Modulation of Mutational Landscape in HER2-Positive Breast Cancer after Neoadjuvant Chemotherapy

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ABSTRACT

Introduction: In early-stage HER2 positive breast cancer (BC) patients, tumor response to neoadjuvant chemotherapy (NACT) predict survival outcomes. Patients achieving less than pathological complete response (pCR) have a worse prognosis, however, this group is heterogeneous. Nowadays limited data on predictive/prognostic biomarkers in patients with residual cancer disease are available.
Methods: Using next-generation sequencing technology, we evaluated a panel of 21 cancer genes in a group of HER2 positive BC patients with residual disease after NACT. A control group of patients who achieved the pCR was selected too. The BC mutational profile was analyzed on both the tumor diagnostic biopsy and matched residual disease.
Results: Overall, the detection rate of mutations was 79% in the No-pCR group versus 90% in the pCR cohort and 98% in the residual BC. The most mutated genes were TP53 and PIK3CA. No correlations between single gene mutations and survival outcomes were found. In no-pCR cohort, 52% of patients had different mutational profile after NACT, 69% of them had an increased in the number of mutated genes. Mutational profile changes from diagnostic biopsy to residual BC were a negative prognostic factor in term of relapse free survival: recurrence probability in different gene profile sub-group was 42% vs 0% in the same profile one ($P = .019$).
Conclusions: Treatment selective pressure on tumor cells due to NACT changed the gene mutational profile in more than half of BC patient with residual tumor disease. Treatment-induced gene mutations significantly increase the risk of relapse. Profiling primary and residual BC is a major step in order to further personalized adjuvant treatment strategy.

Introduction

Neoadjuvant chemotherapy (NACT) is the preferred treatment option in patients with early-stage HER2 positive breast cancer (BC). In that setting the addition of trastuzumab +/- pertuzumab to standard chemotherapy resulted in a significantly higher activity when compared to chemotherapy alone [1–3]. The achievement of a pathological complete response (pCR) is a powerful surrogate of long-term outcomes [4]. Patients achieving less than pCR have a worse prognosis; however, this group is heterogeneous, including patients with still good outcomes or patients with primary resistant disease [5]. Results from Katherine trial suggested that among patients with HER2 positive early BC whit residual invasive disease

after neoadjuvant therapy, the risk of recurrence or death was 50% lower with adjuvant T-DM1 than with trastuzumab alone [6].

Nowadays limited and not conclusive data on predictive/prognostic biomarkers in patients with residual disease after NACT are available [7]. This issue in likely to acquire even more relevance in the coming years with the need to further personalize the post-neoadjuvant approach in early BC setting.

We have evaluated by next-generation sequencing technology, a panel of 21 cancer related genes in a group of HER2 positive BC patients treated with NACT. We analyzed the BC mutational profile on tissue biopsy samples according to tumor response to NACT. In patients with residual tumor disease, gene analysis was performed on the matched surgical

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specimen as well. An exploratory analysis in terms of treatment outcome and genes status was performed too.

Material and Methods

Patient Population and Samples

Patients with diagnosis of HER2 positive early BC with residual disease after anti-HER2 NACT were identified and screened for the study. A control group of patients with HER2 positive disease who achieved a pCR was selected too. In order to be enrolled, patients must had tissue samples taken from both the diagnostic BC biopsy and matched surgical specimens. All BC tissue samples were formalin-fixed and paraffin-embedded (FFPE) and archived in the Pathology Department of Modena University Hospital.

One hundred ninety-six patients with HER2 positive early BC treated with NACT from 2008 to 2018 in Modena Cancer Center were identified. One hundred seventy-eight of them received an anti-HER2 agent (trastuzumab +/- pertuzumab) as part of their neoadjuvant systemic treatment. Among these women, 31 underwent to BC surgery outside Modena University Hospital. Considering patient with known breast surgery outcome, 111 did not achieve a pCR. Among these, 83 women had available FFPE tissues taken from both diagnostic biopsy and surgical specimens suitable for biomarker analyses. In order to select the real treatment-resistant BC, we decided to perform the gene analysis only in samples with at least 1 cm of residual invasive tumors—in fact we decided to exclude from the present work all the cases with residual invasive tumor lower than 1 cm in longest diameter and/or scattered pattern, in order to avoid preanalytical bias of genomic analysis of the samples (contouring of the cellular areas, manual microdissection, etc.) potentially able to distort the preliminary results of this research. The final study population included 32 patients with residual BC after NACT (**No-pCR group**) and a control group of 32 patients who achieved the pCR (**pCR group**) (Figure 1). Of note, all breast biopsy samples were taken before the initiation of any systemic treatment.

