2 tumours, it has been shown that the percentage of ER positive cases decreases with the increasing age. Foulkes et al. (2004) analyzing BRCA2 tumours from patients <45 years, found a statistically significant difference in the percentage of ER-positive tumours among BRCA2 carriers and non-carriers. The percentage of ER positive tumours was higher in BRCA2 tumours, however there was no difference found in a sub analysis of tumours from patients >55 years. On the contrary, Eerola et al. (2005a) did not find any difference in the percentage of ER-positive tumours between BRCA2 carriers and non-carriers <50 years, instead they found that ER-negative tumours were more frequent in BRCA2 carriers >50 years. These data need to be confirmed in additional series, since they could have implications if histopathological data were routinely used in clinical practice.
BRCA2 associated tumours are also characterized by a younger age of onset of the disease and frequent bilateral occurrence (Anderson, 1971; Ligtenberg et al., 1999; Noguchi et al., 1999). Age is among the best-established risk factors for breast cancer. In the general population, the incidence of breast cancer is extremely low before 30 years of age (<25 cases per 100,000), after which it increases linearly until the age of 80 years, reaching a plateau of slightly less than 500 cases per 100,000 (Ries et al., 2003). In BRCA mutation carriers, it is estimated that the risk for early-onset breast cancer is increased up to 20-fold (Kainer et al., 1997). In pooled pedigree data from 22 studies, the relative risk (RR) of breast cancer in BRCA2 mutation carriers was approximately 11-fold in all age groups at less than 40 years of age, and it was not significantly higher at older age groups (Antoniou et al., 2003). In that study, it has been found that in the 40-49 year age group, the breast cancer incidence in BRCA2 mutation carriers showed a pattern similar to that in the general population, where as for ovarian cancer, the RR increased to a maximum of 19 in the 50-59 years of age group and then decreased (Antoniou et al., 2003).

The risk of developing bilateral breast cancer is high for patients with a positive family history (Bernstein et al., 1992; Gajalakshmi et al., 1998), though few studies have failed to show an association between these two (Fisher et al., 1984; Adami et al., 1981b). In women with a BRCA2 mutation, the risk of a bilateral breast carcinoma is over 50% up to age 70 years (Klijn and Meijers-Heijboer, 2003). Contralateral breast cancers were significantly more common in the BRCA2 group (12%) compared with 2% in controls (P=0.02) (Verhoog et al., 1999).

In the present study a higher percentage of the BRCA2 mutation positive HBOC cases belonged to early age group (52.6%) with larger tumour size (36.8%), node involvement (63.2%), bilaterality (16.7%), presence of metastasis (15.8%), higher histological grade (72.7%), ER and PR negative (66.7%) and with higher disease stage (47.4%) compared to the mutation negative cases (Table 16). However, none of the clinicopatholgical parameters analyzed showed a significant
association with the *BRCA2* gene mutation status. Similar were the results for sporadic cases also (Table 17). The results of the present study were comparable to another study from Italy in which *BRCA2* positive patients tended to have more often positive lymph nodes, larger tumour size, negative estrogen receptors and a higher histological grade (Veronesi *et al.*, 2005). Some studies have reported that *BRCA2* associated cancers occur at a more advanced stage, on the basis that patients exhibit a more advanced axillary status (Robson *et al.*, 1998). However other investigations have not found any difference in axillary status (Agnarsson *et al.*, 1998; Armes *et al.*, 1998) or tumour size (Armes *et al.*, 1998). Another study by Verhoog *et al.* (1999) compared the tumour characteristics in *BRCA2* mutated and sporadic controls, and observed no statistical significance in disease stage and distant metastasis between the groups. However, in their study, the *BRCA2* associated tumours showed a borderline significance for larger size (*P*=0.05) compared to the sporadic controls. More over, on contrary to the findings of the present study, *BRCA2* tumours were slightly more often node negative compared to controls (63% vs 53%) in their study, but these results were not statistically significant (*P*= 0.34) (Verhoog *et al.*, 1999). Yet another study in which even though the *BRCA2*-related tumours were not significantly larger, other factors such as axillary nodal involvement (*P*= 0.03), higher disease stage (*P*= 0.02) and bilaterality (*P*=0.02) significantly varied between *BRCA2* mutated cases and controls (Loman *et al.*, 2000)

It is possible that relatively small numbers of study subjects and low numbers of the true pathogenic protein truncation mutations accounts for the present findings in Kerala population. These results could also be partly due to the fact that various factors such as ER-PR status and grade of *BRCA2* tumours in this study may not be representative of all *BRCA2* tumours as these factors remained unknown for 39%, 46% and 53% of the entire study subjects. For sporadic cases, the analysis was restricted to the available four parameters such as tumour size, nodal status, metastasis and stage of the disease, of which data were available for only 61% of the total cases.
SURVIVAL ANALYSIS

