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Original research

Landscape of clinically actionable mutations in breast cancer 'A cohort study'

Mithua Ghosh [a,*,**,1], Radheshyam Naik [b,1], Sheela Mysore Lingaraju [a,1], Sridhar Papaiah Susheela [b], Shekar Patil [b], Gopinath Kodaganur Srinivasachar [b], Satheesh Chiradoni Thungappa [b], Krithika Murugan [b], Srinivas Belagutty Jayappa [b], Somorat Bhattacharjee [b], Nalini Rao [b], Mahesh Bandimegal [b], Roopesh Krishnappa [b], Shashidhara Haragadde Poppareddy [b], Krishna Chennagiri Raghavendrachar [a], Yogesh Shivakumar [a], Sunitha Nagesh [a], Ramya Kodandapani [a], Ashwini Rajan [a], Urvashi Bahadur [a], Pooja Agrawal [a], Veena Ramaswamy [a], Tejaswini Bangalore Nanjaiah [a], Sateesh Kunigal [a], Shanmukh Katragadda [a], Ashwini Manjunath [a], Amritanshu Ram [b], Basavalinga S. Ajaikumar [b]

[a] Strand Life Sciences Pvt. Ltd., 560027, India
[b] HealthCare Global Enterprises Limited, Bangalore, Karnataka 560027, India

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ABSTRACT

Breast cancer (BC) is a heterogeneous disease. Numerous chemotherapeutic agents are available for early stage or advanced/metastatic breast cancer to provide maximum benefit with minimum side effects. However, the clinical outcome of patients with the same clinical and pathological characteristics and treated with similar treatments may show major differences and a vast majority of patients still develop treatment resistance and eventually succumb to disease. It remains an unmet need to identify specific molecular defects, new biomarkers to enable clinicians to adopt individualized treatment for every patient in terms of endocrine, chemotherapy or targeted therapy which will improve clinical outcomes in BC. Our study aimed to identify frequent hotspot mutation profile in BC by targeted deep sequencing in cancer-related genes using Illumina Truseq amplicon/Swift Accel-Amplicon panel and MiSeq technology in an IRB-approved prospective study in a CLIA compliant laboratory. All the cases had pathology review for stage, histological type, hormonal status and Ki-67. Data was processed using Strand NGS™. Mutations identified in the tumor were assessed for 'actionability' i.e. response to therapy and impact on prognosis.

Introduction

Breast cancer (BC) is the most frequently diagnosed malignancy worldwide and is the second leading cause of cancer death among females accounting to approximately 40,000 deaths every year [1]. There is a significant increase in the incidence and cancer-associated morbidity and mortality in Indian subcontinent as described in global and Indian studies [2]. The disease is very heterogeneous and differs among different patients leading to intertumoral heterogeneity as revealed by pathological and radiological staging and histopathological classification in terms of expression of prognostic and predictive biomarkers, hormone receptors (HR) as Estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (Her2) oncoprotein [3]. BC is also characterised by diverse genetic tumor heterogeneity and warrants further molecular classification to stratify the patient into low- and high-risk groups. Moreover, the intra-tumoral heterogeneity within each individual along with tumor morphology, molecular and cellular mechanisms including genomic, transcriptomic, and proteomic levels of tumors also vary creating diagnostic and therapeutic challenges [3]. Classification of BC at the molecular level based on gene expression studies started in the year 2000 and explained that BC is not one single entity but can be subdivided into distinct subtypes [4]. Of all the subtypes, luminal A and B subtypes account for 65% to 70%, triple negative breast cancer (TNBC) about 10% to 15% and Her-2 overexpressing about 10% to 20% [5]. The ER positive groups are about three quarters of all breast cases treated using ER modulators or aromatase inhibitors [6]. Several genomic tests are available to predict the outcome of ER + patients receiving

* Corresponding author at: Strand Life Sciences Pvt. Ltd., India.
** Corresponding author at: Department of Molecular and Clinical Genomics, HealthCare Global Enterprises Limited, Bangalore, 560027, Karnataka, India.
E-mail address: mithuaghosh@strandls.com. (M. Ghosh).
[1] Mithua Ghosh, Radheshyam Naik, Sheela Mysore Lingaraju have equal contribution to the paper.

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endocrine therapy and only about 20% to 40% respond to the treatment [7]. Among the subtypes, the luminal A has good outcome with 95% 5-year survival whereas luminal B tend to have worst outcome as they are less sensitive to endocrine therapy and are more sensitive to chemotherapy [7]. TNBC, also known as basal like breast cancers subgroup, associated with germline BRCA mutations cannot be regarded as a single disorder but rather as a trunk of heterogeneous diseases characterised by different prognosis and response to drug treatments [8]. Further, these findings are supported by gene expression studies which have thrown light on prognostic and predictive subgroups in TNBC [9]. HER-2 subtypes may be eligible for targeted therapy but still the rate of metastasis is high, and prognosis is poor as they are resistant to current chemotherapies [10].

Despite recent advances in the treatment of BC with the advent of better surgical expertise, radiotherapy techniques and chemotherapeutic advances over the past 20 years, the outcome of metastatic BC has increased marginally and remains the second leading cause of female-specific cancer-related mortality [11]. Many major drugs have initial response but the non-responder rates vary between 30% and 70%. The variation in the clinical outcomes of patients can be accounted to inherent variability because of the genomic instability, which generates somatic mutations/alterations leading to clonal expansion. For eg. although trastuzumab has revolutionized the treatment of Her2 positive BC, a significant number of patients with HER2-overexpressing BC do not benefit from it due to acquired or de novo resistance.

