RESEARCH ARTICLE

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Targeted Sequencing of *HER2*-Positive Breast Cancer Mutations Revealed a Potential Association between *PIK3CA* and Trastuzumab Resistance

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Abstract

Background: Different molecular subtypes, including *HER2*-positive, have been identified in breast cancer. The overexpression of *HER2* triggers downstream signaling pathways such as the PI3K/AKT/mTOR pathway. Until recently, trastuzumab has been used as a single HER2-targeted therapy in Egypt. However, resistance to trastuzumab has been reported. Previous studies have demonstrated the genetic variants that affect the trastuzumab response. However in Egypt, few studies investigated molecular biomarkers such as p53 that might affect the trastuzumab response. Therefore, we aimed to extend the genetics workup of Her2 + BC to include important oncogenes and other vital cancer pathways. **Methods:** Formalin-fixed paraffin-embedded samples were collected from 24 *HER2*+ BC Egyptian patients, twelve patients in complete remission for 2 years or more from the start of trastuzumab and twelve resistant patients who relapsed or developed metastasis within 2 years from the start of trastuzumab. Somatic mutations in hotspot regions of 17 genes were further investigated using next-generation sequencing. **Results:** Among the total number of identified variants (106 variants), *PIK3CA* showed the most frequent variants, with more variants occurring in the resistant group than in the responsive group (P= 0.004). The frequency of *PIK3CA* mutations was greater in resistant patients than in responsive patients (P= 0.036). Additionally, there was a significant correlation between *PIK3CA* mutations and pathological complete response (pCR) (P=0.036). Most of *PIK3CA* variants in resistant patients were detected in exon 9 and 20. The *PIK3CA* variants His1047Tyr, Glu545Lys, His701Pro, Lys111Glu, Val344Gly and Tyr1021Cys were found only in the resistant patients, suggesting that they are associated with trastuzumab resistance. **Conclusion:** *PIK3CA* variants were more frequent in resistant HER2+ BC patients than in responsive patients, with a significant correlation between *PIK3CA* mutation and a lower pCR rate. *PIK3CA* variants within exon 9 and 20 (such as Glu545Lys and His1047Tyr respectively) were associated with trastuzumab resistance.

Keywords: *PIK3CA*- *HER2*-targeted therapy- NGS- genetic variants- trastuzumab resistance

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Introduction

HER2 overexpression occurs in around 20% of breast cancer (BC) cases [1]. Patients diagnosed with *HER2*+ BC exhibit an aggressive phenotype, early metastasis and chemoresistance [2]. It is thought that amplification of the *HER2* gene is associated with the proliferation and progression of certain aggressive breast cells. This effect results from signal transduction mediated by the activation of the PI3K/AKT, Ras/Raf/MEK/MAPK or other pathways, causing adverse biological characteristics, tumorigenesis and cancer spread [3, 4].

Advances in *HER2*-targeted therapies have improved outcomes in *HER2*+ BC such as neoadjuvant and adjuvant treatment and also in patients with metastatic disease [4-6]. There are many FDA-approved *HER2*-targeted therapies, e.g., trastuzumab, pertuzumab and lapatinib. Trastuzumab is the first FDA-approved *HER2*-targeted therapy for *HER2*+ BC [7]. Until the end of 2022, trastuzumab was used alone as a monoclonal antibody both as an adjuvant and neoadjuvant agent for stage 2 and stage 3 *HER2*+ BC patients. However, resistance to trastuzumab occurs in approximately 60–80% of patients when it is used as a single therapy or in 20–50% of patients when it is combined with chemotherapy [8].

The mechanism of resistance to trastuzumab is multifactorial [9] Accordingly, distinct genetic alterations associated with trastuzumab resistance, such as mutations in *Akt, PDK, PTEN, TP53, ATM, STK11*, and *RB1*, were investigated [10]. Many previous studies have

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been conducted to clarify the association between gene encoding phosphatidylinositol-4,5-bisphosphate 3 kinase catalytic subunit p110α (*PIK3CA*) and trastuzumab resistance in *HER2*+ BC patients [11-19].