The study was approved by the local ethics committee (protocol number 0024589/19). Written informed consent was obtained from each living patient.

Gene Analysis

DNA extraction was performed in the Molecular Biology Laboratory of Modena Pathology Department. DNA extraction was performed with QIAamp DNA Mini Kit (Qiagen) from 10-μm-thick sections of FFPE tissues. Tumor-representative areas containing at least 20% to 50% tumor cells were selected by pathologist and isolated by manual microdissection, as suggested from guidelines. Extracted DNA was quantified with Qubit fluorometer (Life Technologies) and 10 ng from each sample were amplified in single-tube multiplex PCR. Mutational analysis was performed with targeted amplification-based NGS panel 'Oncomine Solid Tumour DNA' (ThermoFisher Scientific). The analyzed genes were: EGFR, ALK, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, MET, DDR2, KRAS, PIK3CA, BRAF, AKT1, PTEN, NRAS, MAP2K1, STK11, NOTCH1, CTNNB1, SMAD4, FBXW7, and TP53. Analysis of raw sequencing data was conducted with Ion Reporter software. Only variants with a minimum coverage of 500X were considered to ensure variant specify. Integrative Genomic Viewer was used for variant visualization.

Statistical Analyses

Statistical analysis was performed using STATA 13 (Stata Statistical Software: Release 13; StataCorp LP, College Station, TX). Baseline clinical and tumor characteristics were compared between the two tumor-response subgroups (No-pCR vs pCR groups) by chi-square test for categorical variables (i.e. breast cancer subtypes, grading, histotype, neoadjuvant treatment, stage, recurrence and death) and by Wilcoxon Mann-Whitney test for continuous variables (i.e. age at diagnosis). Survival outcomes of interest were OS defined as the time from the diagnosis of BC to the death/last follow up and Relapse Free Survival (RFS) defined as the time from the date of the diagnosis of BC to the date of the first documented recurrence/death. OS and RFS were addressed by the Kaplan–Meier method and log-rank test. A P value $< .05$ was considered statistically significant; hazard ratio was estimated with 95% of confidence limits.

Results

Patients' and Samples' Characteristics

Sixty-four patients were enrolled in the study: 32 with at least 1 cm of residual BC disease after NACT (No-pCR group) and 32 patients with pCR (pCR group). Tumor and patient characteristics are described in Table 1. The groups were well balanced according to baseline clinical and tumor characteristics. As expected, the percentage of hormone receptors positive BC were higher in No-pCR group than in pCR cohort ($P = .002$). All the enrolled patients received trastuzumab combined with systemic chemotherapy, in six cases with addition of pertuzumab too. The median follow up was 45 months in both groups. Considering survival outcomes, two patients in the pCR group relapsed and are still alive while eight recurrence and four deaths were observed in the No-pCR cohort.
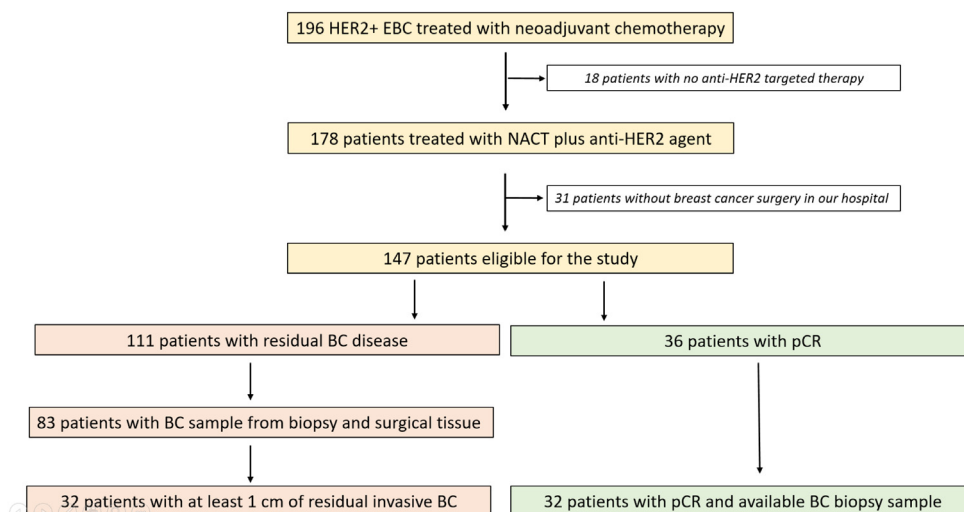