Understanding the mechanisms of action and resistance to trastuzumab is therefore crucial for the development of new therapeutic strategies [12]. The phosphatidylinositol3′-kinase/protein kinase B/mammalian target of rapamycin (PI3KCA, AKT, mTOR) pathway, cross-talk with ER receptors, immune response, cell cycle control mechanisms, and other tyrosine kinase receptors such as insulin-like growth factor I receptor are potential pathways involved in trastuzumab resistance [13]. Drug resistance, which is common in all BC types despite the different treatment modalities applied is not mutually exclusive. It seems that tumor could be resistant to multiple treatment strategies, such as being both chemo resistant and monoclonal antibody resistant. However, the underlying mechanisms are complicated and need further investigation. It is well known that cancer progression is due to the genetic alteration in the cancer genome and hence understanding the genomic drivers is essential to develop new therapies [13]. Evidences suggest that the risk assessment, treatment and patient outcome are not just by the biology of the tumor but are also influenced by somatic and germline mutations [14]. Somatic mutations in AKT1, PI3KCA, PTEN and TP53 genes have been found at high frequency in BC with PICK3CA as 26.4%, TP53 as 24.7%, PTEN as 3.8% and AKT1 as 2.8% as per Catalogue of Somatic Mutations in Cancer (COSMIC) database [15]. Large genomic landscape studies have shown that the mutations in these four genes showed subgroup specificity with clinical implications which helped extensively in cancer classification and treatment [16,17]. PI3KCA/AKT/mTOR signalling pathway is critical to both normal and malignant cellular processes, like proliferation, apoptosis, and metabolism [18]. Spontaneous mutations in PIK3CA are very common with almost 25% of breast cancer patients harbouring a mutation in this gene [19]. Particularly, the p110α catalytic component of phosphoinositide-3-kinase (PI3K) termed as PIK3CA is commonly mutated in approximately 36% of BC, and 80% to 90% of mutations which aggregates in exon 9 (E545K and E542K) and exon 20 of PIK3CA into 3 hotspot regions [20]. PI3K phosphorylates membrane phosphatidylinositols leading to recruitment of AKT and its activator (phosphoinositide dependent kinase-PDK1) to the cell membrane via pleckstrin homology domain and 3′ phosphorylated lipids. This enforced co-localization of AKT and PDK1 phosphorylates the latter which in turn activates the former. PDK1 and PDK2, likely components of TORC2 complex, phosphorylate the AKT initiating a downstream signalling cascade [21]. The same site is dephosphorylated by the tumor suppressor or PTEN on membrane phosphatidylinositols that is phosphorylated by PI3K, which reverses the effect of PI3K signalling.

Preclinical and clinical studies support that PIK3CA mutations may predict sensitivity to treatment with PI3K/AKT/mTOR inhibitors in breast tumors in particular and in a multiple tumor types [22]. Additionally, because of crosstalk between the PI3K-AKT-mTOR and MAPK pathways, KRAS and BRAF activate the members of PI3K-AKT-mTOR pathway [23,24]. Activation of PI3K lipase get aggravated by PIK3CA mutation and in turn upregulates the downstream AKT signalling. There are reports of negative feedback regulation of the PI3K pathway, in which PTEN plays an important role. PTEN is absent in 25% of BC patients and mutated in 5% of the patients and this inactivation leads to phosphorylation of AKT, mTOR and S6K1 [25]. PIK3CA is the second most commonly mutated gene in hormone receptor positive BC [26] for which the mTOR inhibitor everolimus is recommended as a therapeutic option for metastatic BC [27]. Though PIK3CA mutations have been associated with good prognosis for few subtypes, they have also been shown to impart resistance to trastuzumab which is a common treatment option for Her-2 overexpressing BC's [28]. The therapeutic efficacy of several inhibitors of AKT, PIK3CA and mTOR inhibitors are under clinical investigations for breast as well as other cancers [29]. TP53 (p53) is another frequently mutated gene in invasive BC and occurs in 30–35% of all cases. However, the prevalence of p53 mutation is very high and is approximately 80% in TNBC [30].

The repertoire of somatic genetic alterations is very complex and varies according to BC subtype, HR and HER status and other factors. Several studies have interrogated the complex genomic profile of BC by massively parallel sequencing analyses using targeted panels, whole exome/genome sequencing [16,31]. In addition to most common mutations like PI3K and TP53, other genetic alterations reported in BC patients are ERBB2, ERBB3, KRAS, NRAS, ATM, CDH1, GATA3, MAP3K1, CDKN2, RB1, ESR1 [32,33].

It is noteworthy that the current guidelines for reporting of biomarkers aim to maximize patient eligibility for targeted therapy, but do not take into account inter/intratumoral heterogeneity. Hence, it is very important to establish the genetic profile of BC, identify the gene mutations for treatment options and avoid unnecessary drug toxicities [16]. Moreover, in case of TNBC, since there are no targeted treatments, so identifying gene mutations for therapy option becomes important. Also, the mutation spectrum of these genes and other driver mutations is not well studied in Indian population. Therefore, it remains an unmet need to identify specific molecular defects and new biomarkers for Indian BC patients to enable the clinician to adopt individualized treatment for every patient in terms of endocrine, chemotherapy or targeted therapy which will improve clinical outcomes. The advance of next-generation sequencing (NGS) technology enables massively parallel deep sequencing of patients with BC at 'one go' to analyse the mutation profile of tumors and identify genomic markers as response predictors to therapy thus making personalized treatment a reality for patients. This breaks the cycle of 'trial and error' medicine and links the test to patient tailored action and evidence based therapy/treatment plan in breast cancer. However, there are some challenges of less referrals and low adoption of these tests because of higher cost compared to other diagnostic tests, limited awareness and understanding of the clinical implication of the findings.

In the present study, we have analysed the frequent hotspot mutation profile in BCs by targeted deep sequencing in cancer-related genes using Illumina TruseqAmplicon/Swift Accel-Amplicon panel and MiSeq technology in an IRB-approved prospective study in a CLIA compliant laboratory. Further, the clinical and pathological features of BC associated with the mutations detected have been analysed. The goal of this study is to establish clinical utility of the mutations, improve clinical outcome of BC patients and reduce treatment cost with an approach of 'right treatment at the first time' based on the genomic markers. This is particularly important in emerging economic country like India where availability and access to cancer drugs, rational combination therapies, and enriched clinical trials have been some of the challenges in adopting Genomic medicine.

