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ORIGINAL ARTICLE

Genomic alterations associated with *HER2*+ breast cancer risk and clinical outcome in response to trastuzumab

Heena Singla[1] · Raman Preet Kaur[1] · Gowhar Shafi[2] · Rajesh Vashistha[3] · Raja Paramjeet Singh Banipal[4] · Vinod Kumar[5,7] · Anjana Munshi[1,6]

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Abstract

Human epidermal growth factor receptor 2 positive (*HER2*+) breast cancer (BC) is an aggressive BC subtype characterized by *HER2* overexpression/amplification. Genomic alterations of *HER2* and others have been reported to be associated with, *HER2* overexpression and prediction of trastuzumab-response. Here, we aimed at identifying germline and somatic alterations associated with *HER2*+ BC and evaluating their association with clinical outcome in response to trastuzumab therapy given to *HER2*+ BC patients. Global Sequencing Array (GSA) and polymerase chain reaction-restriction length polymorphism (PCR-RFLP) techniques were used to determine alterations in *HER2* and other *HER2*-interacting as well as signaling-related genes in *HER2*+ BC. In addition, 20 formalin fixed paraffin-embedded tissue samples were also evaluated by GSA for identifying significant variations associated with HER + BC as well as response to trastuzumab therapy. A germline variant in *HER2* (I655V) was found to be significantly associated with the risk of the disease ($p < 0.01$). A nonsense mutation in *PTPN11* (K99X), a pathogenic *CCND1* splice site variant (P241P), a hotspot missense mutation in *PIK3CA* (E542K) and a hotspot missense mutation in *TP53* (R249S); were observed in 25%, 75%, 30% and 40% of the *HER2*+ BC tissue samples, respectively. Mutant *CCND1* (P241P) and *PIK3CA* (E542K) were found to be significantly associated with reduced disease-free survival (DFS) in patients treated with trastuzumab (p: 0.018 and 0.005, respectively). These results indicate that *HER2, PTPN11, CCND1* and *PIK3CA* genes are important biomarkers in *HER2*+ BC. Moreover, the patients harboring mutant *CCND1* and *PIK3CA* exhibit a poorer clinical outcome as compared to those carrying wild-type *CCND1* and *PIK3CA*.

Keywords *HER2* positive breast cancer · Genomic alterations · *PIK3CA* · Clinical outcome · Trastuzumab

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✉ Vinod Kumar
vpathania18@gmail.com

✉ Anjana Munshi
anjanadurani@yahoo.co.in

[1] Department of Human Genetics and Molecular Medicine, Central University of Punjab, Bathinda, India

[2] Department of Clinical Genomics and Bioinformatics, Positive Bioscience, Mumbai, India

[3] Max Super-Speciality Hospital, Bathinda, India

[4] Guru Gobind Singh Medical College and Hospital, Faridkot, India

[5] Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, India

[6] Centre for Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab, Bathinda, Punjab, India

[7] Centre for Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, Punjab, India

Introduction

Breast cancer (BC) is a heterogeneous disease. Apart from biological characteristics, the clinical behavior of BC also varies in accordance with the expression levels of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (*HER2*). BCs that positively express ER and/or PR are classified as hormone-receptor positive, whereas BCs showing *HER2* overexpression/amplification are classified as *HER2* positive (*HER2*+). On the contrary, BCs that fail to express all the three receptors- ER, PR and *HER2* are termed as triple-negative BCs. *HER2*+ BCs have the tendency to grow and spread with more aggression, relative to other BC subtypes [1]. These cancers constitute 15–30% of total BC cases and are often accompanied by metastasis and poor clinical outcome [2, 3]. *HER2*/ErBb2/neu gene occupying human chromosomal locus 17q12-21.32 encodes *HER2*/ErBb2/neu tyrosine kinase protein, which is a member of epidermal growth factor receptor (EGFR) family. *HER2*+ status implies overexpression of *HER2* protein and/or amplification of the *HER2* gene. *HER2* overexpression/amplification causes enhanced *HER2* homo-/hetero-dimerisation which, in turn, stimulates a variety of cell-signaling pathways including RAS/MAPK, PI3K/Akt and JAK/STAT pathways. Activation of these pathways elicits enhanced cell-proliferation, reduced apoptosis eventually transforming the cells towards oncogenesis [2]. Genomic alterations play significant roles in conferring risk of increased *HER2*+ BC susceptibility. Some germline variants of *HER2*, as well as other genes CYP19A1, CYP1A1*2, CYP17, ESR1, KRAS, VEGFA, BRCA1, MACC1, SULT1A1, Cdx2 VDR, and CCR2, are reported to be associated with *HER2*+ BC susceptibility [4].