Figure 1. Flowchart of the study population. NACT: neoadjuvant chemotherapy; BC: breast cancer; pCR: pathological complete response.

Table 1
Tumor and patient characteristics

Characteristic	No-pCR		pCR		P value
	32		32		
	N	%	N	%	
Age (range)	50 (28-70)		50 (26-80)		
Clinical stage					
II	24	(75)	23	(72)	1
III	8	(25)	9	(28)	
BC subtypes					
HER2+ and HR+	20	(63)	7	(22)	0.002
HER2+ and HR-	12	(24)	25	(78)	
Histotype					
ductal	29	(93)	32	(100)	0.238
lobular	3	(7)	0		
Grading					
2	4	(10)	2	(6)	0.671
3	28	(90)	30	(94)	
Neoadj pertuzumab					
Yes	3	(7)	3	(7)	1
No	29	(93)	29	(93)	
Neoadj chemotherapy					
Taxane	5	(6)	4	(10)	1
Anthracycline + Taxane	27	(84)	28	(90)	
Recurrence					
Yes	8	(25)	2	(6)	0.081
No	24	(75)	30	(94)	
Death					
Yes	4	(10)	0		0.113
No	28	(90)	32	(100)	

Among the 64 enrolled patients, 28 with residual BC disease and 30 with pCR had successfully gene analysis extraction performed on the diagnostic biopsy. Considering the No-pCR group, in 25 cases the gene analysis was successfully performed in both biopsy and residual BC tissue, the other seven resulting not suitable for DNA extraction procedures.

Overall, the detected mutations were 113: 37 in the pCR group, 35 in the No-pCR group and 41 in the residual BC samples (Table 2). The majority

Table 2
Number and type of detected mutations in the breast cancer biopsy and residual disease

Mutations	Breast cancer biopsy		Residual BC tissue
	pCR (N = 30)	No–pCR (N = 28)	(N = 25)
	N. detected mutated genes		
Number	**37**	**35**	**41**
Type			
TP53	20 (54%)	12 (34%)	13 (32%)
PIK3CA	8 (21%)	8 (23%)	8 (19%)
MET	1 (3%)	2 (6%)	3 (6%)
KRAS	0	2 (6%)	1 (3%)
SMAD4	0	2 (6%)	1 (3%)
NOTCH1	0	1 (3%)	2 (5%)
FGFR3	1 (3%)	2 (6%)	4 (9%)
ERBB2/4	2 (5%)	1 (3%)	2 (5%)
PTEN	0	1 (3%)	3 (6%)
DDR2	2 (5%)	1 (3%)	1 (3%)
ALK	1 (3%)	0	1 (3%)
BRAF	1 (3%)	0	0
STK11	1 (3%)	1 (3%)	0
NRAS	0	1 (3%)	0
EGFR	0	1 (3%)	1 (3%)
AKT1	0	0	1 (3%)

Primary Tumor Mutation (No-pCR Group vs pCR Group)