Materials and Methods

Ethics statement

The study has been approved by the Human Research Ethics Committee of the institute (HCG Central Ethics Committee/EC Registration No: ECR/386/Inst/KA/2013/RR-19, a tertiary comprehensive cancer care Hospital in Bangalore, India).The institutional ethics committee recommended the need for consent for formalin-fixed, paraffin-embedded (FFPE) tumor samples obtained from the tumor tissue bank at the hospital's department of Pathology. All samples and medical data used in this study have been irreversibly anonymized.

Patient / study subjects and sample preparation

570 women with BC (early diagnosed or advanced/metastatic) aged 26–75 yrs. (median age 50.5 yrs) diagnosed at a tertiary hospital in India from April 2014–17 were consulted to be profiled by targeted deep sequencing for somatic hotspot mutations in 56 cancer-related genes. For the study, Swift Accel-Amplicon panel was used on MiSeq technology in an IRB-approved prospective study in a CLIA compliant laboratory. Out of 570 BC cases,275 (48%) patients consented for undergoing the genetic testing. Formalin-fixed, paraffin-embedded (FFPE) tumor samples were obtained from the tumor tissue bank at the hospital's Department of Pathology. All the cases had pathology review for stage, histological type, hormonal status and Ki-67.

DNA preparation

FFPE tissue samples were first deparaffinized in xylene, 3–5-μm-thick sections were sliced, and DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN) as per the manufacturer's instructions. DNA was quantified using Qubit, as well as a qPCR assay.

Histopathology and immunohistochemistry (IHC)

IHC for ER, PR and Her-2/neu was done on tissue sections using primary and secondary antibodies from Biocare, USA (ERSP1 clone for ER, PRSP2 clone for PR and EP3clone for Her-2/neu). The stained slides along with appropriate controls were reviewed and interpreted by two pathologists as per Allred Score for ER, PR and as per American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using a cutoff of >1% stained tumor nuclei and evaluated by Allred score guidelines for Her-2/neu [34]. Fluorescent in situ hybridization (FISH) testing was recommended for a score of 2 + (equivocal) where there was incomplete membrane staining in >10% tumor cells.

Her2/neu by fluorescence in situ hybridization (FISH)

Her2/neu by FISH was tested using an FDA-approved PathVysion HER-2 DNA Probe Kit (Abbott PathVysion Kit Catalogue number 06N46-03) as per manufacture's protocol. The slides were visualized for signals under an epifluorescence microscope (Nikon Eclipse 80i) fitted with appropriate filter set. Enumeration of the signals is done in accordance with the manufacturer's guidelines and interpretation based on recent ASCO and CAP guidelines [35]. The signals for Her2/neu gene to the centromere enumeration probe (CEP) on chromosome 17 was counted to provide a comprehensive report to the clinicians. Her2/neu gene was reported to be amplified if, Her2/CEP17 ratio was greater than or equal to two, and the average copy number of her2/neu gene was less than, equal to or greater than four or Her2/CEP17 ratio was less than two and the average copy number of her2/neu gene was greater than or equal to six. Her2/neu gene was not amplified when the Her2/CEP17 ratio was reported to be less than two and the average copy number of her2/neu was less than four. Her2/neu gene ratio equal to or greater than two indicates amplification or over expression, whereas a ratio less than two does not indicate amplification of the gene.

Next generation sequencing (NGS) - multigene panel selection

NGS was performed using Illumina Tru Seq Amplicon/Swift Accel Amplicon 56G for comprehensive and hotspot coverage of 56 clinically relevant oncology-related genes. The panel utilizes a 263-amplicon design, covering over 16,000 COSMIC mutations (Forbes et al. Oxford Journals. 2014), to generate targeted libraries compatible with Illumina MiSeq sequencing. The genes in the panel are ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, DDR2, DNMT3A, EGFR, ERBB2, ERBB4, EZH2,FBXW7, FGFR1, FGFR2, FGFR3, FLT3, FOXL2, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MAP2K1, MET, MLH1, MPL, MSH6, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET,SMAD4, SMARCB1, SMO, SRC, STK11, TP53, TSC1, VHL.

Library preparation and sequencing

About 10-100ng of DNA depending on the quality of DNA was used for library generation. Individually amplified libraries were tagged with adaptors/barcodes and quantified using NEBNext Library Quant Kit (NEB, USA). Upto 20 libraries were pooled and loaded on Illumina MiSeq platform using a V2 kit to yield multitude of reads for each region of interest.

Analysis

The trimmed FASTQ files were generated using MiSeq Reporter from Illumina. The amplicon primers were trimmed from the reads using cut adapt v1.13. The reads were processed using STRAND® NGS v2.6 (http://www.strand-ngs.com) via the analysis pipeline 'Swift_56G_ST_v4'. In this pipeline, reads were aligned against the whole genome build hg19 (UCSC). Before aligning, one base pair from the 3′ end of the reads was trimmed, as were 3′ end bases with quality below 20. Reads which have length less than 25 bp after trimming were not considered for alignment. A maximum of 5 matches of alignment score of at least 90% were computed. Reads that failed vendor QC, reads with average quality less than 20, reads with ambiguous characters, and reads with alignment score less than 95% were all filtered out. The Strand®NGS binomial variant caller was used to detect variants. Variants are called at locations in the target regions covered by a minimum of 10 reads having at least 2 variant reads. Variants with a decibel score of at least 50 were reported. Substitution variants with a StrandBias >50%, and InDel variants in homopolymer stretches longer than 4 bp with supporting reads <10% were filtered out. Also, all variants with supporting reads <2% were filtered out. All the variants were then imported into StrandOmics for further interpretation and reporting. Annotation and prioritization of variants was done by automated pipelines in StrandOmics. The StrandOmics user interface was then used for identifying variants of interest and for reporting these variants. All variants reported were verified to have good read quality using the Strand® NGS v2.6 genome browser before final reporting. The Limit of Detection (LOD) for this test is 3% variant allele frequency (VAF) at coverage of 200×. It has a sensitivity of ~96% and a specificity of ~100%.

