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Novel Sequence Variants and a High Frequency of Recurrent Polymorphisms in *BRCA1* Gene in Breast Cancer Women of North Coastal Andhra Pradesh

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ABSTRACT Owing to its high incidence coupled with relatively good prognosis, breast cancer is the most prevalent cancer in the world today. Germ line mutations in the susceptibility gene *BRCA1* in hereditary breast/ovarian cancer, though low in prevalence, are highly penetrant and show geographical variations. Most cancer-associated *BRCA1* mutations identified to date result in the premature translational termination of the protein. However, the molecular and genetic effects of missense mutations remain largely unknown. There have been only a few reports from India on mutations in *BRCA1* and none from North Coastal Andhra Pradesh. We have analyzed 114 breast cancer patients with (N = 9) and without (N = 105) a family history of breast cancer, 22 at risk relatives from familial (n=11), sporadic (n=11) cases and 97control subjects for mutations in exons 2 and 11 and their intron-exon boundaries of *BRCA1 gene* by direct sequencing. Sequence alignment was carried by CLUSTAL W and PSI-BLAST. Out of eight sequence variants found, one novel deleterious frame-shift mutation (c.2717insA), one novel polymorphism (c.1400A>G), five previously reported common polymorphisms in exon 11 and one intronic (intron 1) variant (base1822C>T) were observed. All the identified polymorphisms in exon 11 fall in DNA binding domain of BRCA1 protein.

INTRODUCTION

Breast Cancer is the most common malignancy in women worldwide, accounting for 23 percent of all cancer across genders (Parkin et al. 2005). Germline mutations within the breast and ovarian cancer susceptibility gene *BRCA1* predispose carriers to early-onset breast and breast-ovarian cancers (Nathanson et al. 2001). Tumor-associated mutations occur throughout the *BRCA1* coding sequence, but cluster to sequences encoding the N-terminal RING finger domain and the BRCT1 domains (Couch and Weber 1996; Shattuck et al. 1995; Shen and Vadgama 1999). An increased relative risk for the development of colon, cervix, uterus, pancreas and prostate cancer has been suggested in *BRCA1*-mutation carriers (Shih et al. 2000). Accumulating evidence points to a role for the BRCA1 protein product in the regulation of multiple nuclear functions including transcription, recombination, DNA repair and checkpoint control (Chapman and Verma 1996; Monteiro 2000; Scully and Livingston 2000; Venkitaraman 2002).

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To date, more than 1600 sequence variants of *BRCA1* have been described (Breast Cancer Information Core database 2006). The prevalence of *BRCA1* mutations is variable among different populations (Neuhausen 1999) and varies from 1.8 – 13 percent (Bonadona et al. 2005; Esther et al. 2007; Southey et al. 1999) in developed countries. The incidence of breast cancer is high among Indian females in metropolitan cities (ICMR Delhi 2001; ICMR Bangalore 2001, 2002, 2006; Jussawalla and Jain 1977) and the pathogenic mutations account for 16-25 percent at a median age of 35-40 years in familial breast cancer patients (Hedau et al. 2002; Kumar et al. 2002; Pestonjamasp and Mittra 2000; Saxena et al. 2002, 2006; Rajkumar et al. 2003; Valarmathi et al. 2003, 2004).

Information on *BRCA1* mutations in north coastal Andhra Pradesh, India is still lacking. In this study, we carried a detailed analysis of exons 2 and 11 of *BRCA1* gene by direct sequencing, as this approach would cause the least bias in the type of mutation identified while theoretically being the most sensitive method.

MATERIALS AND METHODS

Patient/Family Series

Of 10,128 cancer patients who were registered in the three main cancer hospitals - Lion's Cancer Hospital (2001-2008), King George Ho-

spital (2001-2004) and Mahatma Gandhi Cancer Hospital (2006-2008), Visakhapatnam during August 2001 to September 2008, 1920 (19%) were cytologically and histologically confirmed breast cancer patients. All Patients were from three districts of North Coastal Andhra Pradesh viz., Visakhapatnam, Vizianagaram and Srikakulam. About 80% of the patients had low socioeconomic background.