Breast cancer survival rates have been found to vary among different communities and geographical areas, but factors related to breast cancer survival are not fully understood. Previous investigations have found the following to be related to survival: Stage at diagnosis, age at diagnosis, ethnicity, socioeconomic status (SES), histology, marital status, obesity, oestrogen receptor status, treatment received and nutritional and hormonal status (Meng et al., 1997). Differences in lifestyle, culture and environment may explain the differences in cancer survival among ethnic groups. Moreover, the overall survival rate for breast cancer could be modified by a large variety of clinical and pathologic factors. An accurate appraisal of the survival according to the hereditary or genetic status is essential for counseling at risk individuals or breast cancer gene carriers. The prognosis for BRCA2 mutation-related tumours is important for the elaboration of preventive and therapeutic strategies, and for counseling women at increased risk of breast cancer measures (Hartmann et al., 1999). Therefore, the present study investigated the association of BRCA2 gene mutations as well as various clinicopathological features on overall survival of the breast/ovarian cancer patients. This study have addressed the overall survival as it reflects factors other than biologic curability, such as death resulting from complications of treatment, and subjective factors related to the willingness of the patient to continue treatment and resources available (Nair et al., 1993). The survival analysis on BRCA2 gene status was carried out separately among 3 groups of the breast/ovarian cancer patients: HBOC patients (mutated vs wild), Sporadic patients (mutated vs wild), and finally HBOC vs Sporadic breast cancer patients.

There are only a very few studies which have looked at survival of BRCA2 mutation carriers with breast cancer as a separate entity. These have reported rather controversial results. One study has reported a significantly better prognosis for the BRCA2 mutation carriers (Armakolas et al., 2002). A borderline improvement in the overall survival of BRCA2 mutation carriers has been
reported by Phillips et al. (1999). On the contrary one recent study by Rennert et al. (2007) and several other studies have shown no difference in overall survival between the mutated and non mutated groups (Gaffney et al., 1998; Lee et al., 1999; Verhoog et al., 1999), whereas two other studies have reported a trend towards a worse prognosis (Sigurdsson et al., 1996; Loman et al., 2000).

In the study by Lee et al. (1999) the figures for overall survival of BRCA2 associated cases are those of breast cancer patients who tested positive for the presence of the Ashkenazi BRCA2 founder mutation, 6174delT, and the design of the study was that of a combined BRCA1/2 study with an inferred mutation carrier status using the kin-cohort method (Struwing et al., 1997). In this way, no data were available on clinical presentation and stage of the disease. No significant prognostic differences were detected. A recent study from Poland on 151 women with invasive ovarian carcinoma, BRCA1/2 carriers showed a distinctly longer overall survival than sporadic cases (log-rank P = 0.01) (Brozek et al., 2008). Another recent study by Chetrit et al. (2008) confirmed that, among Ashkenazi ovarian cancer patients, BRCA1/2 Ashkenazi Jewish founder mutations (BRCA1-185delAG, BRCA1-5382insC, BRCA2-6174delT) are associated with improved long-term survival. Median survival for carriers was significantly longer than for non-carriers (53.7 v 37.9 months, respectively; P = 0.002), which could be due to distinct clinical behavior and/or to a better response to chemotherapy.

Differences in the definitions of a positive family history for breast cancer and in the ethnic backgrounds of the populations investigated may have resulted in a varied contribution of genetic susceptibility, and this apart from disparate control groups, might explain conflicting results regarding prognosis of familial breast cancer. Methodological differences too are likely to play a role in the diverging results from these studies.
Survival according to BRCA2 gene status

HBOC patients: In the present study, survival analysis of patients with a proven BRCA2 mutation revealed that hereditary breast/ovarian cancer patients who were inferred carriers of BRCA2 mutations harboured a significantly worse prognostic outcome than those in the non mutated groups (Log rank P = 0.02) (Figure 20). However in the multivariate analysis, when the data was corrected for clinical stage, which is in general the most important prognostic factor, the hazard ratios for mortality in the group of BRCA2 associated breast cancer patients reduced substantially compared with the wild type: HR = 2.3 (95% CI = 0.971 - 5.432; P = 0.06) (Table 20) ie. Higher clinical stages of the disease (Stage III and IV) seem to influence the prognosis greatly in the BRCA2 mutated patient group. On contrary, correction for the differences in bilateral disease did not reduce the risk of death from breast cancer for the BRCA2 associated cases to that extent: HR = 2.6 (95% CI = 1.092 - 6.098; P = 0.03) (Table 21). The survival rates and comparisons of the present study agree generally with the study by Loman et al. (2000). In their study also, a trend towards a shorter overall survival for the BRCA2 associated cases was noted with a border line significance (RR=1.6, 95% CI = 0.98-2.7, P=0.05) and significantly more BRCA2 associated cases presented with axillary lymph node involvement or stage IV breast cancer at the time of diagnosis. After adjusting for stage, the breast cancer-specific survival was no longer significantly worse for the BRCA2-associated cases (RR = 1.6; 95% CI = 0.85 - 3.1, P=0.14). However, corrections for the differences in bilateral disease did not reduce the risk of death from breast cancer for the BRCA2 associated cases to the same extent (RR = 1.8; 95% CI = 1.0 to 3.1, P = 0.03). On contrary, no significant difference in survival was detected between the BRCA2 mutated and non-mutated groups by Gaffney et al. (1998) who studied overall survival in 20 BRCA2-associated breast cancer patients from five families that previously had shown positive linkage for BRCA2.