Overall, 72 mutations were found in the diagnostic BC biopsies. Thirty-seven of them were in the pCR group and 35 in residual disease cohort (Table 2). Considering the mutational burden, in No-pCR cohort six patients had no detected mutation (21%), 9 one detected mutation (32%), 12 two detected mutations (43%) and 1 three detected mutations (4%) (Table 3). In the pCR group, we found three patients with no mutation (10%), 18 with one mutation (60%), 8 with two mutations (27%) and 1 with three mutations (3%) (Table 3). Regarding the meaning of the detected mutations, in both groups, 15 mutations were known to be pathogenic/likely pathogenic ones (PI3KCA, TP53, SMAD4, DDR2, PTEN) (Table 2). Moreover, a MET mutation with uncertain significance was found in the pCR cohort. Considering the detected genes, in both cohorts, TP53 and PIK3CA were the most mutated ones. In particular, mutations of TP53 and PIK3CA were detected in 67% and 27% of pCR women versus 43% and 28% of No-pCR patients, respectively (P value not significant).

In the pCR cohort, both ERBB2/ERBB4 and DDR2 were mutated in 5% of cases; all the other mutated genes were detected in the 3% of women. Considering the No-pCR group, SMAD4 and FGFR3 were detected in 6% of cases followed by KRAS and MET in 5% of cases. Of note, no difference in the rate of PIK3CA mutation according to hormone receptors status was found (22% HR positive and 27% of HR negative). As expected, TP53 mutation was higher in HR negative BC compared to HR positive ones (59% vs 37%).

Comparison Between Primary Tumor and Residual Disease

In the No-pCR group, twenty-five women had DNA analysis performed on both diagnostic tumor biopsy and matched residual BC. Seven out of 32 cases were not suitable for DNA extraction procedures. Overall, 41 mutations were found (Table 3). The total number of the detected mutations has increased from the biopsy to the matched surgical specimen (32 vs 41 respectively) (Figure 2). Among those cancers that changed their mutational profile from breast biopsy to residual disease after NACT, in 69% of cases there was an increase in the absolute number of mutations. In particular, in nine cases the number of mutated genes increased in the surgical tissue while in two cases the primary mutations were lost. In two cases the mutation profile of residual BC was completely different from the primary biopsy one. Considering the mutational burden in the residual tumors, only two patients had no mutation detected, 10 had one mutation, nine had two mutations, three had three mutations and 1 BC presented four mutations (Table 3). Regarding the meaning of the detected mutations, 14 mutations found in the surgery tissues were known to be pathogenic/likely pathogenic (PI3KCA, TP53). Comparing the detected mutated genes in breast biopsy and matched surgical sample, we found an increase number of PIK3CA, TP53, MET, NOTCH1, FGFR3, and PTEN mutations in the residual BC tissue (Figure 3).

Table 3
Patients' mutational burden in the three groups: pCR, No-pCR and residual disease

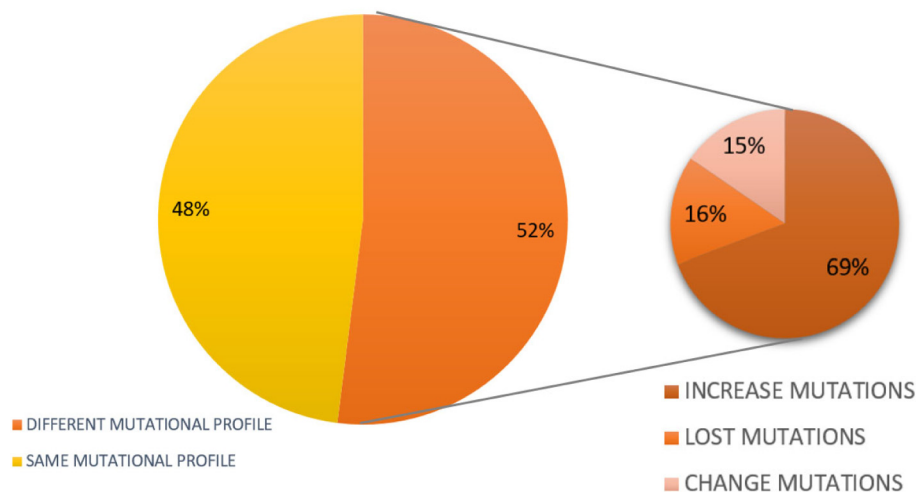|	pCR	No-pCR	Residual disease
	N. of patients (%)	N. of patients (%)	N. of patients (%)
No mutation	3 (10%)	6 (21%)	2 (8%)
1 mutation	18 (60%)	9 (32%)	10 (40%)
2 mutations	8 (27%)	12 (43%)	9 (36%)
3 mutations	1 (3%)	1 (4%)	3 (12%)
4 mutations	0	0	1 (4%)

Figure 2. Different mutational profile among diagnostic breast cancer biopsy and matched residual breast cancer.