To date, there are no reliable predictive markers for the response to *HER2*-targeted therapy worldwide and in Egypt [20]. Therefore, it is crucial to identify potential biomarkers to predict patients who may be responsive or resistant to *HER2*-targeted therapy to improve therapeutic effectiveness and avoid adverse drug [1, 21].

Next-generation sequencing allows simultaneous parallel sequencing of multiple genes. Targeted sequencing can be used to investigate hotspot mutations associated with cancer drivers in BC [22, 23]. Subsequently, this allows the identification of mutations that influence response to *HER2* targeted therapies and increasing the knowledge to the development of therapies targeting special genetic alterations towards personalized medicine [24].

Materials and Methods

This retrospective study included 24 *HER2*+ BC female patients older than 18 years who received trastuzumab combined with chemotherapy with no metastasis at initial time of diagnosis. Patients with metastasis at the time of diagnosis were excluded. The aim of this study was to detect somatic mutations that might be associated with the response of *HER2* targeted therapy (trastuzumab) using NGS in *HER2*+ BC patients. Ethical approval was obtained from the Alexandria Ethics Committee of the Faculty of Medicine. Patients were recruited from the Clinical Oncology and Nuclear Medicine Department at Alexandria Main University Hospital from December 2020 to September 2021. The drug protocol involved administering trastuzumab as a neoadjuvant agent for one month before surgery and continuing after surgery for one year. According to the response to trastuzumab, the twenty-four patients were divided into two groups: 12 patients in complete remission for 2 years or more from the start of trastuzumab treatment and 12 disease-resistant patients who relapsed or developed metastasis within 2 years from the start of trastuzumab treatment. *HER-2* status was determined using immunohistochemistry (IHC) and fluorescence in situ hybridization.

DNA extraction

Twenty four formalin-fixed paraffin-embedded (FFPE) tissue samples were collected. DNA was then extracted from FFPE tissue samples using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany). The concentration of DNA was determined by a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, USA).

Then, the extracted DNA was subjected to the following steps; DNA library preparation, clonal amplification, chip loading, sequencing and finally data Analysis.

Library preparation

Manual library preparations were conducted using an Ion AmpliSeq™ Cancer Hotspot Panel v.2, Ion Xpress barcoded adapters and an Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, Inc.). The panel consisted of approximately 800 COSMIC mutations from hotspot regions of 17 genes. The genes included in this study were *PIK3CA, HRAS, KRAS, NRAS, BRAF, CSF1R, PTPN11, PTEN, AKT1, SRC, ABL1, MPL, NOTCH1, JAK2, JAK3, CTNNB1* and *APC*.

Multiplex PCR was performed using 10 ng of genomic DNA with a premixed primer pool and Ion AmpliSeq HiFi master mix using the Ion AmpliSeq™ Library Kit Plus (Thermo Fisher Scientific, USA). Two microliters of FuPa reagent was added to the amplicons to partially digest the primer sequences and phosphorylate the amplicons. The ligation of barcode adaptors to the amplicons was performed using an Ion Xpress™ Barcode Adapters Kit (Thermo Fisher Scientific, USA). The adapter ligated amplicons (library) were purified using Agencourt™ AMPure™ XP Reagent (Beckman Coulter, USA). The quantification of the libraries was performed using an Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific, USA) on an Applied Biosystems® 7500 Real Time PCR System, according to the manufacturer's instructions, and the results were normalized to ~100 pM and then combined to form one library pool.

Clonal Amplification and chip loading

The clonal amplification of the barcoded DNA library onto ion sphere particles was performed on the Ion OneTouch™ 2 System (Thermo Fisher Scientific, USA) using the Ion 520™ and Ion 530™ Kit OT2 (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The enrichment of ISPs was performed using the Ion OneTouch™ ES instrument (Thermo Fisher Scientific, USA). The enriched, template-positive ISPs were then loaded on an Ion 520™ Chip (Thermo Fisher Scientific, USA) and sequenced using an Ion S5™ next generation sequencing system (Thermo Fisher Scientific, USA).