However, the present study failed to identify a significant difference in overall survival of HBOC patients with BRCA2 polymorphic variants N289H and S455S
To the best of knowledge, this is the first study comparing the prognostic role of these \textit{BRCA2} polymorphic variants in breast cancer patients and as a consequence, currently no references are available to compare this data. Reports regarding the prognostic significance of several other polymorphisms in breast cancer patients are widely reported. The present study results were comparable to the findings of Ishitobi \textit{et al.} (2003), who reported that the N/H372 polymorphism was not significantly associated with patient prognosis. On contrary, the very same study found out that another \textit{BRCA2} heterozygote polymorphic variant (M/V784) was significantly associated with poor prognosis (Ishitobi \textit{et al.}, 2003). These inconsistencies might be explained by the difference in the type of the variant allele, difference in the sample size of the study conducted and also due to racial difference to some extent.

\textbf{Sporadic Breast Cancer patients:} Contrary to the results of the HBOC cases, the present study failed to detect a significant survival difference between the \textit{BRCA2} mutation carriers vs non-carriers in sporadic breast cancer patients (Log rank \( P = 0.18 \)). Similar were the results for the \textit{BRCA2} polymorphic variants also (Log rank \( P = 0.3 \)). However, currently no studies are available to compare the prognostic outcome of \textit{BRCA2} gene mutated vs non mutated among sporadic breast cancer cases. To generate data on survival of breast cancer patients with a \textit{BRCA2} mutation, most of the studies had made comparisons between hereditary breast cancer cases and sporadic controls (Verhoog \textit{et al.}, 1999; Loman \textit{et al.}, 2000) or were primarily limited to a few mutations that were characteristics for specific populations (Sigurdsson \textit{et al.}, 1996; Lee \textit{et al.}, 1999) and few studies failed to discriminate between \textit{BRCA1} and \textit{BRCA2} mutations (Robson \textit{et al.}, 1998; Osin \textit{et al.}, 1998; El-Tamer \textit{et al.}, 2004; Veronesi \textit{et al.}, 2005).

\textbf{HBOC Patients vs Sporadic patients:} It has been postulated that the prognosis of patients with hereditary breast cancer differs from that of sporadic cases. However, results have been conflicting (Chappuis \textit{et al.}, 1999; Phillips \textit{et al.}, 1999). A positive family history for breast cancer has been established as risk
factor for the disease (Pharoah et al., 1997) and has also been extensively investigated as a prognostic feature. A positive family history for breast cancer has been correlated with better, similar, and worse prognosis relative to sporadic breast cancer (Wobbes et al., 1987; Lees et al., 1989, Malone et al., 1996; Marcus et al., 1996; Chappuis et al., 1999).

In the present study, the overall survival of HBOC patients was similar to those of sporadic breast cancer patients. And these results were in agreement with the results of a previous study in which patients with hereditary breast cancer due to BRCA2 have a similar prognosis compared with the sporadic breast cancer patients (Verhoog et al., 1999). A Finnish study by Eerola et al. (2001) reported that the overall survival rates of breast cancer patients in the BRCA2 families did not differ from that of sporadic cases, with an overall 5 year relative survival rate of 77% (95% CI = 61%-93%) for BRCA2 mutated cases and 78% (95% CI = 77%-78%) for sporadic cases. On contrary, another study found a significantly worse disease-free survival for BRCA2-associated cases as compared with sporadic age matched controls (Sigurdsson et al., 1996). However, in that study, controls were not matched for year of diagnosis, and when the controls were matched for the same, there was a trend towards more favorable outcome (Agnarsson et al., 1998). More over, as a result of endogamy and the geographic isolation of Icelandic population, only one single specific mutation was present in the Icelandic breast cancer families linked to the BRCA2 gene (Thorlacius et al., 1996). The position of this mutation in the gene as well as its genetic and environmental background might have very well influenced patient characteristics and subsequent survival (Neuhausen et al., 1998).