Statistical analysis

One objective of the statistical analyses was to evaluate if there was an impact of gene mutation on the proportions of hormonal subtype and pathological response to Neo-adjuvant treatment. This was ascertained by Fisher's Exact Test and χ2-Sqaure test with Bonferroni Corrections on post-hoc analyses for subgroups. All inferential tests were two-tailed, and α was considered to be 0.05. All statistical tests were done using SPSS version 18.

Results

275 patients of Indian ethnicity with a known diagnosis of early stage/or advanced/metastatic BC diagnosed at a tertiary hospital from April 2014–17 were consented to be profiled by targeted deep sequencing for hotspot mutations in 56 cancer- related genes. The cohort comprised of 2 male and 273 female breast cancer patients. The clinicopathological features of the patients are summarized in Tables 1A and 1B. The maximum number of patients was in the age group of 40–65 years (67%), whereas 24% of patients were below the age of 40 years. There were only 25 (9%) patients above 65 years of age. The median age of patients was found to be 52 years. The cohort was predominantly of common histological type of infiltrating ductal carcinoma (IDC) (73%) and presented at stage III (18%) or IV (55%) of BC. About 60% of patients were hormonal positive (HR + ive) out of which 12% were triple positive (TPBC- HR + ve Her2 + ive-) and 48% were HR + ve and Her2-ive (HR + ve Her2-ive). Among the 40% hormonal negative (HR-ve) cohort, 10% of patients were Her2 + ive (HR-ive, HER2 + ive) and 30% were TNBC (HR-ive, HER2-ive). Notably, the number of TNBC cases reported in the tertiary cancer centre in India is higher than the global data [5]. Based on hormonal status and Ki-67, the cohort was classified into Luminal A (7%), Luminal B (34%), basal like/TNBC (30%) and HER 2 enriched (11%) (Table 1B). The stage and hormonal status were not available in the medical records for 33 patients.

Detection of mutations by NGS

The somatic mutation landscape was analysed in these patients (Table 2). Somatic variants were detected in 195 BC patients (71%) comprising of mutation in single gene or co-mutation in multiple genes. "Actionable" mutations were found in 46% BC cases with direct impact on therapy or prognosis. Genetic aberrations were identified in PI3KCA/AKT/PTEN signalling pathway in substantial fraction (57.8%) in Indian BC patients. PI3KCA was found to be altered in 42% cases whereas deletion in pTEN was found in 12.8% and mutation in AKT in 3% of BC patients.

Several different PIK3CA mutations were identified: p.N345K in the C2 domain encoded by exon 4, p.E542K and p.E545K in the helical domain encoded by exon 9, and p.H1047R and p.H1047L in the kinase domain encoded by exon 20 (Table 3). Mutations in p.H1047R was found to be most prevalent (40/82cases) followed by p.E542K, p.E545K. Three patients had other variants of PIK3CA (p.Glu 542 Gln, p.Glu 542 Gln and p. Pro 539 Arg) along with p.H1047R. These three mutations have been found in previous studies to be the most prevalent in human breast cancers, associated with an increase in kinase activity in the PI3K pathway [36,37], and accounted for 88.5% of all PIK3CA mutations in our study. Disruptive and non-disruptive mutations in TP53 alone and co-mutated with other genes were found in 31% and 62% of BC. TP53 was also found to be commutated with PI3KCA in 19% BC patients. P53 mutation had been found to be significantly higher in Indian patients compared to what has been reported in TCGA data [31]. Furthermore, somatic mutations were also detected in cKIT indicating sensitivity to imatinib and therefore enrolled on a clinical trial. 16 patients (8.2%) in the cohort were found to have other variants like RB1 (n = 4), ERBB2 (n = 6), ERBB3 (n = 1), FGFR amplification, KRAS (n = 4), NRAS (n = 2), CDH1 (n = 1), FBXW7 and EGFR (n = 1), ATM (n = 4) either as single mutation or co-mutated with other genes. All these variants identified indicated resistance to conventional therapy and suggested sensitivity to alternative targeted therapy, either approved or in clinical trials.

Table 1A
Statistics of total number of cases.

	Total cases	Percentage
Ca breast cases from 2014 to 2017	570	
Ca breast cases who have undergone Somatic testing	275	48%
Female patients	273	99%
Male patients	2	1%
Cases with mutations detected	195	71%
Cases with actionable mutations detected	126	46%
Cases with no mutation	80	29%

Table 1B
Clinicopathological characteristics of 275 breast cancer patients.

Sl no.	Characteristics			Number of patients	n (%)
	Age at diagnosis		< 40	65	24
			40–65	185	67
A			>65	25	9
	Tumor grade		1	4	1
			2	79	29
			3 or 4	67	24
B			Unknown	125	45
	Tumor stage		1	15	5.5
			2	25	9.1
			3	50	18.2
			4	151	54.9
C			Unknown	34	12.4
	Tumor type		IDC	201	73.1
			ILC	2	0.7
			Mucinous	1	0.4
			Mixed	6	2.2
			DCIS	4	1.5
D			Unknown	61	22.2
	Hormonal status				
	HR		Positive (+)	147	60.7
	HR	Her2	Positive (+) positive (+)	29	12.0
	HR	Her2	Positive (+) negative (−)	118	48.8
	HR	Her2	Negative (−) positive (+)	22	9.1
	HR	Her2	Negative (−) negative (−)	73	30.2
E	HR	Her2	Unknown	33	13.6
	Molecular subtype		Luminal A	19	7
			Luminal B	93	34
			Basal like/TNBC	75	30
			HER 2 enriched	29	11
F			Unknown	61	22

Table 2
Frequencies of most prevalent somatic mutations compared with TCGA data.