Bioinformatic analysis

Torrent Suite™ Software (Thermo Fisher Scientific, USA) was used to plan and monitor sequencing runs, view sequencer activity, analyze barcode reads, align reads to the hg19 reference genome and generate run metrics to determine the quality of the run. The samples were evaluated for genomic alterations, including single nucleotide variants (SNVs) and, insertions and deletions, using Ion Reporter™ Software (Thermo Fisher Scientific, USA). All genetic variants with a minimum depth of coverage of 30× were included in the study. The allelic frequency ranged from 1 to 10. The basic parameters of analysis included a minimum quality alignment threshold of 20 (corresponding to a 1% error rate) and a minimum sequencing depth of 300.

Results

The age of the patients ranged from 35 to 61 years. The histopathological subtype of BC in all patients was infiltrating ductal carcinoma. The demographic and clinicopathological data are summarized in Table 1.

Sequencing of patient samples revealed a total of

Table 1. Demographic and Clinicopathological Data of Both Studied Groups

Response to trastuzumab Baseline characteristics	Responsive (n = 12)	Resistant (n = 12)	p
Age (years)	49.42 ± 9.35	50.17 ± 8.31	0.837
Family history	1 (8.3%)	2 (16.7%)	^{FE}p =1.000
Menstrual history			
Menopausal	7 (58.3%)	8 (66.7%)	^{FE}p =1.000
Menstruating	5 (41.7%)	4 (33.3%)	
Parity			
Nullipara	1 (8.3%)	0 (0%)	^{MC}p= 0.221
Unipara	2 (16.7%)	0 (0%)	
Multipara	9 (75%)	12 (100%)	
Oral Contraceptive Pills	6 (50%)	7 (58.3%)	0.682
Hormone receptor status			
ER-,PR +,HER2+	1 (8.3%)	0 (0%)	^{MC}p= 0.521
ER-,PR-,HER2+	6 (50%)	8 (66.7%)	
ER+,PR-,HER2+	0 (0%)	1 (8.3%)	
ER+,PR+,HER2+	5 (41.7%)	3 (25%)	
TNM Stage			
I	2 (16.6%)	0 (0%)	^{MC}p= 0.365
II	6 (50%)	5 (41.6%)	
III	4 (33.3%)	7 (58.4%)	
IV	0 (0%)	0 (0%)	
Grade			
Grade I	0 (0%)	0 (0%)	^{MC}p= 1.000
Grade II	5 (41.7%)	6 (50%)	
Grade II/III	1 (8.3%)	0 (0%)	
Grade III	6 (50%)	6 (50%)	
Type of relapse or metastasis			
Free	12 (100%)	0 (0%)	^{MC}p= 0.001*
Local deposits	0 (0%)	4 (33.3%)	
Bone deposits	0 (0%)	4 (33.3%)	
Brain deposites	0 (0%)	1 (8.3%)	
Left side recurrence	0 (0%)	1 (8.3%)	
Pulmonary deposits	0 (0%)	1 (8.3%)	
Brain - pulmonary deposits	0 (0%)	1 (8.3%)	
Lines of treatment			
HER2 Targeted Therapy (Trastuzumab)	12 (100%)	12 (100%)	1
Chemotherapy (Adriamycin/Cyclophosphamide, Taxol, and/or Taxotere)	12 (100%)	12 (100%)	1
Hormonal Therapy (Tamoxifen)	6 (50%)	4 (33.3%)	0.68

MC, Monte Carlo; FE, Fisher's exact; p, p value for comparison between responsive and resistant groups

106 genetic variants in the two groups: 50 variants in the responsive group and 56 variants in the resistant group. The most frequently observed variants were detected in *PIK3CA* (29.2%), followed by *PTEN* (21.7%) and *KRAS* (16%). Figure 1 Comparison of the distribution of *PIK3CA* variants between the two studied groups revealed a statistically significant difference (chi-square test, P= 0.004); *PIK3CA* variants were more frequent in the resistant group (23 variants) than in the responsive group (8 variants). Table S1, Figures S1 & S2

