Analysis of Pathogenic Variants in *BRCA1* and *BRCA2* Genes using Next-Generation Sequencing in Women with Triple Negative Breast Cancer from South India

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Abstract

The frequency of triple-negative breast cancer (TNBC) incidence varies among different populations, suggesting the involvement of genetic component towards TNBC development. Previous studies have reported that *BRCA1/2* germline mutations confer a lifetime risk of developing TNBC. However, there is hardly any information regarding the common pathogenic variants (PVs) in *BRCA1/2* genes that contribute to TNBC in the Indian population. Hence, we screened for PVs in *BRCA1/2* and their association with clinico-pathological features in TNBC patients. Among the 59 TNBC genomic DNA samples sequenced, 8 *BRCA* mutations were detected in 59 TNBC patients (13.6%). Almost 50% pre-menopausal TNBC patients had a *BRCA* mutation. Of the 8 *BRCA* mutations, we observed *BRCA1* mutations in 6 TNBC patients, and *BRCA2* mutations in 2 TNBC patients. Among the 6 *BRCA1* mutations, three were c.68_69delAG (185delAG) mutation. Remarkably, all the TNBC patients with *BRCA* mutations exhibited higher-grade tumors (grade 2 or 3). However, among the *BRCA* mutation carriers, only one patient with a *BRCA2* mutation (p.Glu1879Lys) developed metastasis in the observed cohort. Our data advocates that South Indian women with higher grade TNBC tumors and without hereditary breast and ovarian cancer should be considered for *BRCA* mutation screening, thereby enabling enhanced decision-making and preventive therapy.

Introduction

In women, the three most common cancers are breast, lung and colorectal cancer, where breast cancer (BC) alone contributes to 30% of female cancers [1]. BC accounted for an estimated 2.1 million cancer cases in 2018 and is the fifth leading cause of death globally [2, 3]. Triple-negative breast cancers (TNBC) is an aggressive BC subtype that lacks the expression of estrogen receptors (ER), progesterone receptors (PR) and have minimum or no HER2 protein expression [4]. TNBC accounts for 10–15% of BC, which is more likely to affect younger premenopausal women and present as poorly differentiated advanced-stage tumors [5]. TNBC is common among African Americans (AA) and intermediate between White/Caucasian Americans (WA) and West Africans, suggesting a correlation between African ancestry and TNBC [6]. TNBC incidence ranged from 6.7–27.9% in different countries, however India reported the highest TNBC percentage (27.9%) compared to other countries [7]. There are numerous clinical studies on TNBC incidence in African and Caucasian women, which documented TNBC to be more prevalent in African women. However, concerning TNBC incidence, India was found to be ahead of Africa, and the age at TNBC diagnosis was found to be 40–55 years in Indian women compared to 50–70 years in women from other countries [7]. The higher rate of TNBC incidence in India is due to various prominent risk factors such as deprivation status, obesity, lifestyle, high mitotic indexes, family history and *BRCA1* mutations. *BRCA1* and *BRCA2* are crucial tumor suppressor genes and mutations in *BRCA* genes play a pivotal role in the development of TNBC and hereditary breast and ovarian cancer syndrome (HBOC) [8, 9]. It has been reported that approximately 70% of breast tumors in *BRCA1* mutation carriers and around 16–23% of breast tumors in *BRCA2* mutation carriers resulted in TNBC [10]. Initially, the National Comprehensive Cancer Network (NCCN) guidelines have suggested germline *BRCA1/2* testing for BC patients of young age (≤ 45 years), male breast cancer, bilateral breast cancer and HBOC [11]. However, the recent NCCN guidelines have additionally included TNBC patients as an eligibility criteria for genetic testing [12]. Earlier studies have established the association of *BRCA1/2* mutations with elevated cancer risk in Ashkenazi Jewish and European women [13–15]. Moreover, a recent study identified that *BRCA1/2* mutations were associated with prolonged survival in women with TNBC but not in non-TNBC women [16]. There exists a wide variation in the *BRCA1/2* mutational landscape and frequency with respect to ethnicity and geographical background. Ashkenazi Jewish [17, 18], Polish [19], Slovenian [20] and Icelandic [21] populations were identified to have relatively high frequency of *BRCA1/2* founder mutations. However, in India very few studies [22–26] have investigated the germline *BRCA1/2* PVs in early-onset BC patients and HBOC. The exact germline mutational burden conferred by PVs in the Indian sub-continent is largely unknown, due to unaffordability and lack of awareness. Moreover, there is scarcity of information regarding the common PVs in *BRCA1/2* that contributes to TNBC phenotype in the Indian population. It is crucial to identify the PVs for better characterization of TNBC in India, and for genetic counseling. Hence, the present screened for *BRCA1* and *BRCA2* gene mutations in TNBC patients from South India using next-generation sequencing (NGS) technology.