The sporadic breast cancer patients were categorized with respect to their family history of with/without any other cancers (18 with a history and 87 without any history of cancer) (Table 1). Age of the patients' ranged from 25 to 89 years with 54.8 percent being premenopausal.

Blood samples from multiple affected individuals and unaffected relatives were collected with informed consent. A total of 136 samples were analyzed which included 9 primary familial breast cancer patients, 105 sporadic breast cancer patients (index cases) and 22 at risk individuals (includes relatives of index cases with a family history of breast and/or other cancers) derived from first-/second-/ third degree relative(s).

Control Population

Ninety-seven age and sex matched healthy unrelated individuals with no history of cancers were selected from Visakhapatnam as the control group to estimate the frequency of the detected *BRCA1*variants.

Molecular Studies

Genomic DNA was extracted from peripheral blood mononuclear cells (Lahiri and Nurnberger 1991). Exons 2 and 11 of *BRCA1* and their intron/exon splice junctions were amplified by polymerase chain reaction (Denaturation 95^0C; annealing 58^0C; extention 72^0C). Six sets of primers were designed using primer3 software (http://www.genome.wi.mit.edu/cgibin/ primer/primer3-www.cgi) for exons 2 and 11 of human *BRCA1* gene (accession # U14680; version # U14680.1 GI: 555931). Primers were supplied by Sigma Genosys. The primer sequences were as follows.

Exon 2: F. 5'–AGCTAAGGCTACCACCACCTAC- 3'
R. 5'-CAGAGTGGATGGAGAACAAGG -3'
Exon 11: F. 5'-GTGAAAGAGTTCACTCCAAATCAG-3'
R. 5'-TTGGGGTCTTCAGCATTATTAG-3'
F. 5'-AGACATGACAGCGATACTTTCC-3'
R. 5'CATTTCCCATTTCTCTTTCAGG-3'
F. 5'-AGAAAGGAGAGCTTAGCAGGAG-3'
R. 5'-ACGTCCTAGCTGTGTGAAGG-3'
F. 5'-GCAACGAAACTGGACTCATTAC-3'
R. 5'-CCAAATAACAAGTGTTGGAAGC-3'
F. 5'-AGAAAGGAGAGCTTAGCAGGAG-3'
R. 5'-ACGTCCTAGCTGTGTGAAGG-3'

BRCA1 Mutation Analysis

Direct Sequencing

Direct sequencing was carried out on amplified PCR products by forward and when necessary reverse sequencing by ABI 3730 automated

Table 1: Characteristics of breast cancer patients and at risk individuals

Characteristics	*Familial breast cancer patients (N = 9)*	*Sporadic breast cancer patients (N=105)*	*At risk individuals (N = 22)*
Age (years) at Diagnosis (patients)/Sample Collection (at risk individuals)			
Mean± SD	57.4±4.97	50.2±10.43	36.4±9.67
≤ 40	0	23	18
> 40	9	82	4
Number of Family Members With Breast Cancer			
N=0	0	105	0
N=1	5 (1^{st}: 5)	0	13
N=2	3 (1^{st}: 1, 1^{st} and 2^{nd} :1, 1^{st} and 3^{rd}: 1)	0	2
N=3	1(1^{st} and 3^{rd} :1)	0	7
*Number of Family Members with Other Cancers**			
N=1	3	10	2
N=2	1	4	5
N=3	1	2	4
N=4	2	2	1

* ovarian, uterine, colon, kidney, brain, bone, lung, oral, ovarian, thyroid, uterine cervix cancers
$1^{st,}$ 2^{nd} and 3^{rd} degree relative affected with breast cancer.