Mutation Pattern	In this study of 275 patients (195 harbouring somatic mutation		In TCGA data (n = 507)	
	# patients	Percentage	# patients	Percentage
Mutation in gene				
AKT1	6	3	23	4.5
PIK3CA	82	42	179	35.3
PTEN	25	12.8	16	3.2
TP53	121	62	179	35.3
Others	16	8.2	NA	NA
Mutation in single gene				
AKT1	2	1	9	1.8
PIK3CA	35	18	130	25.6
PTEN	4	2	6	1.2
TP53	61	31	127	25.0
Co-mutation in two genes				
AKT1 + PIK3CA	0	0.0	1	0.2
AKT1 + PTEN	1	0.5	0	0.0
AKT1 + TP53	3	1.5	2	0.4
PIK3CA + PTEN	3	1.5	4	0.8
PIK3CA + TP53	37	19	44	8.7
PTEN + TP53	7	3.6	6	1.2
PI3K + others	43	22	NA	NA
Co-mutation in three genes				
AKT1 + PIK3CA + TP53	0	0.0	0	0.0
PIK3CA + PTEN + TP53	1	0.5	0	0.0

Multiple – gene and recurrent mutations

Among the 195 somatic mutation carriers, 102 (52%) harboured mutation in single gene, 94 (48%) harboured co-mutation in two genes

Table 3
Pathogenic mutations of PIK3CA gene (single mutation + co-mutation) detected in 82 Ca breast cases of 275 cases.

Gene	# of patients	Frequency of mutation	Status	Nucleotide change	Variant	Mutation type	Previously reported
	1	1.2	Somatic	c.3140A>T	p.His 1047 Leu	Missence	dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.3132T>A	p.Asn 1044 Lys	Missence	dbSNP[COSMIC]ClinVar
	10	12.2	Somatic	c.1035T>A	p.Asn 345 Lys	Missence	dbSNP[COSMIC]ClinVar
	3	3.7	Somatic	c.1258T>C	p. Cys 420 Arg	Missence	dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.1637A>G	p.Gln 546 Arg	Missence	dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.1636C>G	p. Gln 546 Glu	Missence	dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.1636C>A	p. Gln 546 Lys	Missence	dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.1625A>G	p. Glu 542 Gly	Missence	dbSNP[COSMIC]ClinVar
	7	8.5	Somatic	c.1624G>A	p. Glu 542 Lys	Missence	dbSNP[COSMIC]ClinVar
	2	2.4	Somatic	c.1634A>C	p. Glu 545 Ala	Missence	dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.1634A>G	p. Glu 545 Gly	Missence	dbSNP[COSMIC]ClinVar
	7	8.5	Somatic	c.1633G>A	p. Glu 545 Lys	Missence	dbSNP[COSMIC]ClinVar]ExAC
	37	45.1	Somatic	c.3140A>G	p. His 1047 Arg	Missence	dbSNP[COSMIC]ClinVar]ExAC
	1	1.2	Somatic	c.3140A>G c.1624G>C	p.His 1047 Arg p.Glu 542 Gln	Missence Missence	dbSNP[COSMIC]ClinVar]ExAC dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.3140A>G c.1633G>A	p. His 1047 Arg p.Glu 545 Lys	Missence Missence	dbSNP[COSMIC]ClinVar]ExAC dbSNP[COSMIC]ClinVar
	1	1.2	Somatic	c.3140A>G c.1616C>G	p. His 1047 Arg p. Pro 539 Arg	Missence Missence	dbSNP[COSMIC]ClinVar]ExAC dbSNP[COSMIC]ClinVar
	4	4.9	Somatic	c.3140A>T	p. His 1047 Leu	Missence	dbSNP[COSMIC]ClinVar]ExAC
	1	1.2	Somatic	c.3129G>C	p. Met 1043 Ile	Missence	dbSNP[COSMIC]ClinVar
PIK3CA	1	1.2	Somatic	c.1633G>A	p. Pro 278 Ser	Missence	dbSNP[COSMIC]ClinVar

and 1 patient (0.005%) harboured co-mutation in three genes. The mutation landscape was correlated with the clinicopathological characteristics of the patients (Table 4). Comparing mutation carriers with activating mutations in PI3KCA/AKT/PTEN signalling pathway and non-carriers, mutations were found to be the common genetic event in hormone receptor-positive HR + ve BC (38%). Mutations in PI3KCA/AKT/PTEN signalling pathway was found to be significantly higher in HR + ve BC compared to HR –ve cohort of IDC histology ($p = 0.001$). It is also important to note that PI3KCA/AKT/PTEN signalling pathway was also found to be altered in 18% of TNBC. Also, the mutations in PI3KCA/AKT/PTEN signalling pathway was found to be significantly higher in HR + ve, HER2-ive BC ($\chi 2 = 11.180$; $p = 0.011$).

Pathological complete response rates (pCR) with PI3KCA mutation status

We retrospectively selected patients with invasive breast cancer who underwent surgery at the surgery unit of the hospital after neoadjuvant chemotherapy (NACT) and had undergone somatic mutation testing in our laboratory. Information on the treatment protocol was obtained from the medical records. Out of total 46 selected patients, follow up data were

Table 4
Clinicopathological characteristics and associations with PIK3CA and TP53 in 275 breast cancer patients.