Trastuzumab is a monoclonal antibody targeting *HER2* tyrosine kinase receptor and the most-commonly recommended effective first-line treatment for *HER2*+ BC. However, resistance towards the therapy remains the major issue [5]. The genetic make-up of the *HER2*+ patients has been cited as an important factor towards this differential response towards treatment. Alterations in genes involved in the PI3K/Akt pathway and some other genes have been associated with trastuzumab resistance [4].

The current study was carried out with an aim to identify germline and somatic alterations in *HER2*+ BC patients from the Malwa region of Punjab, where BC is widely feared. In addition, we also evaluated the association of significant genomic alterations with clinical outcome in *HER2*+ BC patients on trastuzumab therapy.

Materials and methods

Collection of samples and clinical data

109 BC patients (90 confirmed *HER2*+ and 19 equivocal), evaluated by IHC at Max Super-speciality Hospital, Bathinda and Guru Gobind Singh Medical College and Hospital, Faridkot, Punjab, the major referral hospitals for Malwa region of Punjab were included in the study. Equivocal cases were further subjected to FISH using PathVysion *HER2* DNA Probe Kit II (Abbott Laboratories, USA) according to the manufacturer's recommendations. *HER2* status was assigned as per ASCO-CAP 2013 guidelines [6]. 10 of the 19 equivocal cases were detected *HER2* amplified as per FISH analysis and the remaining 9 patients designated negative by FISH were excluded from the study. 70 of the study cases (n = 100) were treated with trastuzumab therapy. 100 healthy controls with no personal/ family history of BC or any other types of cancer from the same geographical setup were included in the study. The study was approved by institutional ethics committees of the Central University of Punjab. 5 ml of blood was collected in EDTA vacutainers from all *HER2*+ BC patients (n = 100) and controls (n = 100) with their written informed consent. On the other hand, formalin-fixed paraffin-embedded (FFPE) tissue samples were collected for 20 *HER2*+ BC patients to whom trastuzumab treatment was given. However, matched tumor controls couldn't be collected on account of some ethical issues (Suppl. Figure 1). Information on demographic features and risk factors was collected using a structured questionnaire.

DNA isolation and genotyping

DNA was extracted from blood and tissue samples using the phenol–chloroform method. DNA concentration and purity were determined using a NanoDrop ND-1000 UV–Vis Spectrophotometer (Thermo Scientific). The integrity of DNA samples was validated by electrophoresis. Genotyping was performed on Illumina Infinium HD assay platform using Global Screening Array (GSA) microchip (Illumina Inc.) with 200 ng of genomic DNA as per manufacturer's instructions. DNA collected from blood samples of 60 patients and 20 controls were subjected to GSA. In addition, 20 tissue samples of the patients who were on trastuzumab therapy were also analyzed by GSA. GSA microchip was chosen for study due to the presence of updated markers (more than 700,000) optimized for human genome-wide backbone for unparalleled genomic coverage, including clinically relevant content and pharm GKB markers. Subsequent sample processing and array hybridization were performed according to the manufacturer's instructions (Illumina, Inc.).

The most common significant germline variant of *HER2* (I655V) which emerged from GSA analysis was further validated by polymerase chain reaction and restriction fragment length polymorphism in all patient (n = 100) and control (n = 100) samples including the remaining samples which couldn't be analyzed by GSA on account of financial constraints, as per the protocol described by Pinto et al. using *BsmA1* restriction enzyme that digests the 148 bp PCR product into 116 and 32 bp fragments [7].