Table 2: Mutation and polymorphisms in breast cancer patients

Category	*No. of screened patients*	*No. of relatives screened*	*BRCA1 mutations*				*BRCA1 polymorphisms*			
			Frequency		*%*		*Frequency*		*%*	
			PT	*RT*	*PT*	*RT*	*PT*	*RT*	*PT*	*RT*
Familial breast cancer cases	9	11	0	0	0	0	8	7	88.9	63.64
Sporadic cases with family history of other cancers	18	5	1	0	5.6	0	14	4	77.8	80.0
Sporadic cases without any family history of other cancers	87	6	0	0	0	0	58	3	66.7	50.0
Total summary	**114**	**22**	**1**		**0.9**	**0.0**	**80**	**14**	**70.2**	**63.64**

PT- patients; RT-relatives

sequencer (version 2.5 - SeqScape), to ascertain the position and type of sequence variation for 233 individuals including 105 sporadic, 9 familial, 22 at risk individuals and 97 control group.

Mutation Nomenclature

Approved nomenclature for the description of sequence variants was adopted. All nucleotide numbers refer to the wild type cDNA sequence of BRCA1 (accession # U14680; version # U14680.1GI: 555931) as reported in Gen Bank. The approved nucleotide numbering system uses the A of the ATG translation initiation start site as nucleotide +1.

Multiple Sequence Alignment

The multiple sequence alignment (MSA) of orthologous *BRCA1* gene from six species including Homo sapiens (Gene Bank accession number U14680), Pan troglodytes (AF207822), Mus musculus (U68174), Ratus norvegicus (AF036760), Gallus gallus (AF3552273), Cannis familiaris (U50709), were obtained by using program ClustalW (Thompson et al. 1994). According to PSI-BLAST (Altschul et al. 1997), these five sequences are the only sequences in the nonredundant protein sequence data based at National Center for Biotechnology Information (NCBI) that have 90% sequence identity to the human BRCA1 DNA-binding domain (residues 452-1092).

RESULTS AND DISCUSSION

This is the first report on *BRCA1* mutations in breast cancer patients from north coastal Andhra Pradesh, India. The number of breast cancer cases screened and the mutation status in each category are shown in Table 2. We have identified eight sequence variants of which two are novel variants: one deleterious frame-shift mutation at c. 2717-2718insA and one polymorphism at c.1400 A>G in exon 11 (Fig. 1). Five previously reported polymorphisms (c.2082C>T), (c.2311T>C) (c.2612C>T), (c.3113A>G), (c.3548A>G) in exons 11, and one known intronic variant at base 1822C>T in intron1 of *BRCA1* were found (Table 3). These sequence variations were identified in eight out of 9 (89%) familial and 72 out of 105 (68.6%) sporadic breast cancer patients analyzed in this study.

Table 3: *BRCA1*gene mutations/polymorphisms in North Coastal Andhra Pradesh

Exon/ Intron	*NT**	*Basechange*	*Codon*	*Amino acid change*	*Designation*	*Variation*	*BIC entry*
11	**c.2717**	**+A**	**906**	**fs 914stop**	**-**	**Frame shift**	**Novel**
	c.1400	**A>G**	**467**	**Lys – Arg.**	**K467R**	**polymorphism**	**Novel**
	c.2082	C>T	694	Ser – Ser	Silent	polymorphism	Reported
	c.2311	T>C	771	Leu - Leu	Silent	Polymorphism	Reported
	c.2612	C<T	871	Pro - Leu	P871L	polymorphism	Reported
	c.3113	A>G	1038	Glu - Gly	E1038G	polymorphism	Reported
	c.3548	A>G	1183	Lys – Arg	K1183R	polymorphism	Reported
Int.1	1822	C>T	-	-	-	polymorphism	Reported

*approved nucleotide number

Electrophorogram of BRCA1 showing c. 2717insA codon in 906:fs914 stop/polimorphism c. 1400A>G