Hence, from the present study, it could be concluded that the worse survival observed in BRCA2 mutated HBOC cases might be due to the higher tendency of the BRCA2 associated tumours to present with higher clinical stage at the time of diagnosis. One could also speculate the possibility that families with more breast cancer deaths might have preferably been included in the present study, but this
seemed to be an unlikely explanation. This is not surprising, because selection bias is generally believed to lead to the inclusion of families with breast cancer patients who have a better prognosis. However, because of the low numbers, all figures for survival have to be regarded with caution.

**Main predictors of survival other than the gene mutation**

For HBOC cases, the main predictors of survival other than the *BRCA2* gene mutation status were, tumour size (Log rank $P = 0.01$), stage (Log rank $P = 0.03$), metastasis (Log rank $P = 0.01$) and laterality (Log rank $P = 0.02$), where as for sporadic cases, factors such as tumour size (Log rank $P = 0.03$), stage (Log rank $P = 0.01$) and metastasis (Log rank $P = 0.02$) were emerged as the major survival predictors. The present study results are in agreement with a previous study from Kerala (Nair *et al.*, 1993) in which various clinical factors including tumour size, higher disease stage and metastasis were significantly associated with poor overall survival in breast cancer patients.

In *BRCA2* mutation carriers the lifetime risk of developing contralateral breast cancer by the age of 70 years is 32 to 64% (Nicoletto *et al.*, 2001). In the present study, $8.8\%$ (9/102) of HBOC patients developed bilateral breast cancer and analysis of the effect of laterality on overall survival revealed a significantly poor survival prognosis for the bilateral cases. More specifically, another study showed that after 2 and 5 years follow-up, contralateral breast cancer was diagnosed in $4\%$ and $12\%$, respectively, of the *BRCA2* associated breast cancer patients versus $1\%$ and $2\%$, respectively in sporadic breast cancer patients, with an overall poor clinical outcome obtained for the contralateral cases (Log rank $P = 0.02$) (Verhoog *et al.*, 1999; 2000). Studies from various populations have shown that bilateral breast cancer occur more frequently in *BRCA2* associated patients than in general patient populations (Robson *et al.*, 1998; El Tamer *et al.*, 2004, Pierce *et al.*, 2006), and also that they are at elevated risk of developing other cancers (Stratton and Wooster, 1996; Rahman and Stratton, 1998; Jakubowska *et al.*, 2003).
Consistent with a previous report by Malone et al. (1996), this study also observed that advanced stage of disease and larger tumour size were all associated with increased mortality. The results obtained from the present study are in agreement with the findings of Takahashi et al. (2005). In their study, advanced clinical stage at diagnosis (P<0.0001), tumour size (P<0.0001) and lymph node status (P<0.0001) emerged as significant predictors for overall survival of breast cancer patients. In another hospital based series of 310 women younger than age 60 with non metastatic breast carcinoma, the stage specific survival analysis showed a relative risk of 1.5 (95% CI = 0.8-2.6) for death by breast cancer in patients with advanced disease versus localized stage (RR: 0.9; 95% CI = 0.3-2.6) (Ruder et al., 1988). Hill et al. (1999) reported that tumour size and axillary lymph node involvement were important prognostic factors for male breast carcinoma. In their study, tumour size was found to be significant in univariate analysis (P= 0.02), where as axillary lymph node involvement significantly affected survival in both univariate (P= 0.0004) and multivariate analysis (P= 0.01). In another study, the stage and the number of involved axillary lymph nodes were found to be the factors affecting the overall survival rate (Kim et al., 2005). On the contrary, axillary node involvement revealed no significant correlation with survival of the HBOC/sporadic cases in the present study. Similar were the results obtained for higher histologic grade in the HBOC cases, though these factors emerged as important prognostic factors for breast cancer survival in various other studies (Nair et al., 1993; Mustafa et al., 1998).

Regarding metastasis, the results of the present study are contrary to results from the study by Andre et al. (2004) which showed improved survival for breast cancer patients presenting with synchronous metastases over time (1987 to 2000). In another study, BRCA1 mutation status was found to be correlated with increased incidence of brain, bone and lung metastasis (Albiges et al., 2005). Hence the present finding that various clinicopathological factors analyzed has a key influence on prognosis in hereditary breast cancer patients imply the utilization of these factors as proxy for predicting patient outcome in breast
cancer screening as well as surveillance programmes. Moreover, tumour size has been used as a surrogate measure for the prediction of mortality reduction in screening trials in hereditary breast cancer patients (Brekelmans et al., 2006).