Follow-up

Follow-up of the patients treated with trastuzumab was done at an interval of 3, 6, 9, 12, 15, 18, 21, 24 and 27 months from the last administered cycle of trastuzumab, telephonically and with the help of clinician during their visits to the hospitals.

Statistical analysis

GenomeStudio (Illumina, Inc.) was used for data pre-processing and analysis. Genotypes were called within GenomeStudio with the GenCall algorithm of Genotyping Module v1.0. The final sample call rate was 99.99%. The data was subsequently exported to R/Bioconductor to calculate X^2 and odds ratio. The results were annotated using databases including ClinVar, 1000 Genomes, ExAC, Cosmic and dbSNP. A p-value $\leq 5 \times 10^{-8}$ was considered statistically significant while analyzing GSA data.

Association of the disease and the *HER2* (I655V) with various demographic/clinico-pathological features was estimated by odds risk ratio with 95% confidence interval (CI) and χ^2 analysis using Open Epi software (Open Epi version 2.3.1 from Department of Epidemiology, Rollins School of public health, Emory University, Atlanta, GA 30322, USA). Kaplan–Meier method and log-rank test were performed using SPSS (Chicago, IL, USA) to determine the association of gene variants with disease-free survival of patients in response to trastuzumab treatment. A p-value ≤ 0.05 was considered significant, here.

Results

Demographic profile

100 patients with clinically confirmed *HER2*+ status from the Malwa region of Punjab were included in the study. Mean age at the time of diagnosis was 53.23 ± 12.1. Mean age at the time of menarche and first pregnancy was 14.92 ± 1.32 and 22.05 ± 2.42, respectively. None of the patients in the study cohort was pregnant at the time of diagnosis. 52% (52/100) of patients were from the rural background and 48% (48/100) were residing in the urban area. Postmenopausal *HER2*+ BC was observed in 46% (46/100) of females.

53% (53/100) of the study subjects were obese and 42% (42/100) displayed normal BMI with the remaining 5% (5/100) being underweight. 8% (8/100) of patients displayed the family history of cancers especially breast. Since Punjab is a leading grain producer in India with maximum use of pesticides, around 43% (43/100) patients had an exposure to pesticides. None of the patient characteristics was found to be significantly associated with *HER2*+ BC ($p > 0.05$, Table 1).

Death, recurrence and metastasis were observed in 15.71% (11/70), 11.43% (8/70) 22.86% (16/70) of the patients on trastuzumab therapy. However, in 20 patients whose tissue samples were also analyzed 15% (3/20), 10% (2/20) and 20% (4/20) of the patients suffered from death, recurrence and metastasis, respectively.

Evaluation of germline genomic alterations

Genes namely *HER2, EGFR, ESR1, KRAS, VEGFA, TP53, PIK3CA, IL6, CYP2C19, CYPA452, CYP3A5, CYP2A7P1, CYP2B6, CYP3A4, CYP2C9, CYP2C8, CYP4A2, CYP19A1, CYP4B1, CYP2D6, CYP7A1, CYP2E1, CYP3A5, CYP2A6, CYP1A2* and *CYP11B2* were screened by GSA in *HER2*+ BC

Table 1 Comparison of demographic characteristics between *HER2*+ BC patients and healthy controls

Characteristics	Patients	Controls	p
Age at first pregnancy (years)			
≥ 25	22	39	> 0.05
< 25	78	61	
Age at menarche (years)			
< 14	35	42	> 0.05
≥ 14	65	58	
Menopausal status			
Premenopausal	39	45	> 0.05
Postmenopausal +	46	51	
Hysterectomy cases	15	4	
Locality			
Urban	48	42	> 0.05
Rural	52	58	
Obesity			
Yes	53	61	> 0.05
No	47	39	
BC family history			
Yes	5	3	> 0.05
No	95	97	
Pesticide exposure			
Yes	43	47	> 0.05
No	57	53	

patients and age-matched healthy controls. A germline polymorphism in *HER2* (I655V) was found to be significantly associated with *HER2*+ BC ($p < 0.01$; Table 2), also confirmed by PCR-RFLP. These results indicate Val allele to be significantly associated with *HER2*+ BC.