The most prevalent affected genes were the PIK3CA, *PTEN* and *KRAS* genes in the two studied groups (58.33%, 50% and 50%, respectively). Figure (2) Additionally, there was a statistically significant difference between the two studied groups regarding the prevalence of *PIK3CA* mutations, which were more frequent in the resistant group than in the responsive group (Fisher's exact test, P= 0.036). Table S2

However, *PTPN11, AKT1, MPL, NOTCH1, JAK2* and *CTNNB1* did not show any identified mutations in the 2

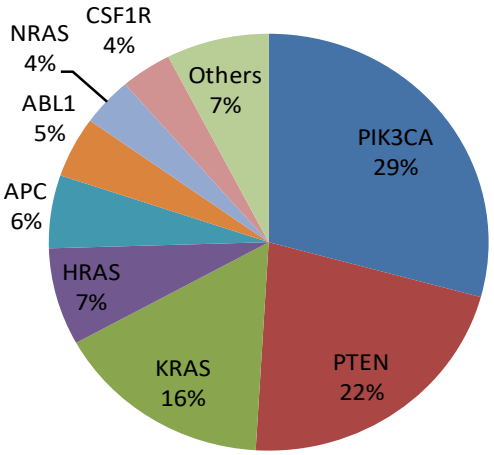

Figure 1. Pie Chart Showing the Distribution of the Studied Genes Variants (n =106) in the 24 Patients.

Table 3. Univariate Analysis of Genetic Mutations and Response to Trastuzumab.

Gene	pCR no. (%)	NonpCR no. (%)	ORa (95% CI)	p
PIK3CA				
Wild	8 (66.6%)	2 (16.6%)		
Mutant	4 (33.3%)	10 (83.3%)	10 (1.44-69.2)	0.036*
PTEN				
Wild	5 (41.6%)	7 (58.3%)		
Mutant	7 (58.3%)	5 (41.6%)	0.51 (0.1-2.58)	0.684
KRAS				
Wild	5 (41.6%)	7 (58.3%)		
Mutant	7 (58.3%)	5 (41.6%)	0.51 (0.1-2.58)	0.684
APC				
Wild	7 (58.3%)	11 (91.6)		
Mutant	5 (41.6%)	1 (8.3%)	0.13 (0.01-1.33)	0.155
ABL1				
Wild	8 (66.6%)	11 (91.6)		
Mutant	4 (33.3%)	1 (8.3%)	0.18 (0.01-1.95)	0.316
HRAS				
Wild	8 (66.6%)	11 (91.6)		
Mutant	4 (33.3%)	1 (8.3%)	0.18 (0.01-1.95)	0.316
NRAS				
Wild	11 (91.6)	9 (75%)		
Mutant	1 (8.3%)	3 (25%)	3.66 (0.32-41.5)	0.59
CSF1R				
Wild	11 (91.6)	9 (75%)		
Mutant	1 (8.3%)	3 (25%)	3.66 (0.32-41.5)	0.59
SRC				
Wild	11 (91.6)	10 (83.3%)		
Mutant	1 (8.3%)	2 (16.6%)	2.2 (0.17-28.1)	1
BRAF				
Wild	11 (91.6)	10 (83.3%)		
Mutant	1 (8.3%)	2 (16.6%)	2.2 (0.17-28.1)	1
JAK3				
Wild	11 (91.6)	11 (91.6)		
Mutant	1 (8.3%)	1 (8.3%)	1 (0.05-18)	1