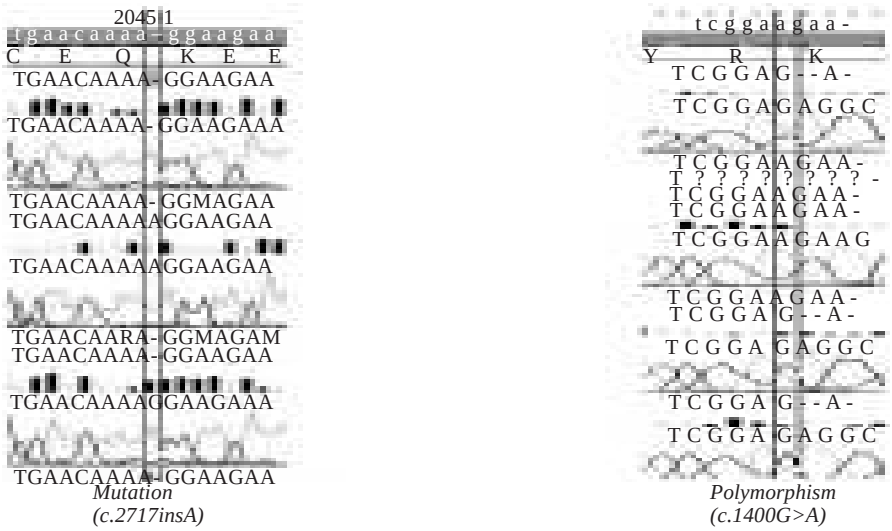

Fig. 1. Electropherogram of *BRCA1* gene showing frameshift mutation.

Frameshift Mutation

The frameshift mutation at *c*.2717insA (codon 906:fs914stop) occurs due to an insertion (A) in the normal sequence AA† G GAA GAA AAT CAA GGA AAG AAT GAG at codon 906 in exon 11 of *BRCA*. This mutation changes the reading frame of the mRNA and causes a premature termination codon at position 914. This mutation was detected in one of the sporadic patients of late-onset breast cancer (aged 64) with only one daughter (aged 40) affected with uterine cancer. The c.2717-2718insA frameshift mutation occurs at a frequency of 0.9% and was observed only in a sporadic breast cancer patient but not in familial cases, at risk individuals or in control chromosomes studied. This mutation has not been reported in Breast cancer Information Core database (BIC) or Human Gene Mutation Database (HGMD).

Neither mutations nor polymorphisms were observed in exon 2 in the present study. The founder Ashkenazi Jewish mutation, 185delAG, in exon 2 was reported in four Indian studies (Kumar et al. 2002 in South India and Valarmaathi et al. 2002 ; Hedau et al. 2004; Saxena et al. 2006 in North India) and missense mutations at 145 G>A, 186G>A in ex-on 2 were observed by Valaramathi et al. (2004) in North India. Lack of mutations in exon 2 may be because of small sample size or due to ethnic diversity.

Polymorphisms

Common polymorphisms in *BRCA1* gene appear to be highly prevalent in north coastal Andhra Pradesh breast cancer patients. In this study, the polymorphism at c.1400 A>G (p. K467R-28%) in exon 11 (Fig. 1) has not been previously reported. The six known nucleotide substitutions which include two synonymous (c.2082C>T: p. S694S, 24.3%), (c.2311T>C: p.L771L, 17.6%), three non synonymous (c.2612C>T: p.P871L, 25%), (c.3113A>G: p.E1038G, 39%), (c.3548A>G: p.K1183R, 22%) amino acid changes of unknown functional importance in exon 11, and one nucleotide substitution (base 1822 C>T (20.8%); rs.37655640) in intron-1 of *BRCA1* were detected in breast cancer patients. The six amino acid changes in exon 11 have occurred in N-terminus DNA-binding domain (residues 452-1092), that bind to different proteins such as P53, RAD50, RAD51, c-Myc (Aihara et al. 1999; Narod and Foulkes 2004) and also function as nuclear localization signal domain.