Further, *HER2* I655V was found to be significantly associated with pesticide exposure ($p < 0.05$; Table 3). However, no significant association was found between *HER2* I655V genotype and other clinico-pathological features of *HER2*+ BC (Table 3).

Evaluation of tissue-specific alterations

A PTPN1 nonsense mutation (K99X), a pathogenic *CCND1* splice site variant (P241P), a hotspot missense mutation in *PIK3CA* (E542K) and a hotspot missense mutation in *TP53* (R249S) were observed in 25%, 75%, 30% and 40% of patients, respectively.

CCND1 (P241P) and *PIK3CA* (E542K) were found to be significantly associated with poor clinical outcome in these patients (p: 0.018 and 0.005, respectively). A significantly lower DFS was observed in *HER2*+ BC patients treated with trastuzumab, who harbored mutant *CCND1* and/or *PIK3CA* than those carrying wild-type *CCND1* and/or *PIK3CA* (Figs. 1, 2). However, *PTPN11* (K99X) and *TP53* (R249S) were not found to be significantly associated with clinical outcome in *HER2*+ BC patients ($p > 0.05$).

Although, the germline variant I655V of *HER2* was also evaluated in association with outcome in patients on trastuzumab therapy. However, no significant association was observed.

Discussion

The present study was carried out with an aim to understand the burden of pathogenic genomic alterations in *HER2*+ BC. To the best of our knowledge, this is the first study from India evaluating the association of genomic alterations with *HER2*+ BC. *HER2*+ BC constitutes about 20% of total BC cases from the Malwa region of Punjab, where BC is widely threatening. Department of Health & Family Welfare in 2013 reported higher cancer prevalence (1089/million/year) in Malwa, as compared with other two regions of the state Majha (647/million/year) and Doaba (881/million/year) [8].

Table 3 Association of *HER2* I655V polymorphism with clinico-pathological features of *HER2*+ BC patients

Characteristics	AA (Ile/Ile)	AG + GG (Ile/Val + Val/Val)	p
Age at diagnosis			
≤ 45 years (n = 45)	23 (51.11%)	22 (48.89%)	> 0.05
> 45 years (n = 55)	37 (67.27%)	18 (37.73%)	
Age at first pregnancy			
≥ 25 years (n = 22)	12 (54.55%)	10 (45.55%)	> 0.05
< 25 years (n = 78)	48 (61.54%)	30 (38.46%)	
Age at menarche			
≥ 14 years (n = 35)	24 (68.57%)	11 (31.43%)	> 0.05
< 14 years (n = 65)	36 (55.38%)	29 (44.62%)	
Menopausal status			
Premenopausal (n = 39)	25 (64.1%)	14 (35.9%)	> 0.05
Postmenopausal (n = 46) + Hysterectomy cases (n = 15)	35 (57.38%)	26 (42.62%)	
BC family history			
Yes (n = 5)	3 (60%)	2 (40%)	> 0.05
No (n = 95)	57 (60%)	38 (40%)	
Obesity			
Yes (n = 53)	32 (60.38%)	21 (39.62%)	> 0.05
No (n = 47)	28 (59.57%)	19 (40.43%)	
Pesticide exposure			
Yes (n = 43)	18 (41.86%)	25 (58.14%)	< 0.01
No (n = 57)	42 (73.68%)	15 (26.32%)	

Germline variants

HER2 (I655V) is the common *HER2* variant that has been detected in blood as well as tumor tissues of BC [4, 9–11]. I655V, the germline variant of *HER2* gene alters the conformation thereby rendering the receptor continuously active, promoting *HER2* homo-dimerization and tyrosine kinase signaling [12]. The role of Val allele in enhanced cell proliferation is also supported by in vitro experiments that demonstrated that *HER2*/Val-expressing cells possess a higher growth capacity and lower apoptosis as compared to *HER2*/Ile-expressing cells, and tumor formation was only seen among *HER2*/Val-expressing nude mice [13]. Many studies have established a significant association of *HER2* (I655V) with BC susceptibility [4, 14, 15], whereas others have failed [16, 17]. Han et al. found that *HER2* (I655V) is an important prognostic marker in *HER2*+ BC that contributes to