Table 2. Pathogenic Variants Possibly Related to Trastuzumab Resistance

Gene	Case No.	Exon No.	Type of mutation	Variant	Heterogeneity	Inheritance	SIFT	Provean	Amino acid substitution	Disease	Reference
PIK3CA	1	14	SNV	c.2102A>C	Het	Somatic	Damaging	Damaging	p.His701Pro	Breast neoplasm	rs121913282
PIK3CA	2	2	SNV	c.331A>G	Het	Somatic	Damaging	Damaging	p.Lys111Glu	Breast neoplasm	rs1057519933
PIK3CA	4, 8, 12	9	SNV	c.1633G>A	Het	Somatic	Damaging	Damaging	p.Glu545Lys	Breast adenocarcinoma	rs104886003
PIK3CA	6	5	SNV	c.1031T>G	Het	Somatic	Damaging	Damaging	p.Val344Gly	Breast neoplasm	rs1057519941
PIK3CA	6, 10, 11	20	SNV	c.3139C>T	Het	Somatic	Damaging	Damaging	p.His1047Tyr	Breast neoplasm	rs121913281
PIK3CA	12	20	SNV	c.3062A>G	Het	Somatic	Damaging	Damaging	p.Tyr1021Cys	Breast neoplasm	rs121913288
PTEN	3, 6, 10, 11, 12	1	Deletion	c.50_51del	Het	Somatic	Damaging	Damaging	p.Gln17fs	Hereditary cancer-predisposing syndrome	rs587781912
PTEN	10	5	Deletion	c.315del	Het	Somatic	Damaging	Damaging	p.Cys105fs	Hereditary cancer-predisposing syndrome	rs1114167626
PTEN	12	5	SNV	c.389G>A	Het	Somatic	Damaging	Damaging	p.Arg130Gln	Breast neoplasm	rs121909229
KRAS	3, 6,10,12	4	SNV	c.437C>T	Het	Somatic	Damaging	Damaging	p.Ala146Val	Neoplasm of the large intestine	rs1057519725
JAK3	4	4	SNV	c.394C>A	Het	Somatic	Damaging	Damaging	p.Pro132Thr	acute Lymphoblastic leukemia	rs3212723
BRAF	3	15	SNV	c.1761C>G	Het	Somatic	Damaging	Damaging	p.Asp587Glu	Neoplasm	rs121913336
BRAF	11	15	SNV	c.1799T>A	Het	Somatic	Damaging	Damaging	p.Val600Glu	Melanoma	rs113488022
HRAS	3	2	SNV	c.38G>T	Het	Somatic	Damaging	Damaging	p.Gly13Val	Breast neoplasm	rs104894226

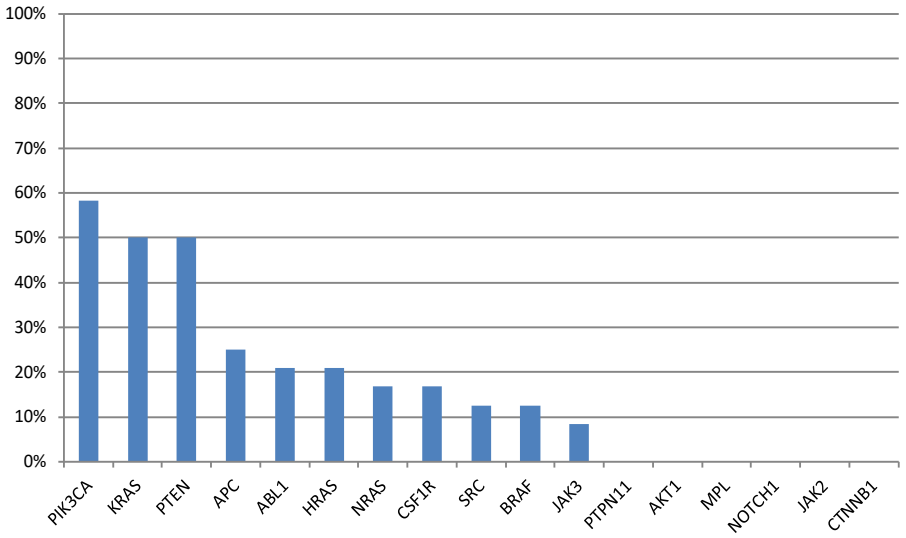

Figure 2. The Mutational Frequency of the Studied Genes in the 2 Studied Groups (n = 24)

studied groups.

Pathogenic variants possibly related to HER2-targeted therapy (trastuzumab) resistance: (Table 2)

A comparison of the identified pathogenic/likely pathogenic variants between the 2 studied groups revealed 14 variants that were detected only in the resistant group. These 14 variants were identified in *PIK3CA* (6 variants), *PTEN* (3 variants), *KRAS* (1 variant), *BRAF* (2 variants), HRAS (1 variant) and *JAK3* (1 variant) Table 2.