	Total (275)	PIK3CA co-mutation (82)		TP53 co mutation (121)		PIK3CA + TP53 (39)	
Characteristic	Number	Mutant (%)	WT	Mutant (%)	WT	Mutant (%)	WT
Age at diagnosis							
<40	65 (23.6)	11 (13.4%)	54 (28.0%)	29 (23.9%)	36 (23.4%)	4 (10.8%)	61 (25.8%)
40–65	185 (67.3)	63 (76.8%)	122 (63.2)	84 (69.4%)	101 (65.6%)	31(83.7%)	154 (65.3%)
>65	25 (9%)	8 (9.8%)	17 (8.8)	8 (6.6%)	17 (11.0%)	2 (5.4%)	23 (9.7%)
Hormonal status							
HR (+)	147(60.7%)	56 (38%)	91 (62%)	41 (33.9%)	95 (61.7%)	28 (71.8%)	108 (45.8%)
HR (+) Her2 (+)	29 (19%)	11 (38%)	18(62%)	9 (7.4%)	12 (7.8%)	4 (10.3%)	17 (7.2)
HR (+) Her2(−)	118 (83%)	45 (38%)	73 (62%)	32 (26.4%)	83 (53.9%)	20 (51.3%)	95 (40.3)
HR (−)Her2 (+)	22 (11%)	4 (18%)	18 (82%)	16 (13.2%)	13 (8.4%)	2 (5.1%)	27 (11.4%)
HR (−)Her2 (−)	73 (30%)	13 (18%)	60 (82%)	51 (42.1%)	25 (16.2%)	8 (20.5%)	68 (28.8%)
HR Her2 (unknown)	33 (13.6)	25 (13.0%)	9 (11%)	13 (10.7%)	21 (13.6%)	5 (12.8%)	29 (12.3%)
Ki 67 expression							
<15	25 (9.1%)	6 (24%)	19 (9.8)	4 (16%)	21 (13.6%)	3 (12%)	22 (9.3%)
>15	162 (58.9%)	54 (33%)	108 (56.0%)	75 (46.3%)	87 (56.5%)	23 (14.2%)	139 (58.9%)
Unknown	88 (32.0%)	22 (25%)	66 (34.2%)	42 (47.8%)	46 (29.9%)	13 (14.8%)	75 (31.8%)
Age at diagnosis							
<40	65 (23.6)	11 (13.4%)	54 (28.0%)	29 (23.9%)	36 (23.4%)	4 (10.8%)	61 (25.8%)
40–65	185 (67.3)	63 (76.8%)	122 (63.2)	84 (69.4%)	101 (65.6%)	31(83.7%)	154 (65.3%)
>65	25 (9%)	8 (9.8%)	17 (8.8)	8 (6.6%)	17 (11.0%)	2 (5.4%)	23 (9.7%)
Hormonal status							
HR (+)	147(60.7%)	56 (38%)	91 (62%)	41 (33.9%)	95 (61.7%)	28 (71.8%)	108 (45.8%)
HR (+) Her2 (+)	29 (19%)	11 (38%)	18(62%)	9 (7.4%)	12 (7.8%)	4 (10.3%)	17 (7.2)
HR (+) Her2(−)	118 (83%)	45 (38%)	73 (62%)	32 (26.4%)	83 (53.9%)	20 (51.3%)	95 (40.3)
HR (−)Her2 (+)	22 (11%)	4 (18%)	18 (82%)	16 (13.2%)	13 (8.4%)	2 (5.1%)	27 (11.4%)
HR (−)Her2 (−)	73 (30%)	13 (18%)	60 (82%)	51 (42.1%)	25 (16.2%)	8 (20.5%)	68 (28.8%)
HR Her2 (unknown)	33 (13.6)	25 (13.0%)	9 (11%)	13 (10.7%)	21 (13.6%)	5 (12.8%)	29 (12.3%)
Ki 6 7 expression							
<15	25 (9.1%)	6 (24%)	19 (9.8)	4 (16%)	21 (13.6%)	3 (12%)	22 (9.3%)
>15	162 (58.9%)	54 (33%)	108 (56.0%)	75 (46.3%)	87 (56.5%)	23 (14.2%)	139 (58.9%)
Unknown	88 (32.0%)	22 (25%)	66 (34.2%)	42 (47.8%)	46 (29.9%)	13 (14.8%)	75 (31.8%)

available for 35 cases. All patients underwent at least three cycles of NACT before surgery. The NACT regimens without trastuzumab included anthracycline based (doxorubicin with cyclophosphamide) or anthracycline and taxane-based (doxorubicin and docetaxel with or without cyclophosphamide) regimens. The NACT regimens including trastuzumab were anthracycline-based, taxane-based (docetaxel), or anthracycline and taxane-based (doxorubicin with docetaxel and cyclophosphamide) regimen. The histopathology reports of sequential FFPE blocks including pre-NACT and post-NACT, and recurrent specimens of each patient were collected. Patients were excluded if they did not have either pre-NAC or post-NAC specimen blocks. Tumor recurrence/progressive disease (PD) was defined as involving both local recurrence and distant metastasis, while pCR was defined as no invasive tumor in the breast or lymph nodes, and no lymphovascular invasion. The patients were categorized into pathological partial response (PR) if the invasive tumor in the breast or lymph nodes was more than 25% and stable disease (SD) if invasive tumor in the breast or lymph nodes was less than 25%. In total 5 patients (14%) achieved pCR after NACT, 15 patients had PR (43%), 7 patients(20%) were found to achieve SD while 8 patients (23%) had PD. Overall 27 patients (77%) responded to NACT either with a pCR or a stable disease whereas 8 patients (23%) had progressive disease. PIK3CA mutation in post-NACT specimens correlated with a lower pCR rate than that observed in the post-NACT wild-type specimens. Majority of these patients were HER2 + ve and had undergone NACT regimen that included trastuzumab.

Although the cohort is small to perform a statistical analysis but the overall data clearly indicates that activation of the PI3K/ATK/mTOR pathway could be a potential factor conferring resistance to trastuzumab treatment in HER2 + breast tumors thus suggestive of using combination therapy/alternate therapy.

Discussion

Currently, BC is divided into different molecular subtypes which can effectively predict disease features and prognosis. However, the clinical outcome and response to drugs for patients with the same subtype are also diverse. Tumor heterogeneity, diverse microenvironment, and genetic heterogeneity in the tumors have been proposed as major causes for the failure of drug treatment [38,39]. This study applied NGS to understand the landscape of somatic mutations of different subtypes of BC, identify the clinically actionable mutations and analyse the association between pathological clinical features and gene profiling data.

The results revealed that in general, TP53 and PIK3CA are the most frequently mutated genes in BC in both early and advanced stage BC. Genetic aberrations were identified in PI3KCA/AKT/PTEN signalling pathway in substantial fraction (57.8%) in Indian BC patients. PI3KCA was found to be altered in 42% cases whereas deletion in pTEN was found in 12.8% and mutation in AKT in 3% of BC patients. However, the somatic mutation frequency of these genes reported by TCGA was 35.5%, 3.2% and 2.4% respectively [31]. Overall, the mutation frequency of these genes is higher in Indian patients thus offering important biomarkers for targeted therapy in metastatic setting. The study also revealed that mutations in PI3KCA/AKT/PTEN signalling pathway to be significantly higher in HR + ve BC compared to HR–ve cohort and was altered in 18% of TNBC. It was further reported that mutations in PI3KCA/AKT/PTEN signalling pathway was significantly higher in HR + ve, HER2-ive BC. These findings are consistent with a study by Kalinsky et al. which showed PIK3CA mutations were more in HER2 − tumors than HER2 + tumors (61.5% vs. 38.5%, respectively) and patients with HR + tumors were shown to have higher likelihood of having a PIK3CA mutation than those with HR − tumors. However, no statistical significance was shown in this study [40].