Table 2 Distribution of *HER2* I655V polymorphism in *HER2*+ BC patients

	AA (Ile/Ile)	AG (Ile/Val)	GG (Val/Val)	p (GG vs AA)	p (GG + AG vs AA)	A (Ile)	G (Val)	p (G vs A)
Patients	60	11	29	< 0.01	< 0.01	131 (65.5%)	69 (34.5%)	< 0.01
Controls	87	7	6			181 (90.5%)	19 (9.5%)	

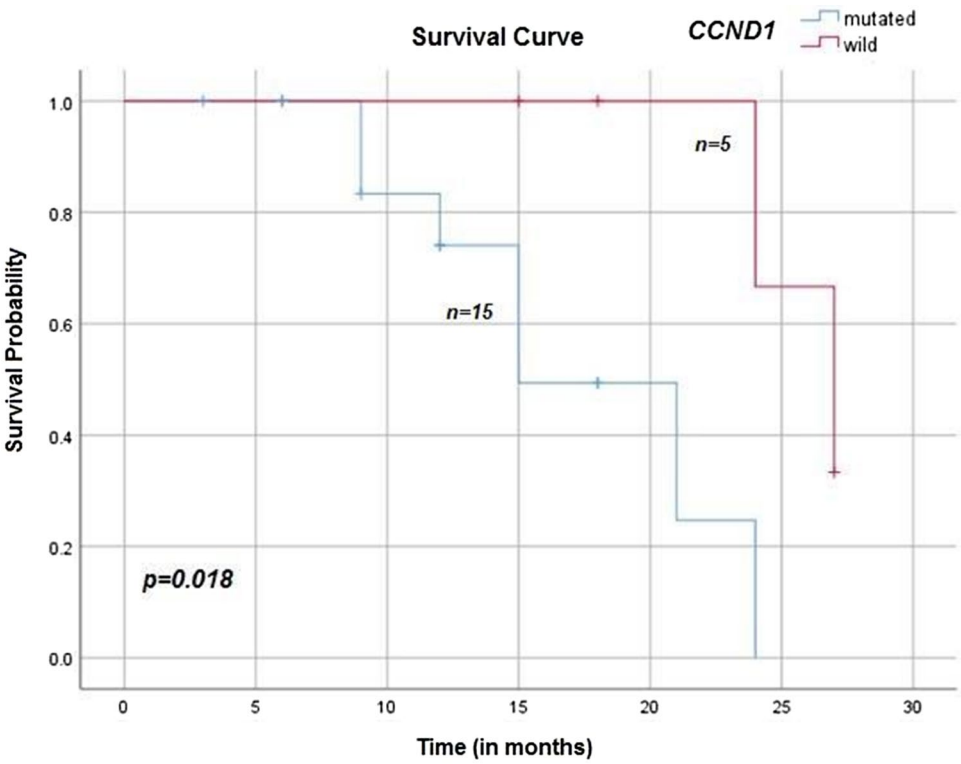

Fig. 1 Kaplan–Meier plot showing DFS according to *CCND1* (P241P) mutation status in *HER2*+ BC patients treated with trastuzumab