In conclusion, the present study results showed that despite the absence of significant difference between the association of various clinicopathologic factors with BRCA2 gene status, the BRCA2 germline mutations have a negative prognostic influence on overall survival among women with hereditary breast/ovarian cancer, and screening for the BRCA2 mutation status might be clinically used to determine patients at high risk. The poor overall survival could be due to delay in diagnosis, advanced stage of disease presentation, and probably inadequate facilities for early diagnosis and treatment. Survival analyses have shown clearly that those with earlier stage disease have better survival rates. It is also possible that the position of the mutation in the gene, as well as genetic and environmental background may very well influence and patient characteristics and subsequent survival.

However, as in many other studies, the present study has certain limitations. Although a significantly worse prognosis was obtained for BRCA2 mutated cases, no far-reaching prognostic conclusions can be drawn from these results because of the small number of mutation positive cases, and hence results have to be interpreted with caution. A significant percentage of families remained negative for mutations in the BRCA2 gene even though many showed a clear predisposition for breast/ovarian cancer. No currently available techniques can guarantee 100% detection of pathogenic mutations in the BRCA2 gene and hence some mutations could also have remained undetected. In particular, all PCR based methods currently available are unable to detect large genomic rearrangements, big deletions or duplications, and regulatory mutations in other parts of the gene (like the promoter region) (Agata et al., 2005; Karhu et al., 2006) that occur frequently and which could also account for a significant proportion of unidentified BRCA2 mutations. Further research work is warranted to investigate these mutation mechanisms. Finally, it is also conceivable that
some of these HBOC families might include mutations conferring breast/ovarian cancer risks in genes other than \textit{BRCA2}.

The data presented here are based on hospital statistics and therefore, the inherent selection bias must be considered in interpreting the information. However, in several developing nations, hospital based data are probably the only available means to study disease pattern and outcome. The hospitals data in developing countries tend to reflect the community pattern to a limited extent, in view of the fact that several patients from vast geographic region report to relatively few cancer treatment facilities. Further studies with larger patient populations is be necessary to confirm the decreased survival in patients with \textit{BRCA2} associated breast/ovarian carcinoma and to better elucidate the biologic basis of this survival disadvantage.

\textbf{ASSOCIATION BETWEEN HEREDITARY BREAST CANCER AND NON GENETIC MODIFIERS}

Breast and ovarian cancer risk is significantly elevated in women who have inherited a germline mutation in \textit{BRCA2} gene. However, there is substantial inter-individual variability in both the age at diagnosis and site of cancer occurrence in \textit{BRCA2} mutation carriers, which even appears among relatives who carry the same \textit{BRCA2} mutation (Levy-Lahad and Friedman, 2007). These observations support the hypothesis that the risk imparted by \textit{BRCA2} mutations is not equal among all mutation carriers, and that certain non genetic modifiers also contribute to cancer risk in \textit{BRCA2} mutation carriers. Several environmental risk factors that may contribute to or hasten the development of breast cancer have been identified, including lifestyle and reproductive factors. These may account for the majority of observed trends and the variation in incidence rates between developed and developing countries. Reproductive factors have been shown to modify the risk of breast cancer in both mutation carriers (Narod, 2002) and in general population (Clavel- Chapelon and Gerber, 2002) and estrogen hormone dependence is the common denominator recognized in pathogenic pathways.
Discussion

leading to breast cancer. The longer a woman is exposed to estrogen, either exogenously or endogenously, the higher her risk of developing breast cancer (Nkondjock and Ghadirian, 2004). BRCA gene expression is up regulated during puberty and pregnancy, when estrogen production is greatly elevated, suggesting BRCA stimulation by estrogen (Welcsh and King, 2001).

Identification of these risk modifiers has a number of implications for cancer prevention and control in women who carry BRCA2 mutations. First, knowledge of these factors may be used to guide recommendations for modification of certain exposures (e.g., oral contraceptive use), which may become a part of standard care in the follow-up of women who are found to carry these mutations. Second, knowledge of risk-modifying factors in these women may lead to tailored cancer screening strategies. This could include recommendations related to the timing, intensity, or organ specific screening in subsets of women. Finally, knowledge of factors that modify cancer risk may direct research efforts to identify relevant breast or ovarian carcinogenesis pathways. This may, in turn, lead to the novel use of existing (e.g., hormonal) agents or the development of new agents that could be applied in chemoprevention or treatment strategies specifically targeted towards women who carry BRCA2 mutations. Hence, in the current study, the association of various available epidemiological/reproductive factors with hereditary breast cancer patients and their BRCA2 gene mutation status was also explored.