The impact of these mutations on the outcome of BC is still not clear. It has been reported in some studies that early hormone receptor positive (HR + ive) /HER2-ive breast cancer with PIK3CA mutation is associated with a better recurrence-free survival [41] and a better disease-free survival (DFS) [42] whereas recent molecular profiling data from metastatic BC patients indicate that in advanced HR + /HER2- BC, a PIK3CA mutation would lead to a certain resistance to chemotherapy and a poor outcome [43]. Another important finding from preclinical study shows that PI3K/AKT/mTOR pathway plays a potential role in secondary endocrine resistance in HR + BC [44]. Also, there have been major advances in BC therapy with the discovery that certain hormone receptor positive (HR +) BC's which become resistant to endocrine therapy can be treated with modified rapamycin [45]. The findings have a potential clinical implication as it led to US Food and Drug Administration (FDA) approval of everolimus (AFINITOR®) in 2012, a rapamycin derivative that inhibits PI3K/ATK/mTOR signalling in combination with the aromatase inhibitor exemestane to treat HR + , HER2 − BC after other treatments have failed and have been shown to increase progression-free survival [46,47]. However, it is noteworthy that early pan-PI3K inhibitors have been shown to target this pathway but the use of such inhibitors is limited due to side-effects. Recent SOLAR-1 trial leading to FDA approval of the first PI3K inhibitor, alpelisib in combination with Fulvestrant, has revolutionized BC therapy for postmenopausal women and men with HR + /HER2-, PIK3CA mutated advanced or metastatic BC's which have progressed on or after endocrine therapy. Combination of alpelisib with fulvestrant showed that the specificity of alpelisib against the p110α catalytic isoform provided additional efficacy and a better toxicity profile [48]. The finding is very important in our cohort which was dominated by HR + ive cases and HR + ive HER2-ive patients with alteration in PI3KCA/AKT/PTEN as common genetic event. Based on the genetic report, combination therapy of everolimus with aromatase inhibitor exemestane has been initiated in 5 postmenopausal patients with HR + ive HER2-ive tumors and PI3KCA mutation which had progressed on other therapies. All these 5 patients were diagnosed in late 2017 and are under constant follow up since the change of therapy based on the genomic findings. Till now, all 5 patients have stable disease with no further progression.

Activation of the PI3K/ATK/mTOR pathway has been shown to confer resistance to trastuzumab treatment in HER2 + breast tumors [49,50] thus offering a potential prognostic value. Previous reports have shown that in early BC patients, mutation in PIK3CA was associated with a reduced pCR rate on treatment with combination of NACT and anti HER2 therapy [51]. However, several studies in advanced BC patients with PIK3CA mutation have reported worst prognosis without any significant predictive benefit to different anti- HER2 agents [52]. In our cohort of 35 cases, PIK3CA mutation correlated with a lower pCR rate than that wild-type specimens in post-NACT specimens. Majority of these patients were HER2 + ve and had undergone NACT regimen that included trastuzumab. Therefore, identifying these mutations in HER + BC patients may serve as a potential tool to stratify patients as responders and non- responders of anti Her2 therapy. This data clearly establishes the fact that although genetic testing is comparatively costly than conventional diagnostic tests like Her2testing by IHC and/or FISH, but there is a huge cost benefit if patients are stratified into responders and non-responders of anti Her2 therapy in improving the clinical outcome. About 12 patients with PIK3CA mutation who had a lower pCR than that of wild-type specimens in post-NACT specimens were started on combination of trastuzumab and everolimus in adjuvant setting and are on follow up.

In TNBC, activating PIK3CA mutations was found to be the second most frequent molecular alteration after TP53 mutations and our study revealed PI3KCA/AKT/PTEN signalling pathway was significantly altered in 18% of TNBC.

Since TNBC is characterised by poor outcomes and a lack of targeted therapies, alteration of PI3KCA/AKT/PTEN signalling pathway opens up a new avenue of using PI3K inhibitors, either as combination of PI3K inhibitors with PARP inhibitors [53], immune checkpoint inhibitors [54] and chemotherapy [55].

While the study of Kalinsky found overall patient survival (OS) to be significantly improved in patients with PIK3CA mutations [40], in a short follow up study, we found that patients with PIK3CA mutations collectively had a roughly equal DFS time to patients with WT PIK3CA. Since the cohort

of patients for whom we had clinical follow up was small, more patients have been enrolled in the investigator initiated trials and are being followed up for OS.

PIK3CA mutations are an early event in BC. In our study, we found mutations in this gene at all stages and all BC subtypes. While previous research has found PIK3CA mutations to be associated with older patients [37], we did not find significant differences between age and PIK3CA mutations among all patients. No correlation was found with stage and Ki-67 index of the tumor also. Notably 67% of BC cases presented with liver metastases at the time of diagnosis were detected with PIK3CA mutation indicating its role as a surrogate marker of organ specific metastasis. Similar findings have been reported in a recent study where PI3KCA mutations have been frequently observed in BC with liver metastasis (LM) and persist along with the recurrence [25]. This finding can be very useful in serial monitoring of the clinical response by identifying the PI3KCA mutations in circulating tumor cells (ctDNA) through "liquid biopsy" of the patient and can serve as a useful biomarker in the routine practice of BC management to prevent tumor recurrence and overcome the problems of intra- and inter-tumoral heterogeneity.