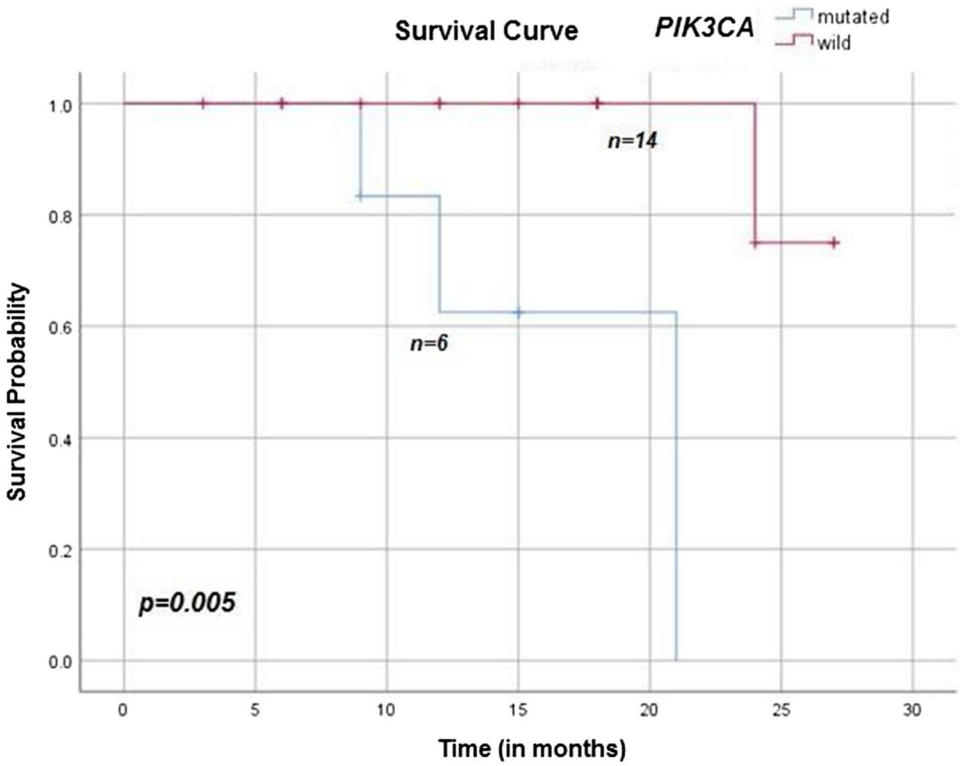

Fig. 2 Kaplan–Meier plot showing DFS according to *PIK3CA* (E542K) mutation status in *HER2*+ BC patients treated with trastuzumab

more aggressive phenotype. However, Han and colleagues didn't report a significant association of this variant with *HER2*+ BC [18]. We identified a significant association of I655V variant with *HER2*+ BC. Malwa region of Punjab is an area where pesticide usage is relatively higher compared to the other regions. It has been reported that people with

higher pesticide exposure are at a greater risk of developing gene mutations as compared to those with lesser pesticide exposure [19]. Cancer is a multi-factorial disease, where both genetic aberrations and environmental factors act in a synergistic manner to promote the disease [20, 21]. There are no reports of the association between pesticide exposure and development of *HER2*+ BC, till date. However, *HER2* I655V was found to be significantly associated with pesticide exposure in the current study. This suggests that patients carrying *HER2* I655V and exposed to pesticides are at a higher risk of developing the disease.

Tissue-specific variants

As far as the tissue-specific variants are concerned, *PTPN11, CCND1, PIK3CA*, and *TP53* were found to be mutated in the study group. *PTPN11* has been known to dephosphorylate *HER2*, hence repressing *HER2* signal transduction [22, 23]. *PTPN11* nonsense mutation (K99X) resulting in non-functional protein product, was found in 25% of patients. This non-functional protein may hamper *HER2* repressing activities as a result of the inhibition of *HER2* dephosphorylation, thus, aggravating the effects of *HER2*+ BC.

PI3K/AKT is a major signaling event, triggered upon *HER2* dimerization. Activated Akt in PI3K/Akt signaling cascade leads to increased cell proliferation by transcriptional factor regulation and enhancement of *CCND1* levels [23]. In the current study, we found *CCND1* (P241P) alteration in 75% of patients. *CCND1* (P241P) has been reported to confer an enhanced risk for BC, previously [24, 25]. P241P is a synonymous variant at amino acid position 241, in exon 4 splicing site that causes alternate splicing of *CCND1* mRNA into transcript-a and transcript-b. The wild-type allele mainly encodes transcript-a, whereas variant allele codes for transcript-b. As transcript-b encoded by the variant has a prolonged half-life, it may lead to enhanced *CCND1* protein levels [26]. Overexpression of *CCND1* permits early G1/S transition in cell-cycle causing abnormal cell proliferation [27].