Patients And Methods

Patients: The study recruited TNBC patients from Dr.G.V.N Cancer Institute, Tamil Nadu. All the patients were histopathologically proven breast carcinomas. Women with metachronous breast cancer, hormone and/or Her2 positive breast cancers, and/or ovarian cancer were excluded from the study. Blood, clinical, and demographic data were collected from 59 TNBC patients, who consented to take part in the study. 3-5ml of venous blood was collected in a EDTA-K2 coated sterile vacutainers from patients through antecubital venepuncture. Informed consent was obtained from the participants. The study was in accordance with declaration of Helsinki and the ethical committee of Dr.G.V.N Cancer Institute approved the present study (GVNCH-EC: ECR/436/INST/TN/2013).

Methods

The genomic DNA was isolated from the peripheral blood lymphocytes of TNBC cases. DNA isolation was performed within three days of sample collection using a commercially available HiPurA SPP blood DNA isolation kit (HiMedia™, Mumbai, India) following the manufacturer's instructions. The quality of the isolated genomic DNA was assessed by performing agarose gel electrophoresis. Quantification of genomic DNA was performed by spectrophotometric analysis by using NanoDrop 2000™ spectrophotometer (Thermo Fischer Scientific, USA). This was followed by DNA purification, and DNA quality control (QC). All the 59 samples were quantified using Qubit ds DNA BR Assay kit (Invitrogen, Cat# Q32850). DNA purity was checked using QIAxpert and DNA integrity was assessed on 1% agarose gel electrophoresis. The entire *BRCA1* (81.2kb) and *BRCA2* (84.8kb) genes were sequenced in all the 59 TNBC samples. All the 59 QC passed samples were taken for the Library preparation and targeted capture. 100ng of the DNA was taken for the library preparation using KAPA HyperPrep Kit (Roche, Cat# KK8504). The DNA samples were fragmented to produce 250bp inserts. The DNA fragments were then end repaired, 3' mono-adenylated, and ligated with indexed adapters. The adapter-ligated fragments were amplified with adapter-specific primers followed by size selection and purification to generate a genomic DNA library. Biotin-labelled probes were custom-designed to capture all the introns and the exons of the *BRCA1* and *BRCA2* genes. The probes were hybridized with the genomic DNA library for 16 hours at 65°C. Post incubation, the hybridized libraries were captured using Streptavidin beads followed by stringent washes to remove unbound DNA molecules. The enriched libraries were then amplified and purified. The final libraries were assessed for fragment size distribution using TapeStation 4200 (Agilent, USA). The prepared libraries were quantified using Qubit dsDNA High Sensitivity Assay kit (Invitrogen, Cat# Q32851). The obtained libraries were pooled and diluted to the final optimal loading concentration before cluster amplification on the Illumina flow cell. Once the cluster generation is completed, the cluster flow cell is loaded on the Illumina HiSeq X instrument to generate 150bp paired-end reads of 100X (mean coverage). Bioanalyzer plots were used at each step to evaluate the library step, and qPCR was used for measuring the library quantity before sequencing. The generated sequence data was analyzed after necessary quality control for variant calling and variant annotation. The essential computational methods such as quality analysis (FASTQC), adapter trimming (fastmcq), alignment with human reference genome hg19 (BWA, Sentioen), duplicate removal (Picard), realignment and recalibration (GATK), variant calling, and variant annotation (VariMat and ANNOVAR) were performed on the raw FASTQ data. The identified *BRCA1* and *BRCA2* variants were compared with cDNA sequences of *BRCA1* and *BRCA2*.

