Somatic alterations of targetable oncogenes are frequently observed in BRCA1/2 mutation negative male breast cancers

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ABSTRACT

Male breast cancer (MBC) is a rare disease. Due to its rarity, MBC research and clinical approach are mostly based upon data derived from its largely known female counterpart. We aimed at investigating whether MBC cases harbor somatic alterations of genes known as prognostic biomarkers and molecular therapeutic targets in female breast cancer.

We examined 103 MBC cases, all characterized for germ-line BRCA1/2 mutations, for somatic alterations in PIK3CA, EGFR, ESR1 and CCND1 genes.

Pathogenic mutations of PIK3CA were detected in 2% of MBCs. No pathogenic mutations were identified in ESR1 and EGFR. Gene copy number variations (CNVs) analysis showed amplification of PIK3CA in 8.1%, EGFR in 6.8% and CCND1 in 16% of MBCs, whereas deletion of ESR1 was detected in 15% of MBCs. Somatic mutations and gene amplification were found only in BRCA1/2 mutation negative MBCs.

Significant associations emerged between EGFR amplification and large tumor size (T4), ER-negative and HER2-positive status, between CCND1 amplification and HER2-positive and MIB1-positive status, and between ESR1 deletion and ER-negative status.

Our results show that amplification of targetable oncogenes is frequent in BRCA1/2 mutation negative MBCs and may identify MBC subsets characterized by aggressive phenotype that may benefit from potential targeted therapeutic approaches.

INTRODUCTION

Male breast cancer (MBC) is a rare disease, representing less than 1% of all breast cancers (BCs) and less than 1% of all cancers in men [1]. Germ-line mutations in the high-penetrance BC genes, BRCA1 and, mainly, BRCA2 play a major role in MBC susceptibility and about 10% of MBCs are associated with mutations in these genes [2].

Due to its rarity, MBC research and patient management are mostly based upon data derived from its largely known female counterpart. To date treatment strategies for MBC patients generally follow those for female patients. As most breast cancers in men are hormone receptor-positive, current therapeutic options mainly include hormone therapy that is usually recommended for MBC patients following the same recommendations as hormone-dependent-BC in women [3].

MBC shares some similarities with post-menopausal estrogen receptor (ER)-positive female breast cancer (FBC), however increasing evidence indicates that, on clinical and molecular level, MBC may be a heterogeneous
disease, different from FBC [4–6]. Compared to FBC, MBC occurs later in life, with higher stage, lower grade and more estrogen/progesterone receptor (ER/PR) positivity [7–8].

To date, the majority of MBC studies focused on germ-line mutational analysis and gene expression profiling, while there are only few studies investigating somatic alterations in MBCs [4–5, 9]. By contrast, accumulating data have provided a landscape of somatic alterations with possible clinical relevance and several somatic mutations with potential prognostic and therapeutic significance are known in FBC [10–13]. In addition to gene mutations, gene copy number variations (CNVs) have been reported as an important mechanism in the development and progression of cancer and could serve as potential prognostic biomarkers and molecular therapeutic targets [14–18].