PIK3CA encodes for p110α, the catalytic component of the PI3K/Akt pathway. *PIK3CA* is found to be frequently mutated in breast cancers [28]. The current study identified *PIK3CA* (E542K) mutation in 30% of the cases. Kalsi et al. reported that E542K mutation in *PIK3CA* significantly alters the conformational behavior of interacting residues, thus preventing the participation of these residues in protein–protein interaction. The regulatory control of p85α (PIK3R1) over p110α is lost on account of deprivation of interaction between p110α and p85α, prolonging the activity of catalytic p110α subunit. Prolonged p110α activity is accounted as one of the main reasons for uncontrolled cell division [29].

TP53 is a tumor-suppressor gene that has been found to be frequently mutated in *HER2*+ BCs [30]. *TP53* (R249S) was found to be mutated in 40% of the patients in our study. *TP53* R249S is a hotspot and gain-of-function mutation promoting tumourigenic events. This *TP53* mutation is related to the amplification of cell-cycle regulatory proteins such as *CCND1* and CDK4. *TP53* mutated at R249S, acts as a substrate of CDK4/*CCND1* in the G1/S phase of cell cycle. Liao et al. found that upon phosphorylation at Ser 249 by CDK4/*CCND1, TP53* promotes cell-proliferation *via* c-Myc activation [31].

A poor clinical outcome in response to trastuzumab-treatment has been reported in many studies and various genomic alterations have been found to play a significant role in conferring poor response in *HER2*+ BC patients treated with trastuzumab [4]. Mutated *CCND1* and *PIK3CA* were found to be significantly associated with poor clinical outcome in *HER2*+ BC cohort treated with trastuzumab in the current study. Patients harboring mutant *CCND1* (P241P) exhibited reduced DFS as compared to the patients who carried wild-type *CCND1* (P241P). *CCND1* (P241P) is associated with *CCND1* overexpression and the latter has been reported to be associated with poor clinical response in patients on trastuzumab therapy [32].

PI3K/Akt pathway has been found to be frequently mutated in trastuzumab-resistant breast cancer [33]. Baslega et al. reported the association of mutant *PIK3CA* with favorable outcome in BC patients, who didn't receive trastuzumab treatment. Hanker and colleagues reported that mutated *PIK3CA* expedites *HER2* driven mammary tumorigenesis [34]. The oncogenic *PIK3CA* mutations activate the PI3K pathway and have been reported to be associated with the poor response of BC patients to trastuzumab [35–39]. In conformity with previously published data, we found a significant association of mutant *PIK3CA* (E542K) with worse clinical outcome in *HER2*+ BC patients on trastuzumab treatment. Patients carrying *PIK3CA* (E542K) showed significantly decreased DFS in response to trastuzumab in the current study. E542K has been reported to be associated with enhanced PI3KCA kinase activity and thus, over-activation of PI3K/Akt pathway [40–42]. The functional implications of the significant genomic alterations observed in the present study have been demonstrated in Fig. 3.

The main drawback of the study was the availability of a very less number of tissue samples. In the current study, we focused to delineate *HER2*-interacting genomic alterations from *HER2*+ BC and impact on clinical outcome. Our results suggest that germline *HER2* (I655V) variant and tissue-specific mutations including *PTPN11* (K99X), *CCND1* (P241P), *PIK3CA* (E542K) and *TP53* (R249S) are associated with *HER2*+ BC. In addition, mutant *CCND1* and PI3KCA showed a significant association with reduced DFS in patients on trastuzumab therapy and the results of the present study need to be confirmed in a larger cohort. If confirmed, the patients might be advised to get screened