Results

The present study recruited 59 TNBC cases for investigating the prevalence and type of *BRCA* mutations in South Indian women. All the samples which were sequenced passed the initial quality control. Among the 59 TNBC genomic DNA samples sequenced, we identified 8 *BRCA* mutations in 59 TNBC patients (13.6%). Among the 8 mutations, we observed *BRCA1* mutations in 6 TNBC patients, and *BRCA2* mutations were observed among 2 TNBC patients. Among all the *BRCA1* and *BRCA2* mutations identified in the present study, most of the mutations were classified as pathogenic by the ClinVar, except for a *BRCA2* mutation which was identified as a benign variant by the ClinVar database. The age distribution of TNBC patients with *BRCA* mutations is represented in Fig. 1. *BRCA1* mutations were observed in all age groups, in contrast to *BRCA2* mutations which were observed frequently in women with the age group of 40–50 years. The mean age of TNBC onset in *BRCA1* mutation carriers was 48.3 ± 10.4 years and that of *BRCA2* mutation carriers was 45 ± 5.7 years. Table 1 summarizes the type of *BRCA1* and *BRCA2* mutations observed in TNBC patients recruited in the present study.

Table 1
BRCA1 and *BRCA2* mutations in TNBC patients of South India

Patient Id	<i>BRCA1/2</i> Mutation	rs ID	Ref.	Alt.	Exonic/ Intronic	Mutation Type	Mutation (Designation)	ClinVar	Age at onset (yrs.)	Reports from South India
GTNBC 02	<i>BRCA1</i>	rs62625308	G	A	Exon 11	Stop Gain	c.3607C > T (p.Arg1203Ter)	Pathogenic	55	Yes
GTNBC 06	<i>BRCA1</i>	rs80358027	C	A	Splicing	-	c.4357 + 1G > T (IVS13 + 1G > T)	Pathogenic	55	No
GTNBC 32	<i>BRCA1</i>	rs80357914	CT	-	Exon 2	Frameshift deletion	c.68_69delAG (p.Glu23ValfsTer17)	Pathogenic	50	Yes
GTNBC 40	<i>BRCA1</i>	rs80357914	CT	-	Exon 2	Frameshift deletion	c.68_69delAG (p.Glu23ValfsTer17)	Pathogenic	31	Yes
GTNBC 48	<i>BRCA1</i>	rs80357234	G	A	Exon 6	Stop Gain	c.220C > T (p.Gln74Ter)	Pathogenic	58	No
GTNBC 59	<i>BRCA1</i>	rs80357914	CT	-	Exon 2	Frameshift deletion	c.68_69delAG (p.Glu23ValfsTer17)	Pathogenic	41	Yes
GTNBC 11	<i>BRCA2</i>	rs80359531	CT	-	Exon 11	Frameshift deletion	c.5718_5719CT (p.Leu1908fs)	Pathogenic	41	No
GTNBC 15	<i>BRCA2</i>	rs55996097	G	A	Exon 11	Missense variant	c.5635G > A (p.Glu1879Lys)	Benign	49	No

A frameshift deletion c.68_69delAG mutation (p.Glu23ValfsTer17) located in the exon 2 of the *BRCA1* gene was observed commonly in TNBC patients. The c.68_69delAG mutation, also called as 185delAG mutation occurred in three out of six *BRCA1* mutation carriers (50%). Other deleterious *BRCA1* pathogenic variants observed in the TNBC patients were stop gain (c.3607C > T; p.Arg1203Ter and c.220C > T; p.Gln74Ter) and splicing variant (c.4357 + 1G > T; aka IVS13 + 1G > T) mutations. Among the two *BRCA2* mutations, we observed a TNBC patient carrying a frameshift deletion mutation in the exon 11 of the *BRCA2* gene (c.5718_5719CT; p.Leu1908fs) and another patient carrying a benign missense variant in exon 11 (c.5635G > A; p.Glu1879Lys). We did not observe any novel *BRCA* mutations in the investigated population. The clinicopathological characteristics of TNBC patients with *BRCA* mutation are represented in Table 2. *BRCA* mutations were observed in 50% of pre-menopausal TNBC patients. Remarkably, all the TNBC patients with *BRCA* mutations exhibited higher-grade tumors (grade 2 or 3). Among all the *BRCA* mutation carriers, only one patient with a *BRCA2* mutation (missense variant; p.Glu1879Lys) developed metastasis in the observed cohort. Another interesting observation made in the present study is that among the eight patients with TNBC, one patient (GTNBC 02) with *BRCA1* pathogenic variant (p.Arg1203Ter) developed ovarian cancer during the course of breast cancer treatment. Invasive ductal carcinoma was observed in most of the patients with *BRCA* mutations. Moreover, TNBC patients with *BRCA2* mutations developed advanced-stage tumors (Table 2).