The six *PIK3CA* variants (His1047Ty, Glu545Lys, His701Pro, Lys111Glu, Val344Gly and Tyr1021Cys) were all missense mutations. Two *PIK3CA* variants found in 2 regions in helical domain (Glu545Lys; exon 9) and catalytic domain (His1047Tyr; exon 20). These hot-spot mutations were known to induce a gain of function in *PIK3CA* and previously mentioned to be associated with *HER2* targeted therapy resistance and poor survival [15-19]. The *PIK3CA* pathogenic variant Glu545Lys was identified in 3 patients with IDs 4, 8 and 12. Furthermore, the pathogenic variant His1047Tyr was detected in 3 patients with IDs 6, 10 and 11. The other exon 20 variant (Tyr1021Cys) was identified in patient ID 12. The remaining 3 *PIK3CA* variants, His701Pro, Lys111Glu and Val344Gly, were detected in exons 14, 2 and 5, respectively (in patients with IDs 1, 2 and 6, respectively) Table 2.

Additionally, three *PTEN* variants were identified only in the resistant group. *PTEN* Gln17fs is a frameshift variant in exon 1 and was identified in five patients with IDs 3, 6, 10, 11 and 12. The other two *PTEN* variants were in exon 5: a frameshift variant, Cys105fs, was identified in patient ID 10, and a missense variant, Arg130Gln, was identified in patient ID 12, Table 2.

Moreover, one missense *KRAS* variant (Ala146Val) was found in exon 4 in four patients (IDs 3, 6, 10 and 12). Additionally, two missense pathogenic *BRAF* variants (Asp587Glu and Val600Glu) were detected in exon 15 in patients 3 and 11, respectively. The *JAK3* p.Pro132Thr variant was found in exon 4 of patient ID 4. Finally, the pathogenic variant HRAS p.Gly13Val was detected in patient ID 3 in exon 2, Table 2.

Analysis of resistant patients who had more than one pathogenic variant mutation revealed early metastasis. Resistant patient ID 3 was a 36-year-old female who was diagnosed with stage IIIA and grade II *HER2*-enriched breast cancer. This patient had a positive family history of BC. After radical mastectomy, she received chemotherapy and trastuzumab. Even so, she developed metastatic brain deposits after the first year of treatment. Her genetic analysis revealed 4 of these pathogenic variants: the *KRAS* variant p.Ala146Val, the *PTEN* variant p.Gln17fs, the HRAS variant p.Gly13Val and the *BRAF* variant p.Asp587Glu.

In addition, the resistant patient ID 11 was a 53 year old female patient, diagnosed as HER2-enriched BC in stage III and grade III. After surgery, she received chemotherapy and trastuzumab. Unfortunately, 10 months later, she suffered from pulmonary metastatic nodules and brain metastatic deposits. Her genetic analysis revealed 3 pathogenic variants: *PIK3CA* p.His1047Tyr, *PTEN* p.Gln17fs and *BRAF* p.Val600Glu.

Moreover, resistant patient ID 12 had five pathogenic variants (*PIK3CA* p.Glu545Lys, *PIK3CA* p.Tyr1021Cys, *KRAS* p.Ala146Val, *PTEN* p.Arg130Gln and frameshift deletion *PTEN* p.Gln17fs). This patient was diagnosed with luminal B breast cancer (ER+, PR+, *HER2*+) of stage IIIA and grade II, underwent radical mastectomy and received chemotherapy, tamoxifen and trastuzumab. However, she developed pulmonary metastasis and died in the second year of treatment.

Associations between the studied genetic mutations and pathologic complete response (pCR)

Among the studied genes, only a *PIK3CA* mutation showed a significant relationship with the trastuzumab response (Table 3). The pCR rate of patients with wild-type *PIK3CA* was significantly greater than that of patients with mutated *PIK3CA* (66.6% vs. 33.3%, odds ratio (OR) 10 and P = 0.036). Among the patients who achieved

pCR in the responsive group, four patients had *PIK3CA* mutations. In contrast, two patients with wild-type *PIK3CA* did not achieve a pCR. Patients with *PIK3CA* mutations had a lower pCR rate (Table 3).