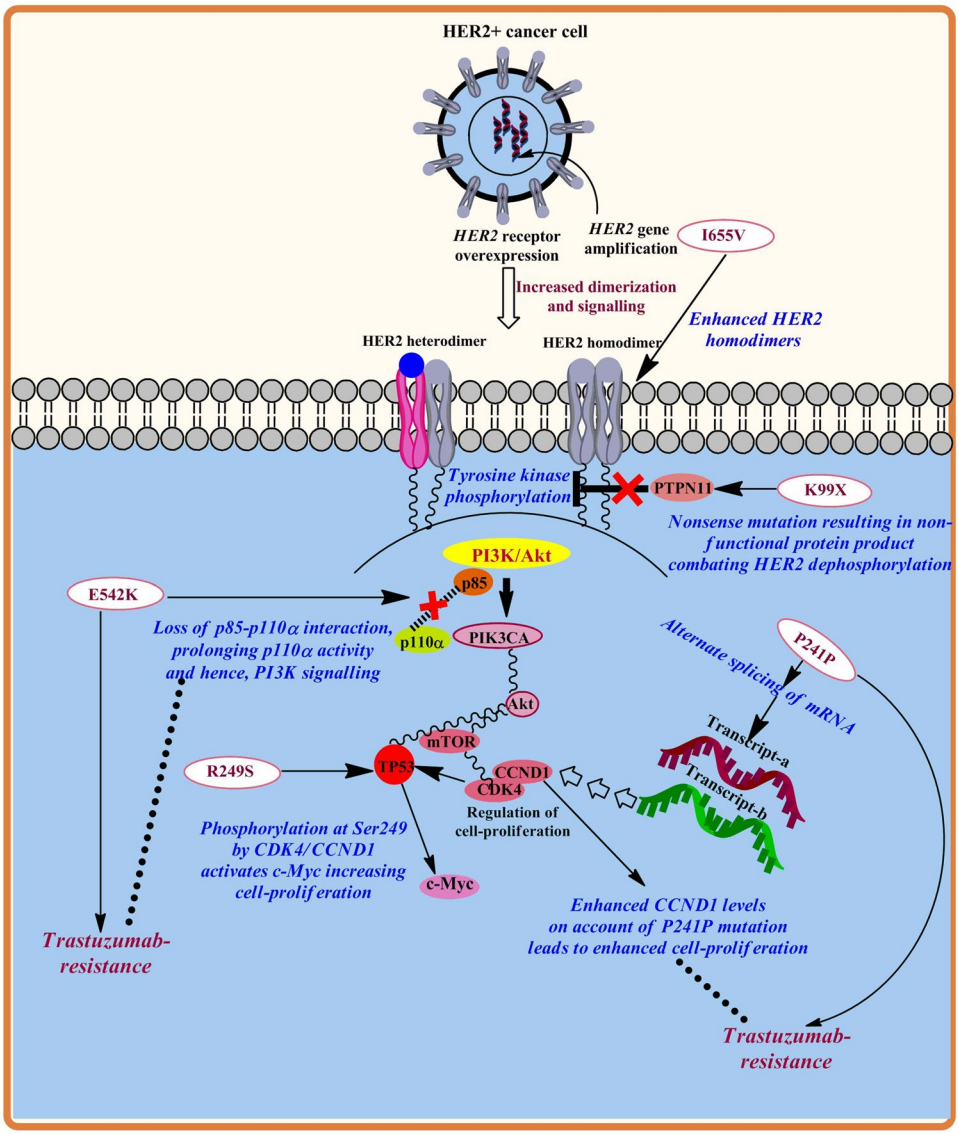

Fig. 3 Functional implications of genomic alterations in *HER2* signal transduction. *HER2* germline variant along with *PTPN11* nonsense mutation exponentially multiplies *HER2*-mediated cell signaling. *CCND1, PIK3CA*, and *TP53* mutations lead to uncontrolled cell division and hence, elevated tumourigenesis. Mutant *CCND1* and *PIK3CA* confer resistance towards trastuzumab therapy (refer text for details)

for *CCND1* and *PIK3CA* mutations before undergoing trastuzumab therapy.

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Author contributions HS and RPK are researchers working in the field of BC and have conducted all the study. GS has undertaken the analysis of GSA results and carried out the statistical analysis. AM along with VK, RV and RPSB designed the work and helped in the interpretation of results. All the authors contributed to manuscript writing and approved it.

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Compliance with ethical standards

Conflict of interest Authors declare that they do not hold any conflict of interest.

Ethical approval The authors declare that the Institutional Ethics Committee of the Central University of Punjab, Bathinda approved this study which was conducted in agreement with the recommendations of the Helsinki Declaration.

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

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