Prognostic Value of Detected Gene Mutations

As expected patients who achieved a pCR had significantly lower risk of relapse compared to patients with residual disease (RFS: 25% vs 6%, respectively; $P = .037$). No statistically significant differences in RFS according to the detected mutational burden of disease have been found. In particular, there were no differences between patients with no detected mutations and patients with at least one gene mutation (33% versus 10%, respectively, $P = .119$) as well as between patients with no or one mutation and those with two or more mutations (11% versus 18%, respectively, $P = .35$). The detection of TP53 or PIK3CA mutations did not significantly influence the risk of relapse too ($P = .68$).

On the contrary, changes in the gene mutational profile during the NACT treatment significantly influenced the risk of relapse. In particular, patients with different mutational profile between diagnostic biopsy and matched residual BC had a significantly higher risk of recurrence compared to patients with no treatment induced gene modifications. In fact, all the recurrences occurred in the subgroup of patients with different gene status after NACT, independently from pCR groups. Forty-two per cent of patients with treatment induced gene changes relapsed (P = .019, log-rank test)

(Figure 4A). In particular, the increase of the mutational tumor burden seemed to be mostly involved in the risk of relapse (Figure 4B).

Survival analysis in term of overall survival was not performed because of the few number of accorded deaths at the time of the analysis. All the deaths were in the subgroup of No-pCR patients.

Discussion

BC is a dynamic and heterogeneous disease. New gene analysis technology showed that there are several BCs with different genomic profiles reflecting in different treatment sensitivity and survival outcomes [8]. Moreover, during cancer progression tumors acquired genomic mutations able to influence treatment sensitivity too [9]. The heterogeneity of the tumors, exacerbated by the selective pressures imposed by the systemic treatments, confers a major resistance to anti-cancer drugs [9]. In this context, emerging evidence from NACT trials showed how the residual tumor genetic landscape is largely involved in anticancer sensitivity/resistance mechanisms [10]. Proper understanding of residual disease mutational landscape may lead to a personalized systemic adjuvant treatment approach. Against this backdrop, we evaluated a panel of 21 genes involved
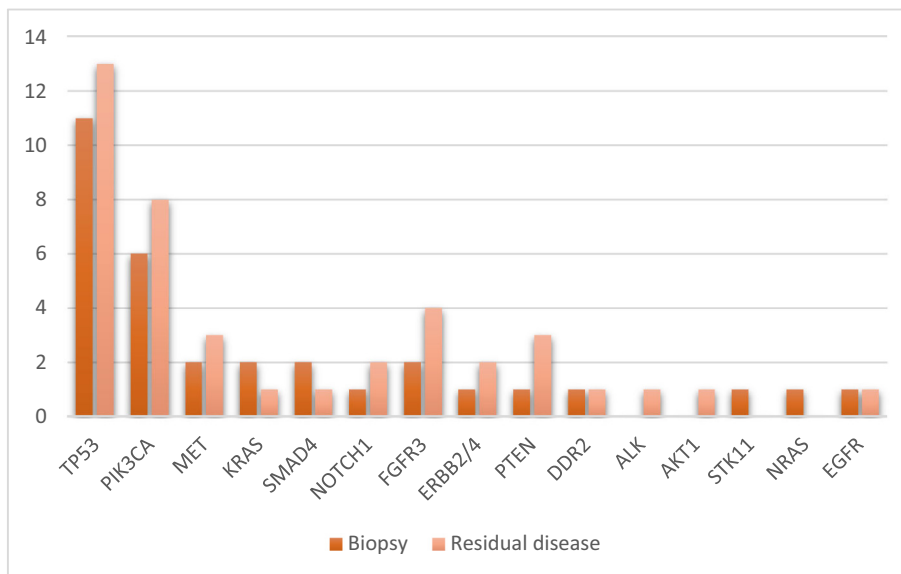