Disruptive and non-disruptive mutations in TP53 alone and co-mutated with other genes were found in significant cohort of BC patients. The prevalence of P53 mutation had been found to be significantly higher in Indian patients compared to what has been reported in TCGA data [31]. Previous in vivo study has confirmed that TP53 and PIK3CA mutation show cooperation in mammary tumor formation in mice [50]. It has been reported in numerous studies that TP53 mutations are a negative prognostic factor and are more likely to be aggressive particularly in HER2 negative and TNBC subtypes rendering resistance to chemotherapy and radiotherapy [56]. It has been reported that TP53-PIK3CA co-mutation carriers had worst disease-free survival comparing with non-mutation carriers, PIK3CA-mutation-only or TP53-mutation-only carriers [57]. Since a high frequency of TP53-PIK3CA co-mutations was detected in our cohort, this mutation pattern needs to be evaluated closely in clinical settings for Indian breast cancer patients in the future. Also, in our cohort, TP53 mutation carriers had a significant higher proportion of patients to be TNBC. A follow up of few cases showed shorter disease-free survival (DFS) and poor outcome in resected BC treated with NACT, indicating its robust prognostic value in NACT setting.

It is also noteworthy that 6 patients in the cohort had mutation in ERBB2(HER2) gene. Out of 6 patients, 2 patients were TNBC, 3 patients were TPBC, one patient was HR-ive/HER2 + ive. All the patients were early stage BC except one of the TNBC cases which had metastasized to pancreas. This is an important finding for clinical management of these cancers. It has been reported that amplification and mutation of HER2 are generally mutually exclusive occurrences in treatment-naïve patients [58]. Cocco et al. also found that there is only a small fraction of either treatment-naïve and/or pre-treated patients with advanced and metastatic BC in which HER2 amplification and mutation were found to concurrent [58]. It has been also reported in preclinical studies that the co-amplified/mutant cells were resistant to HER2-specific and HER2/EGFR-specific inhibitors but were sensitive to the new pan-EGFR inhibitor neratinib. In the cohort of Indian patients, 4 cases had both amplification and mutation of HER2 and were benefitted by neratinib. All of them have stable disease and are on constant follow up. However, the sample size is too small to present the statistical analysis. Previous reports have also demonstrated that HER2 mutations in BC's without HER2 gene amplification may respond to HER2-targeted therapy. This finding has enormous clinical and therapeutic implication for TNBC and HER2-ive patients of using anti Her2 therapy or pan-EGFR inhibitor neratinib [59].

Conclusions

This study provides a comprehensive mutation profile of Indian BC patients and has potential implication in clinical management. However, a major limitation of this study is the relatively small sample size as the referral for genetic testing was very less and only 50% of total BC patients counselled opted for genetic testing within the study period. The reasons of less referral and low adoption of these tests in clinical practice could be manifold. Comparatively higher cost of genetic tests than other conventional diagnostics tests (IHC, FISH), limited awareness and accessibility of these tests are the major hindrances. The clinical utility of the test findings and implementation of genome based personalized therapy is limited due to lack of understanding of etiological and intratumor heterogeneity, characterization of drivers from passenger mutations, lack of sustained response to drugs, and acquired resistance to targeted therapies.

Over the last few years, we have put intensive efforts to develop and improve the advanced protocols of genetic testing which has significantly reduced the cost and turnaround time of these tests, raised awareness on the clinical utility of genetic testing by deploying clinical geneticist to interpret and discuss the clinical utility of the results. The outcomes of the current study have not only reinforced the utility of genetic testing in clinic but also helped to add more data of clinical relevance by identifying hotspot mutations as valuable tool for correct diagnosis, prognosis and clinical intervention. The findings also confirm the utility of multigene profiling in Indian cohort of BC patients, both early diagnosed and advanced cases to identify markers/signature which will help to stratify them based on their molecular profile who could potentially benefit from targeted therapy. This approach will reduce treatment cost and improve clinical outcome. This has a huge impact on the healthcare cost in emerging economic country like India where availability and access to cancer drugs, rational combination therapies, and enriched clinical trials have been the additional challenges in adopting Genomic medicine.

The current study has also enabled the researchers and laboratory to adopt a collaborative approach with the clinicians to initiate prospective studies to confirm the independent prognostic and therapeutic value of the mutations in a larger cohort of Indian population and design randomized, genome specific, next generation clinical trials with a deliverable milestone to develop a large scale Indian population-specific cancer database to achieve better therapeutic and clinical outcome.

CRediT authorship contribution statement

Mithua Ghosh (Corresponding Author): Conceptualization, Methodology, Writing - Original Draft, Review & Editing Visualization, Investigation, Project administration, Supervision. **Radheshyam Naik**: Investigation, **Sheela Mysore Lingaraju**: Conceptualization, Methodology, Project administration. **Sridhar Papaiah Susheela**: Investigation, **Shekar Patil**: Investigation, **Gopinath Kodaganur Srinivasachar**: Investigation **Satheesh Chiradoni Thungappa**: Investigation, **Krithika Murugan**: Investigation, **Srinivas Belagutty Jayappa**: Investigation, **Somorat Bhattacharjee**: Investigation, **Nalini Rao**: Investigation **Mahesh Bandimegal**: Investigation, **Roopesh Krishnappa**: Investigation, **Shashidhara Haragadde Poppareddy**: Investigation, **Krishna Chennagiri Raghavendrachar**: Methodology, Validation, **Yogesh Shivakumar**: Methodology, Validation, **Sunitha Nagesh**: Methodology (Genetic Counselling), **Ramya Kodandapani**: Methodology, Validation, **Ashwini Rajan**: Methodology, Validation, **Urvashi Bahadur**: Soft Ware, Formal Validation, Investigation, **Pooja Agrawal**: Methodology, Validation, **Veena Ramaswamy**: Methodology, **Tejaswini Bangalore Nanjaiah**: Methodology, **Sateesh Kunigal**: Methodology, **Shanmukh Katragadda:** Soft Ware, Formal analysis, Data Curation, **Ashwini Manjunath**: Soft Ware, Formal analysis, Data Curation, **Amritanshu Ram**: Soft Ware, Formal analysis, Data Curation. **Basavalinga S Ajaikumar**: Conceptualization, Investigation, Resources.

Ethical approval

EC Name: HCG Central Ethics Committee.

EC Registration No: ECR/386/Inst/KA/2013/RR-19.

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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