Increased parity is protective for sporadic breast cancer beyond age 40 years, but may increase risk for early-onset breast cancer. In BRCA2 carriers, parity effects may also be age dependent. In BRCA2 carriers, parity caused a borderline increase in risk for breast cancer before age 50 years (OR=1.17 for each pregnancy, 95% CI = 1.01–1.36) (Cullinane et al., 2005). In a case-only study, young age at first pregnancy delayed onset of breast cancer in carriers (King et al., 2003), and a retrospective study of 1601 carriers found that in women over 40 years of age, each full-term pregnancy reduced breast cancer risk by 14% (95% CI = 6–22%). An age effect was noticeable in that BRCA2
carriers with later first pregnancies had increased risk (Andrieu et al., 2006a). Overall, the influence of parity on breast cancer in carriers was similar to that in sporadic cases.

Younger age at menarche is associated with increased risk for breast cancer. Its influence was not observed in BRCA2 carriers, but BRCA1 carriers whose age at menarche was >13 years had a 54% reduction in breast cancer risk compared to those with menarche at <11 years of age (OR=0.46, 95% CI = 0.30–0.69) (Kotsopoulos et al., 2005). With respect to the association between breast cancer risk and age at menarche, the results of another study from Iceland indicated no differences between carriers and non carriers of the founder mutation 999del5 in the BRCA2 gene (Tryggvadottir et al., 2003)

Long-term oral contraceptive use has been associated with a moderate increment of breast cancer risk in the general population. However, its effect on breast cancer risk in carriers was difficult to assess because of changing patterns of use and oral contraceptives formulations with time. In a retrospective case–control study of 330 BRCA2 carrier pairs, there was no effect of oral contraceptive usage on breast cancer risk in BRCA2 carriers (Narod et al., 2002b). Two recent studies based on early-onset breast cancer cases, including small numbers (<100) of carriers, observed no general increase in breast cancer risk with oral contraceptives usage (Jernstrom et al., 2005; Milne et al., 2005). Altogether, these studies suggest that for breast cancer risk, usage of oral contraceptives have similar relative effects in carriers and non-carriers. Oral contraceptive usage has been known to decrease ovarian cancer risk in the general population (Narod, 2002; Whittemore et al., 2004). However, Modan et al. (2001) showed minimal or no reduction in the odds of ovarian cancer in Israeli Jewish women who were BRCA2 mutation carriers and oral contraceptive users.

Regarding the influence of spontaneous or induced abortions on breast cancer risk, Friedman et al. (2006) found no difference on the influence of spontaneous abortions among the BRCA2 mutation carriers and non carriers. However BRCA2
mutation carriers who had histories of two or more therapeutic abortions faced a 64% decrease in the risk of breast cancer (Friedman et al., 2006). Other population based studies from Denmark and China reported no overall increased risk of breast cancer among women with a history of induced abortions (Melbye et al., 1997; Ye et al., 2002).

Exposure to ionizing radiations, particularly in early age, increases breast cancer risk. In a retrospective cohort study of 1601 BRCA2 carriers found that exposure to chest X-rays was associated with an increased breast cancer risk (HR=1.54; P=0.007); risk was increased in carriers aged 40 years and younger (HR=1.97; P<0.001), particularly those exposed only before the age of 20 years (HR=4.64; P<0.001) (Andrieu et al., 2006b). In contrast, in a case-control study of 3200 mutation carriers, previous exposure to mammography did not increase breast cancer risk (OR =1.03, 95% CI = 0.85–1.25) (Narod et al., 2006). Further studies may determine if these results imply differential effects of dose and developmental timing of radiation exposure.

Thus studies on various non genetic risk modifiers of breast cancer are inconsistent, with controversial results. However, in the present study, none of the parameters analyzed such as history of abortions, parity, early age at menarche, oral contraceptive use and history of radiation exposure showed a significant association with BRCA2 gene mutation status in these HBOC cases (Table 27). While no factors seem to significantly influence BRCA2 mutation risks, this could be due to the smaller number of BRCA2 mutation carriers and subsequent lack of power. Moreover, no significant influence of these factors on hereditary cases compared to control subjects were also noted (Table 26). Hence it could be speculated that among patients with a family history of breast cancer, genetic factors like mutations in susceptibility genes that predisposes to breast cancer are more important than other known epidemiological risk factors.

In summary, the results obtained from this study indicate that germline mutations in BRCA2 gene is involved in the development of some (18.6%), but not all,
hereditary breast cancers in the Kerala population as has been reported for other populations (Serova et al., 1997). The present study identified twelve distinct mutations as well as seven distinct silent/intronic alterations in BRCA2 gene, with a unique pool of novel mutations in Kerala population. These results indicated that the pattern of mutations seen in the BRCA2 gene differs slightly from other populations studied with a slight preponderance of population specific missense mutations. Thus, the BRCA2 germline mutations attributed to somewhat smaller fraction of breast/ovarian cancer cases in Kerala population than in other western populations studied. A lower frequency of mutations could be the result of several factors, including a lower penetrance of BRCA2 mutations due to the surrounding environmental and hormonal milieu, and a different spectrum of mutations, supporting the notion that other genes may be important determinants of familial risk. It is necessary to establish the simultaneous effects of environmental factors and of other tumour-associated low penetrant genes in modifying the impact of inherited cancer predisposition genes in breast cancer patients.