The polymorphism at c.1400A>G leads to the substitution of Arginine to Lysine at codon 467 (K467R). Arginine is the evolutionarily conserved amino acid observed in all other species aligned excluding *Homo sapiens* (U14680) where it differs by having Lysine at c.1400 position (Fig. 2). The reverse change from Lysine to

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query      R—KASLPNLSHVTENLIIGAFVTEPQIIQERPL—TNKLKRKRRPTSGLHPEDFIKKA225
U68174     R—KGSRPHLNHVTE—IIGTFITEPQITQEQPF—TNKLKRKR—STSLQPEDFIKKA 514
AF036760   R—KGSRPHLNHVTE—IIGTFTTEPQIIQEQPF—TNKLKRKR—STCLHPEDFIKKA515
U14680     K—KASLPNLSHVTENLIIGAFVTEPQIIQERPL—TNKLKRKRRPTSGLHPEDFIKKA521
AF207822   R—KASLPNLSHVTENLIIGAFVTEPQIIQERPL—TNKLKRKRRATSGLHPEDFIKKA521
U50709     R—KASLPKVSHTTEVLTIGACAIEPQTMQTHPF—MNKAEHKRRTTSSLHPEDFIKKV518
AF355273   RGRKSNPSTILRDILPATKKEDAAAEEGCLNNSR—KDRLKRKRKSACILQPEDFIKKK534
AF416868   RERKAMPPNNITCVAEVHHDSALETGKENMTLEYGTGMSHLMSKRKMVYSLNPENTSKKN492
           : *. .          :      .:  **    *:**:  **
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Fig. 2. Protein sequence alignment of BRCA1 DNA binding domain

CLUSTAL W (1.83) multiple sequence alignment [Mus musculus (U68174); Ratus norvegicus (AF036760); Homo sapiens (Gene Bank accession number U14680); Pan troglodytes (AF207822); Cannis familiaris (U50709); Gallus gallus (AF3552273).

the conserved amino acid (Arginine) was observed in breast cancer patients. This polymorphism was identified in 28 percent of the sporadic, 22 percent of familial cases, 36 percent of at risk individuals and 15.5 percent of control population. The frequency of this novel variant is 23.2 percent. The distribution of allele frequency of the polymorphisms was summarized in Table 4.

Polymorphism at c.3113A>G in exon 11 of *BRCA1* at codon 1038 (E1038G), was identified in 27.6 percent in sporadic cases without any other cancers in the family, 44.4 percent in sporadic cases with a family history of other cancers, 78 percent of familial breast cancer patients, 36.4 percent in at risk individuals and only 0.02 percent out of 97 in control group of this series. Interestingly only E1038G polymorphism was observed to be transmitted to at least a few members of all the families in familial cases and to daughters in two sporadic cases. The frequency of E1038G was found to be increasing with an increasing association with cancer (Table 5) suggesting that it does not represent a common polymorphism. There is a significant discernible difference (P = 0.0000) in the allele frequencies of this polymorphism between familial breast cancer, sporadic breast cancer with and without a history of other cancer, at risk individuals and control population. When physiochemical properties of the amino acids concerned are taken into account, this alteration would be expected to be significant, since it results in the replacement of a large negatively charged amino acid (Glutamic acid) by smaller uncharged (Glycine) amino acid. The low frequency of this variant in

Table 4: Allele frequencies of *BRCA1* mutation/polymorphisms in North Coastal Andhra Pradesh