Figure 3. Biopsy versus surgery tissue detected mutated genes.
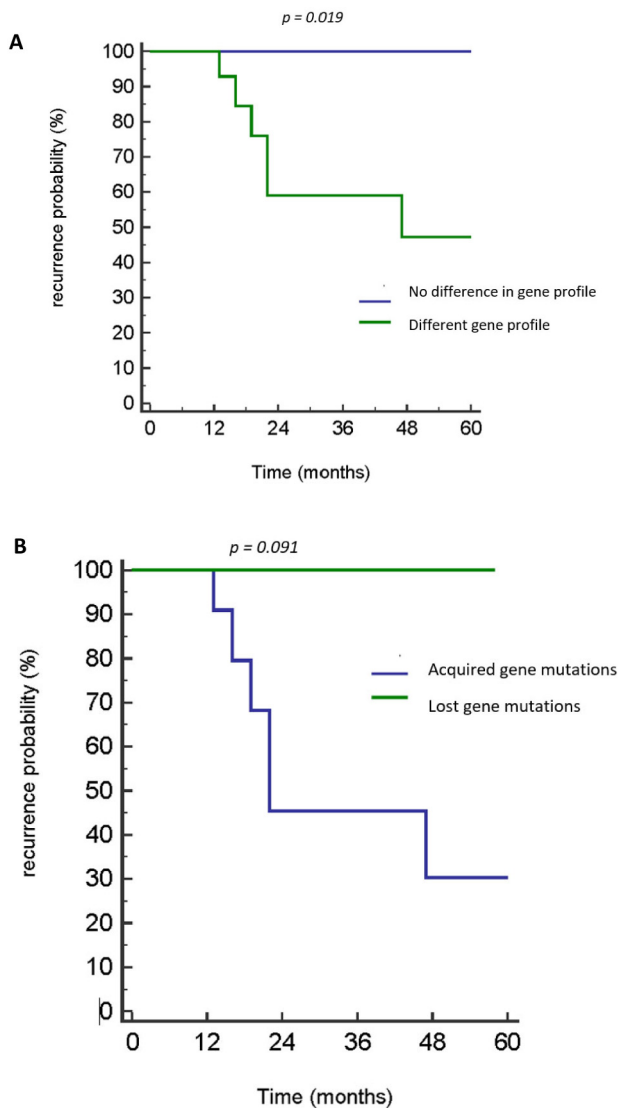
A

B

Figure 4. (A) Kaplan-Meier curves for Relapse Free Survival according to modification in breast cancer mutational profile pre- versus post-NACT. (B) Kaplan-Meier curves for relapse free survival for subgroup of patients with acquired gene mutations versus lost gene mutations, (*P* value, log-rank test)

in treatment resistance, comparing cancer samples taken from the diagnostic biopsy and the residual tumor after NACT selected on the basis of treatment sensitivity (No-pCR versus pCR group). Considering results from the BC diagnostic biopsy analysis, the detection rate of mutations was 79% in No-pCR group versus 90% in pCR one. The high rate of mutations detected on the diagnostic biopsy underlines that the analyzed genes were highly involved in the first steps of cancer progressions. The most frequently mutated genes were TP53 and PIK3CA in both No-pCR and pCR groups of patients. The mutation rate found in our study population was similar to those reported in literature (30% for PIK3CA and 50% for TP53) [11,12].

TP53 mutations were overall present in 55% of the biopsy, mainly in pCR subgroup (67% vs 43%). This finding confirms a higher rate of pCR in TP53 mutated patients according to other published researches [13]. Even if the TP53 status seems to be a predictor for pCR, survival studies showed worse outcomes in TP53-mutated patients [13]. This evidence may depend by the fact that TP53 induces arrest and senescence instead of apoptosis [14]. Several pre-clinical evidence have shown that senescent cells drive relapse by producing cytokines that promote proliferation, survival, angiogenesis and increase in cancer stem cells population [15]. In our analysis, no correlation between TP53 status and risk of relapse/

death has been found likely due to the high rate of TP53 mutated tumors and the low rate of relapse in the study population.