**UTILITY OF THE STUDY**

BRCA2 gene mutation analysis of Hereditary Breast/Ovarian Cancer patients enabled the characterization of the most frequently occurring BRCA2 germline mutations in HBOC families in Kerala. The primary benefit of this BRCA2 mutation screening is the information that can be gained about individual and familial breast and ovarian cancer risk. In future, genetic testing for BRCA2 mutation can be undertaken in unaffected first degree relatives in breast/ovarian cancer families to identify the high risk individuals. Examination of high risk family members can lead to the detection of tumours at a higher than expected rate and at an earlier age than by permitting the institution of therapy earlier than if tumours had been detected after the onset of the symptoms. Moreover, once the mutations prevailing among breast/ovarian cancer families in Kerala is determined, then the screening for specific mutations at the "hot spot" site shall become simpler and cheaper (preferably exon 11 in Kerala population). In addition, information on BRCA2 status will allow us to identify a valuable cohort of
families to search for new genes that predisposes to breast/ovarian cancer and to study both retrospectively and prospectively the cancer risks and potential modifiers of risk, for mutation carriers.

Genetic Testing

The identification of \textit{BRCA2} gene has made predictive genetic testing possible for individuals in families with an identified mutation. This has the obvious advantage of being able to test single individuals, although it is usual to test an affected relative first to identify the mutation prevailing in that family, which can then be screened for, in other family members. Unlike most other medical tests, genetic tests can reveal information not only about the person being tested, but also about that person's first/second degree relatives. The goal of genetic testing is to provide patients and their physicians with information that will allow them to make informed decisions regarding cancer prevention, screening, surveillance, and treatment options. A significant benefit of genetic testing is the ability to quantify cancer risk estimates more precisely, thereby improving the process of determining the most appropriate management strategies in patients who test positive. The possible test outcomes obtained by focusing on testing for \textit{BRCA} gene mutations are described below:

\textbf{Positive}: In a family with a history of breast and/or ovarian cancer, it may be most informative to first test the member who has the disease. A positive result indicates that a deleterious germline mutation has been identified in the gene. This result confirms a greater risk for developing breast or ovarian cancer as a result of inheritance of the mutated susceptibility genes. A positive result also has implications for other family members. Each of the patient's siblings and children, regardless of their gender, has a 50% chance of carrying the same genetic mutation. Members of the affected branch of the family have varying risks of carrying the familial mutation, which depend upon their relationship to the affected individual.
**Discussion**

Negative: A negative result indicates no deleterious mutations identified in BRCA2. But the interpretation of a negative test result is dependent upon the specific case scenario, as described below:

(a) If a genetic mutation has been identified previously within the family, and the patient receives a negative result, the result is considered a “true negative”, meaning that the patient did not inherit the known familial mutation. For such individuals, the risk of developing breast and/or ovarian cancer is similar to that of the general population, and they have no higher risk to pass the mutation on to their children.

(b) In cases where no known mutation in BRCA2 has previously been identified in a family with a history of breast and/or ovarian cancer, a negative test is not informative. It is not possible to tell whether a person has an alteration in BRCA2 that was not identified by the test (a false negative), or whether the result is a true negative. In addition, it is possible for people to have an alteration in a gene other than BRCA2 that increases their cancer risk, but is not detectable by this test.

**The Implications of Genetic Testing in Breast Cancer Families**

Management implications for affected members: Most affected women will have an early age of onset of breast cancer, and the psychological impact on these women will therefore be quite profound and may necessitate considerable psychological counseling. Women who carry a BRCA2 mutation are at an increased risk of developing bilateral disease, and various other cancers (Ovary, pancreas etc). Some estimates have determined that the risk of contralateral breast cancer by the age of 70 may be in the order of 60–80%. Careful assessment and follow up of the contralateral breast and other sites is therefore required in these instances, and some women may opt for a prophylactic mastectomy on the unaffected side. Various studies (including the present study) have suggested that familial breast cancers in general are associated with a worse prognosis. In view of this tendency towards more aggressive tumour characteristics in BRCA2-associated malignancies, there are clear implications
that treatments, both local and systemic, may need to be tailored accordingly. Hence it is likely that treatment by mastectomy rather than breast conservation may need to be undertaken more frequently, or at least the criteria for undertaking breast conservation therapy may need to be more stringently applied and this procedure only performed under clearly favourable circumstances.