PIK3CA mutations currently investigated as part of FDA-approved diagnostic testing in BC

The FDA approved the companion diagnostic therascreen® *PIK3CA* test (QIAGEN Manchester, Ltd.), which is used to detect patients who have *PIK3CA* mutations in BC. The therascreen® *PIK3CA* test detects 11 *PIK3CA* hotspot mutations in exons 7, 9 and 20. Among these Therascreen 11 *PIK3CA* hotspot mutations, 5 *PIK3CA* variants were identified in the two currently studied groups: Cys420Arg, Glu542Lys, Glu545Lys, His1047Arg and His1047Tyr. These variants account for 51% of all detected *PIK3CA* mutations in this study. (Table S3, Figure S5)

Discussion

Many studies have investigated possible genetic biomarkers that affect the response of *HER2*+ BC to trastuzumab (the targeted therapy of choice) [7, 25, 11, 26, 20, 27]. Therefore, we aimed to perform a study to include oncogenes and other important pathways to demonstrate any association between trastuzumab response and frequent oncogenic mutations in Egyptian *HER2*+ BC patients.

Among the 17 studied genes, *PIK3CA* was the most frequently affected gene in patients (58.33%), with the highest percentage of variants (29.2% of the total resultant variants). In line with our findings, a recent Egyptian study [28] that sequenced 46 breast tumors of Egyptian BC patients revealed that *PIK3CA* was the most frequently mutated gene in 27 patients (58.7%). Additionally, Sherene Loi et al. [29] who genotyped 705 *HER2*+ BC patients receiving trastuzumab, *PIK3CA* was the most frequently mutated gene (25.3%). Moreover, *PIK3CA* was the most frequently mutated gene in a Chinese study targeting 520 cancer-related genes in 589 BC patients (45.0%, 265/589) [30]. Phosphoinositide 3-kinase (PI3K) p110α, encoded by the *PIK3CA* gene, is known to be one of the most important kinases activated in response to signaling from *HER2* and other kinases. Abnormal activation of PI3K has long been recognized as one of the main oncogenic drivers in BC. It is thought that as a recombinant monoclonal antibody, trastuzumab binds to the extracellular domain IV of *HER2*, downregulating the PI3K/*Akt* pathway [12].

Additionally, in the present study, *PIK3CA* variants were more frequent in the resistant group (23 variants) than in the responsive group (8 variants). The most involved exons in the *PIK3CA* gene were exon 20 (29.1%, 7/24) and exon 9 (25%, 6/24). *PIK3CA* pathogenic variants (His1047Arg and His1047Tyr) were detected in exon 20, and Glu542Lys and Glu545Lys were detected in exon 9, constituting approximately 48% of all *PIK3CA* mutations. Similarly, in a study performed by Loibl S et al. [13] who evaluated *PIK3CA* mutations and their associations with pCR in 967 primary *HER2*+ BC patients, the number of mutations found in exon 20 was twice as high as that in exon 9 (14.5% vs 7.2%). Jensen JD et al. [14] who conducted a study in 240 *HER2*+ BC patients receiving adjuvant treatment, a total of 61 (26%) patients had either exon 20 or exon 9 mutations (17% vs 9%, respectively). Additionally, in the present study, six *PIK3CA* variants, His1047Tyr, Glu545Lys, His701Pro, Lys111Glu, Val344Gly and Tyr1021Cys, were detected only in the resistant patients. These variants might be related to trastuzumab resistance. The *PIK3CA* His1047Tyr and Tyr1021Cys variants were detected in exon 20, and the Glu545Lys variant was detected in exon 9. Previous studies revealed that *PIK3CA* mutations within exons 9 and 20 are associated with poor survival and *HER2* targeted therapy resistance [13, 15-19].