Table 2
Clinicopathological features of TNBC patients with *BRCA* mutations

Patient Id	<i>BRCA1/2</i> Mutation	BMI	Menopausal status	Tumor Grade	Tumor Stage	LVI	Metastasis	HPE report	Position
GTNBC 02	<i>BRCA1</i>	Overweight	Post-menopausal	2	II	Positive	No	Invasive ductal carcinoma	Left breast
GTNBC 06	<i>BRCA1</i>	Normal	Post-menopausal	3	IV	Positive	No	Invasive ductal carcinoma	Left breast
GTNBC 11	<i>BRCA2</i>	Overweight	Pre-menopausal	3	III	Positive	No	Invasive ductal carcinoma	Right breast
GTNBC 15	<i>BRCA2</i>	Normal	Post-menopausal	2	IV	Positive	Yes	Invasive ductal carcinoma	Right breast
GTNBC 32	<i>BRCA1</i>	Obese	Pre-menopausal	2	IV	Positive	No	Papillary carcinoma	Right breast
GTNBC 40	<i>BRCA1</i>	Normal	Pre-menopausal	3	I	Absent	No	Medullary carcinoma	Right breast
GTNBC 48	<i>BRCA1</i>	Underweight	Post-menopausal	2	II	Absent	No	Invasive ductal carcinoma	Left breast
GTNBC 59	<i>BRCA1</i>	Overweight	Pre-menopausal	3	I	Absent	No	Invasive ductal carcinoma	Left breast

Discussion

The contribution of *BRCA1* and *BRCA2* mutations that predispose to triple-negative breast cancer development in Indian women is largely unexplored. Thus, there is an urgent necessity for population-specific studies to understand the underlying pathogenic variants that contribute to TNBC development in South Indian women. Hence, the present study screened the entire *BRCA1* and *BRCA2* genes for pathogenic variants in 59 TNBC patients using NGS. In the current study, 6 *BRCA1* mutations were identified in TNBC patients. The most common *BRCA1* pathogenic variant was c.68_69delAG (p.Glu23ValfsTer17) mutation, which was observed in three out of six *BRCA1*^{Mut} positive TNBC patients. Among the three c.68_69delAG (also known as 185delAG) mutation-positive TNBC cases, two patients were observed to have a positive family history of cancer. Reports globally suggest that the frequency of *BRCA1* and *BRCA2* mutations vary widely across different ethnicities and geographic regions. The presence of this deleterious variant in the Indian population was first identified in a South Indian family from Trivandrum [27]. Later, two South Indian families from Kerala [23] and two sisters from Goa [28] were reported to have 185delAG mutation. A previous South Indian study by Vaidyanathan *et al.* [25] explored the role of *BRCA1/2* mutations in hereditary breast and ovarian (HBOC) cases in South India. They observed 28% *BRCA* mutations in HBOC patients (24.6% *BRCA1*^{Mut} and 3.3% *BRCA2*^{Mut}). In line with our reports, they observed the presence of *BRCA1*^{Mut} in all age groups; however, *BRCA2*^{Mut} was observed only in women with ≤ 40 years of age. Similarly, they also observed a higher occurrence of 185delAG *BRCA1* (frameshift deletion) mutation in South Indian women. Remarkably, the 185delAG mutation is the founder mutation of the Ashkenazi Jewish (AJ) population and is responsible for the development of HBOC in AJ women. Besides, North Indian [24] and North-East Indian [29] studies also reported the presence of 185delAG mutation in breast cancer patients (non-Jewish ancestry) with no family history of cancer. Similarly, this mutation was also observed in the non-Jewish American population [30]. Interestingly, the 185delAG mutation was reported to have developed around 61 generations ago and arose independently not less than twice in the non-Jewish population [31]. Notably, Drost *et al.* [32] reported that tumors of both murine and breast cancer cell line with *BRCA1*185stop and *BRCA1*185delAG mutations, respectively, expressed a new domain-less *BRCA1* (RING-less) protein that mediates resistance to homologous recombination deficiency (HRD) therapies. Hence, investigation of 185delAG mutation in South Indian women might have better clinical and public health connotations. Moreover, the Indian population is not a completely uni-ethnic population, and it has a high prevalence of conservative mating habits, which prevailed for numerous generations. Thus, the likelihood of founder mutations cannot be entirely ruled out in such a population [33].