**Management implications for unaffected relatives with BRCA2 positive genetic testing:** For the unaffected individuals who are demonstrated to have BRCA2 genetic alterations on mutation analysis, there are a number of management options which could be considered (Table 28) (Domchek and Weber, 2006)

*Cancer screening:* is the preferred management option for gene mutation carriers. Mutation carriers are advised to undergo regular targeted screening for tumours to which the gene predisposes.

**Prophylactic Surgery:** Despite the invasive nature of the intervention, prophylactic mastectomy (PM) is the most effective strategy available. Hartmann *et al.* (1999) first published data on breast cancer risk reduction after PM in women with strong family histories of breast cancer (not restricted to BRCA1/2 carriers) and demonstrated a 90% reduction in breast cancer associated with this procedure. Subsequently, several groups have confirmed that PM reduces breast cancer risk in mutation carriers by 90% (Hartmann *et al.*, 2001; Rebbeck *et al.*, 2004; Domchek and Weber, 2006). Prophylactic oophorectomy can be discussed with women who carry mutations of BRCA2 gene as a strategy to reduce the incidence of both breast and ovarian malignancies (Kauff *et al.*, 2002; Domchek and Weber, 2006). Quite clearly, neither prophylactic mastectomy nor prophylactic oophorectomy provides complete protection against the subsequent development of malignant disease and hence ongoing surveillance is still therefore required. It is important that women be extensively counselled in relation to these matters prior to the performance of prophylactic surgery.
Table 28. Management implications for unaffected members with *BRCA2* positive genetic testing

<table>
<thead>
<tr>
<th>Cancer screening</th>
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<tbody>
<tr>
<td><strong>Breast</strong></td>
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<tr>
<td>• Monthly breast self-examination</td>
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<td>• Annual clinical examination</td>
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<tr>
<td>• Annual ultrasound</td>
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<td>• Mammography at selected intervals (e.g. 30, 35 years)</td>
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<tr>
<td><strong>Ovarian</strong></td>
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<tr>
<td>• Annual trans-vaginal ultrasound</td>
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<td>• Serum CA-125</td>
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<tr>
<td><strong>Colorectal</strong></td>
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<tr>
<td>• Annual faecal occult blood testing</td>
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<tr>
<td>• Flexible sigmoidoscopy/colonoscopy every 3–5 years</td>
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<tr>
<td>• Prostate cancer (from age 50 years)</td>
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<tr>
<td>• Annual digital examination</td>
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<tr>
<td>• Annual prostatic specific antigen (PSA)</td>
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<table>
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<tr>
<th>Preventative strategies</th>
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<tbody>
<tr>
<td><strong>Prophylactic surgery</strong></td>
<td></td>
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<tr>
<td>• Bilateral mastectomy (simple or subcutaneous)</td>
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<tr>
<td>• Laparoscopic oophorectomy</td>
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<td><strong>Chemoprevention</strong></td>
<td></td>
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<tr>
<td>• Tamoxifen</td>
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<tr>
<td>• Avoid hormone replacement therapy</td>
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<tr>
<td>• Oral contraceptive (slight increased risk of breast Cancer, decreased risk of ovarian cancer)</td>
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<tr>
<td><strong>Lifestyle factors (unproven)</strong></td>
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<tr>
<td>• Reduce dietary fat</td>
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<td>• Reduce alcohol consumption</td>
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<tr>
<td>• Consume antioxidants/vitamins</td>
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<td>• Reduce radiation exposure</td>
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<td>• Promote physical exercise</td>
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**Chemoprevention:** Limited data are available on the use of tamoxifen for chemoprevention of breast cancer in *BRCA1/2* mutation carriers, however, the finding of a lower contralateral breast cancer incidence following tamoxifen administration for adjuvant therapy led to the concept that the drug might play a role in breast cancer prevention (Gronwald *et al.*, 2006; Pierce *et al.*, 2006; Domchek and Weber, 2006).

Identification and management of women with hereditary predispositions to breast and ovarian cancers are complex clinical undertakings. Optimally, they require consultation with and collaboration among surgeons, medical oncologists, genetic counselors, gynecologists, and radiologists in a multidisciplinary clinical environment that provides clarification of risk, elucidation of management options, and elaboration of both the risks and benefits of various intervention strategies. Striving for continuous collegial interaction among all involved specialists will assure optimal management of these unique patients. Ultimately, comprehensive cancer genetic testing offered as part of routine clinical practice, will translate into breast cancer prevention at the population level. Thus the *BRCA2* mutation analysis may have significant implications for decisions about cancer surveillance, and it is anticipated that identification of *BRCA2* mutational patterns in Indian population would represent an essential prerequisite for a prevention programme based on DNA analysis. It is hoped that this gene alterations as well as any other discovered in future studies, will provide novel targets for anti cancer drugs.