Notably, our findings showed that among all the studied gene mutations identified by NGS, only a *PIK3CA* mutation had a significant relationship with the trastuzumab response. The pCR rate of patients with wild-type *PIK3CA* was significantly greater than that of patients with mutated *PIK3CA* (66.6% vs. 33.3%; P=0.036). In line with our results, Qiyun Shi et al. [11] sequenced 50 *HER2*+ BC patients receiving targeted therapy. The pCR rate of patients with wild-type *PIK3CA* was significantly greater than that of patients with mutated *PIK3CA* (80.8% vs. 26.3%; P=0.00057). Several additional clinical trials demonstrated that *PIK3CA* mutations were associated with a lower pCR rate in *HER2*+ BC patients receiving *HER2*-targeted therapy [11, 31, 32, 13, 33].

Moreover, the present study revealed other variants that were detected only in resistant patients, e.g., three *PTEN* variants. These *PTEN* variants included two deletions (Gln17fs, Cys105fs) and one SNV (Arg130Gln). In line with this finding, P. Lebok et al. [34] *PTEN* deletion is a frequent event in breast cancer patients with a poor prognosis. Many studies have also shown a relationship between *PTEN* alterations and sensitivity to trastuzumab [35-38, 3, 39, 40]. However, additional studies did not find a relationship between *PTEN* alterations and sensitivity to trastuzumab [41-43].

Moreover, in our study, two RAS variants were identified only in the resistant patients, *KRAS* Ala146val and HRAS Gly13Val, suggesting that they are associated with trastuzumab resistance. Notably, another study of metastatic colorectal cancer revealed that patients with *KRAS* Ala146 mutation had a greater tumor burden and worse clinical prognosis [44]. HRAS was previously reported to be an anti-*HER2* therapy resistance-associated gene [27].

Furthermore, two *BRAF* variants were detected only in the resistant patients: Asp587Glu and Val600Glu. The *BRAF* Val600Glu mutation is known to be associated with poor prognosis due to inappropriate activation of the MAPK/ERK pathway, uncontrolled cell proliferation, migration, angiogenesis, and a lack of apoptosis [45]. The *BRAF* Val600Glu mutation is a target of treatment for different types of malignancies, such as melanoma, non-small cell lung cancer (NSCLC), hairy-cell leukemia and brain tumors [46-51]. In addition, the *JAK3* Pro132Thr variant was found in patient ID 4 in the resistant group. In a recent Egyptian study that investigated the impact of

somatic mutations in 55 BC patients, the *JAK3* Pro132Thr variant was found in 3 patients [52].

In conclusion, collectively, our data suggested that *PIK3CA* mutations were significantly related to trastuzumab resistance. *PIK3CA* variants were more frequent in the resistant group than in the responsive group, with an association between *PIK3CA* mutation and a lower pCR rate. More *PIK3CA* variants within exons 9 and 20 were detected in resistant patients. Some PIK3CA, *PTEN*, *KRAS*, HRAS, *JAK3* and *BRAF* gene variants were detected only in resistant patients, so these variants might be related to trastuzumab resistance. Consequently, these findings clarify the significance of the use of emerged PI3K/mTOR inhibitors to increase trastuzumab sensitivity in *HER2*+ BC resistant patients.

Finally, the limitation of this study was the small sample size. Therefore, further studies are recommended to investigate the genetic variants associated with the response of combined *HER2* targeted therapy in *HER2*+ BC patients using a larger sample size..

Author Contribution Statement

AMM, MHS, DE, TAE, and DH participated in the study design. AMM was responsible for enrollment of the study subjects, and the gathering clinical data. AMM and MHS were responsible for the genetic analysis using Ion S5™ next-generation sequencing system and data analysis using Ion Reporter™ Software. AMM and MHS prepared the manuscript. MHS, DE, TA, and DH reviewed and edited the manuscript. All the authors read and agreed to the final form of the manuscript.

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Data Availability

The data supporting the conclusions are included within the article.

Ethics approval

The study was approved by the Ethics Committee of Alexandria University, Egypt. All methods were conducted in compliance with the appropriate guidelines and standards.

Conflict of interest

The authors declare that there is no conflict of interest.

List of Abbreviations

List of Abbreviations in the supplementary File.

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