The other *BRCA1*^{Mut} observed in a TNBC patient of the present study is the c.3607C > T (p.Arg1203Ter) mutation, which results in *BRCA1* protein truncation. This mutation was reported in a North Indian study by Mehta *et al.* [34], and was observed in two patients with ovarian cancer. Similarly, another Indian study observed the prevalence of the Arg1203Ter mutation in both breast and ovarian cancer patients, irrespective of family history of cancer [35]. Remarkably, in line with our observation, this variation was also reported in Turkish TNBC patients [36]. Besides, this mutation was previously reported in Italian women with breast and/or ovarian cancer [37]. We also observed a splicing variant (c.4357 + 1G > T; IVS13 + 1G > T) in one of the TNBC patients. We did not find any Indian study reporting this variant; however, Ahmad *et al.* [35] reported a nearby intronic variant (c.4357 + 2T > G) in a North Indian breast cancer patient. Nevertheless, c.4357 + 1G > T intronic variant was reported in breast cancer patients from Latvia [38], China [39], mixed ethnicity [40], Brazil [41], and America [42]. Moreover, Lindor *et al.* [43] had classified this variant under the class 5 variant. Individuals carrying class 5 *BRCA1* variant should be considered as a carrier of completely pathogenic mutation and genetic testing should be recommended for all the at-risk relatives. Similarly, we observed the c.220C > T (p.Gln74Ter) *BRCA1*^{Mut} in a TNBC patient. Interestingly, this variant was recently reported in a TNBC patient from North India [44]. This variant was also reported in Chinese breast cancer patients [39].

Regarding the *BRCA2* variants observed in the present study, one patient was observed to carry the *BRCA2* c.5718_5719CT (p.Leu1908fs) pathogenic variant. This variant was reported in a male breast cancer patient recruited in the UNCseq™ study. The patient had a family history of cancer, where his sister and paternal aunt were affected with breast cancer [45]. This is a class 4 variant (likely-pathogenic) and genetic testing is recommended for at-high risk relatives. Moreover, this *BRCA2* variant was also observed in an Italian ovarian cancer patient resistant to platinum therapy [46]. This variant was also reported in two Chinese breast cancer patients [39, 47], Algerian HBOC patients [48], Macedonian [49], and German [50] breast cancer patients. The other *BRCA2* variant observed in the present study is the c.5635G > A (p.Glu1879Lys) missense variant. Even though this variant has been categorized under class 2 (likely not pathogenic) mutation, Zuntini *et al.* [51] observed several families with *BRCA2* Glu1879Lys variant. Moreover, a report by Thirthagiri *et al.* [52] observed the presence of Glu1879Lys missense variant in an Indian breast cancer patient. Interestingly, the WECARE study by Borg *et al.* [53] reported the presence of *BRCA2* Glu1879Lys variant in contralateral breast cancer patients. Pertaining to the clinical characteristics of TNBC patients with *BRCA* mutation, similar to our observation Thirthagiri *et al.* [52] observed invasive ductal carcinoma as the prevalent histological breast cancer subtype in both *BRCA1* and *BRCA2* mutation carriers. They also reported that all of the *BRCA* mutant tumors were mostly grade II or III, and none of them were grade I tumors. Furthermore, in agreement with our report, several studies observed the higher prevalence of *BRCA1* mutant tumors in TNBC patients [52, 54]. Strikingly, Lang *et al.* [39] reported that Chinese women with *BRCA* mutations are at reduced risk of developing breast cancer, suggesting the effect of specific *BRCA* mutations and ethnic background in modulating breast cancer risk. Noteworthy, for *BRCA1/2* mutation detection, NGS confers a comparably accurate and rapid yield compared to Sanger sequencing [55]. This might help the clinicians to make much informed and timely treatment decisions, such as usage of PARP inhibitors; thereby, increasing the survival of TNBC patients. Up to our knowledge, this is the first study to perform *BRCA* mutation screening in TNBC patients, without hereditary breast and ovarian cancer in South India. Hence, our study suggests that *BRCA1* mutation testing could be incorporated for South Indian women diagnosed with TNBC, particularly for patients with a family history of cancer

Declarations

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Conflict of interest: None

Author Contributions: TR: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. AS: Methodology, Resources, Supervision. AJ: Data analysis, Validation, Software. KKR: Methodology, Resources. ST: Investigation, Supervision, Review & editing; SV: Review & editing; NRD: Conceptualization, Funding acquisition, Project administration, Resources, Investigation, Validation, Supervision, Writing - review & editing.

Ethical approval Study proceeded with the approval of ethical committee Dr. GVN cancer Institute (GVNCI-HEC – ECR/436/INST/TN/2013)

Informed Consent: Informed consent was obtained from all the individual participants included in the study.

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Figures

Figure 1

Age distribution of TNBC patients with BRCA mutations