PIK3CA mutations were present in 27% of patients, similar between pCR and No-pCR subgroup (27% vs 28%). Preclinical evidence suggests that PIK3CA mutated cancer cells have an abnormal pathway activation, which lead to resistance to trastuzumab [16]. A meta-analysis on the role of PIK3CA mutations and response to NACT published in 2018 confirmed these results [11]. The predictive value of PIK3CA mutation is less clear in patients selected according to hormone receptors status. Higher pCR rate was found in PIK3CA mutated-HER2 positive-hormone receptors positive BC but not in hormone receptors negative ones [11]. Looking at our pCR subgroup, only seven women had hormone receptors positive BC. This may justify the lack of significant correlation between the PIK3CA mutation and the pCR rate in our study population.

Overall, the lack in the identification of a prognostic and/or predictive mutational gene profile may be justified by the complexity of the BC biology. Knowledge on cancer progression suggested that the carcinogenesis is moved by multiple gene mutations that generate changes in several molecular pathways involved in cell survival [17]. Abnormalities in DNA methylation, microRNA and protein expression increased the molecular architecture of BC too [17].

Considering residual BC disease, it is well clear that NACT is able to change the tumor mutational profile. Since the presence of residual tumor after NACT confers an increased risk of recurrence, a better characterization of these patients is necessary [18]. Our findings showed a potential prognostic value of treatment induced mutational burden modifications. Changes in the residual BC mutational landscape (acquired or lost mutations) were negative prognostic factors in term of RFS. In particular, patients with a different mutational profile between diagnostic biopsy and residual disease had a significantly higher risk of relapse compared to those without gene modifications.

Despite preliminary and to be confirmed in prospective clinical trials, these results may help in the selection of those patients with residual HER2 positive disease after NACT, and therefore candidates for post-NACT treatments—on the model of the KATHERINE study—for further prolong DSF and OS.

Overall, the total number of detected mutations was increased from the matched BC biopsy to the surgical specimen (32 vs 41 respectively) as well as the number of patients with at least one detected mutations (76% vs 92%). More than half of the patients changed the mutational profile during the neoadjuvant treatment, 69% increased the number of the detected mutated genes in the surgical tissue compared to the matched biopsy. In two cases the mutational profile of residual tumor was completed different from the primary biopsy one. Both these patients early relapsed. In particular, even if not statistically significant, the acquisition of new mutations compared to their loss seems to increase the risk of relapse too. All the recurrence occurred in the subgroup of patients with an increased mutational burden from the biopsy to the residual disease. The selective pressure of the NACT on tumor cells can explain these data. In resistant cancer cells, therapy could induce gene mutations able to confer a more aggressive tumor behavior, reflecting in worse survival outcomes. Our results are useful in the present context, when NACT is more and more used to select the best post-operative treatment. Based on the detection of different mutational profile from biopsy to surgery, patients could be selected for different adjuvant treatment strategy. In particular, women with changes in mutational profile could be better candidate for an alternative adjuvant treatment (such as TDM1) [6], whereas patients maintaining the same molecular profile could stay on the same treatment used before surgery (such as trastuzumab).

In spite of our findings, this study presents two main limitations and must be considered hypothesis generating. Firstly, this is a retrospective study with a small sample size. Secondary, due to the retrospective nature of our analysis, samples were fixed and processed for storage in different periods and by different technicians, with no purpose of genomic analysis. This variability might have reduced the quality and preservation of some tissues.

Conclusion

Survival benefit in early BC patients is strictly linkable to the improvement in genomic BC profile knowledge. Findings from our research confirm that mutations on driver genes are present from the first steps of BC carcinogenesis and/or may arise during cancer treatment. In particular, during NACT BC may acquire gene mutations able to confer resistance to anticancer systemic therapy itself. The selective pressures imposed by chemo- and targeted-therapies changes the mutational BC landscape in the majority of patients with residual tumor disease. Our results demonstrated that patients with changes in the gene status, as both gains and losses of mutations, have an increased risk of relapse. The identification of those patients could be useful in the management of adjuvant treatment strategy. Profiling BC sample before and after any interventions is the first critical step in the precision medicine era.

Funding

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