BRCA1 mutations								
Exon / Intron	*Designation approved†*	*Predicted effect*	*Age* Yrs.*	*Method of detection*	*No. detected*			*BIC‡entries*
					Cases n = 136	*Controls n = 97*	*Pvalue*	
11	**c.2717(+A)**	**Fs914stop**	**64**	**DS**	**1**	**0**	**0.3889**	**No**
BRCA1 polymorphisms								
Exon / Intron	*Designation approved*	*Predicted effect*	*Method of detection*	*Allele frequency§%*			*BIC entries*	
				Cases n = 136	*Controls n = 97*	*pvalue*		
11	**c.1400A>G**	**Lys467– Arg**	**DS**	**14.0**	**7.7**	**0.02**	**No**	
11	c.2082C>T	p.Ser694Ser	DS	12.1	7.7	0.0943	Yes	
11	c.2311T>C	p.Leu771Leu	DS	8.8	6.2	0.1944	Yes	
11	c.2612C>T	p.Por871Leu	DS	12.5	7.7	0.0738	Yes	
11	c.3113A>G	Glu 1038Gly	DS	17.0	1.0	0.0000	Yes	
11	c.3548A>G	Lys1183Arg	DS	11.0	11.9	0.8938	Yes	
Int.1	c.1822C>T	-	DS	20.8	8.2	0.0006	Yes	

†The GenBank reference sequences: *BRCA1* ~ accession # U14680; version # U14680.1 GI: 555931;*Age at diagnosis of proband; DS: direct sequencing; ‡The Breast Cancer Information Core Database (BIC); **§** Allele frequency was expressed as the prevalence among 466 chromosomes; **Bold face =Novel variants.**

Table 5: Distribution of non synonymous polymorphisms in exon-11 of *BRCA1* gene

Category	*N*	*K467R%*	*P871L%*	*E1038G%*	*K1183R%*
Sporadic cases without family history of cancers	87	25.3	17.2	27.6	26.4
Sporadic cases with a family history of other cancers	18	39.0	16.7	44.4	5.6
Familial	9	22.2	44.4	77.8	0.0
At risk individuals	22	15.5	22.7	36.4	9.1
Controls	97	15.5	6.2	2.0	23.7

the control group, lack of similarity between normal and the variant amino acid and location in putative functional domain may be indicative of a biologically deleterious effect.

The allele frequencies of some of the polymorphisms observed in this study were higher than that reported by north Indian studies (Saxena et al. 2002; Valarmathi et al. 2004). However, the frequencies reported in a study from south India (Nagaswamy et al. 2009), Malaysia (Balraj et al. 2002; Toh et al. 2008) and Sri Lanka (Wasanthi et al. 2008) were higher than that observed in the present study.

All the six polymorphisms were found to co-exist only in one at risk individual (daughter of a sporadic case) but not in cases or in controls. However, polymorphisms c.1400A>G and c.3113A>G co-existed in 9 sporadic breast cancer patients of which five have family history of other cancers, one familial and two at risk individuals with a family history of breast and uterine/ovarian cancers. Of 18, seventeen sporadic breast cancer patients with a family history of cancers (either/or uterine, ovarian, brain, head and neck, cervix, thyroid in first and second degree family members) had only c.1400A>G polymorphism. Twelve sporadic cases (with either/or bone, throat uterine cancers in five of the families), three familial cases (with uterine cancer in the family) had only c.3113A>G polymorphism. All these six polymorphisms were found to co-occur in other south Indian (Nagaswamy et al. 2009), Malaysian (Balraj et al. 2002; Lim and Halimah 2004; Tang et al. 1999; Toh et al. 2008; Wasanthi et al. 2008) studies as well as Caucasian populations (Durocher et al.1996).

CONCLUSION

Considering the Indian history and taking into account the multi ethnic, multi religion status and population structure of this country, the results of this initial study suggest that the mutational spectrum in exons 2 and 11 of *BRCA1* gene in this population may differ from what has been observed in other Indian populations. Further studies on mutational analysis of the gene will help in identifying the relevant mutation/polymorphisms and the multiple structure-based alignments of the native structure of these polymorphisms may provide the knowledge of the biological effects of the protein corresponding to polymorphisms to help predict the aetiology of breast cancer in different geographical regions. Accompanied by proper counselling, patients and pre-symptomatic mutation carriers would be able to make better decisions about medical and surgical preventive options.

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