A comprehensive portrait of the genetic landscape of FBCs has demonstrated that Phosphatidylinositol 3-kinase gene (PIK3CA) is the most commonly mutated gene and two mutation hotspot regions in exons 9 and 20 have been identified [19–20]. Mutations of PIK3CA have been suggested to have prognostic value and to confer resistance to hormone therapy and to downstream mTOR inhibitors [11, 21]. The proportion of FBCs exhibiting PIK3CA mutations ranges between 20-25%, and, notably, mutations occur approximately in 40% of ER-positive FBCs [11, 19–23]. To date four studies investigated PIK3CA mutations in MBC [6, 24–26]. Overall PIK3CA mutation frequency observed in MBC was lower than FBC. In addition to somatic mutations, gene amplification of PIK3CA is reported in about 10% of FBC [27–29]. Among targetable genes, the epidermal growth factor receptor (EGFR) is one of the first important molecular targets identified in BC [10, 30–31]. EGFR gene CNVs in FBC range from 8 to 10%, [32–33] while the proportion of FBCs exhibiting EGFR somatic mutations ranges from 2 to 15% [34–36]. Notably a higher rate of activating EGFR mutations is detected in BRCA1/2 mutation positive tumors, supporting the hypothesis that carcinogenic processes may be dependent on the germ-line phenotype [34]. CCND1 is an estrogen-responsive gene that enhances ER-mediated gene transcription. The product of CCND1, cyclin D1, provide growth advantage to cancer cells and contribute toward resistance to endocrine therapy in ER-positive cancers. In FBC, CCND1 is amplified in 5–20% of primary BCs, typically ER-positive BC, and is a prognostic biomarker with a potential therapeutic role [12, 13, 15, 37, 38]. As MBC is an estrogen-driven disease and is most frequently ER-positive, the investigation of CCND1 alterations might be relevant in male breast tumors [8]. Recently, CCND1 amplification has been shown to be an independent prognostic factor in MBC [39]. Somatic mutations of CCND1 are not a frequent alteration in cancer, and, in particular have not been reported in BC [19, 40].

Interestingly, crosstalk between ER and PI3K pathway may increase estrogen-induced and ligand-independent ER transcriptional activity [41]. Recurrent activating mutations of ESR1, the gene encoding the estrogen receptor alpha (ERα), have been identified in hormone therapy-refractory ER-positive metastatic FBC [42–44]. CNVs of ESR1 have been largely investigated in FBC, although with controversial results [45]. Data emerging from studies evaluating ESR1 somatic alterations in MBC suggest that ESR1 alterations may be gender specific [39].

Although knowledge on somatic landscape of MBC is increasing, comprehension on the role of somatic alterations of specific genes with potential prognostic and therapeutic significance need to be further investigated in MBCs characterized for BRCA1/2 mutations. In this study, we focused on somatic mutations and CNVs of PIK3CA, EGFR, ESR1 and CCND1 genes by examining a large series of MBCs screened for BRCA1/2 germ-line mutations.

RESULTS

Mutational screening

Mutational screening of PIK3CA was performed in 102 primary breast cancers and one lymph-node metastasis. Two mutations, both located in exon 9, were detected: the c.1633G>A (p.E545K) was identified in two MBC cases and the c.1634A>C (p.E545A) in two additional cases (Table 1). In these latter cases, a second mutation, c.1658_1659delGTinsC (p.S553Tfs*7), also located in exon 9, was detected (Figure 1).

To exclude that PIK3CA pseudogene, retaining high homology with the sequence of exon 9 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), might affect PIK3CA mutation screening, we performed amplification and sequencing analysis using primers specific for the PIK3CA gene and pseudogene (Supplementary Table S1). This analysis showed that both c.1634G>A and c.1658_1659delGTinsC (p.S553Tfs*7) were not a frequent alteration in male breast tumors [8]. Recently, CCND1 amplification has been shown to be an independent prognostic factor in MBC [39]. Somatic mutations of CCND1 are not a frequent alteration in cancer, and, in particular have not been reported in BC [19, 40].

Overall, PIK3CA mutations were identified in 2 out of 102 (2%) primary MBCs examined. Both these MBCs were from BRCA1/2 mutation negative patients with no family history of BC, were invasive ductal carcinomas, and presented with high grade (G3), ER-positive, HER2-negative and lymph node positive status. An additional PIK3CA mutation located in exon 20, the c.3140A>G (p.H1047R), was detected in the metastatic lymph node sample examined from a MBC case whose primary tumor was PIK3CA mutation negative (Table 1).

Mutational analysis of EGFR identified two synonymous variants (Table 1): the c.2361G>A (p.Q787Q) occurring in 15 of 102 cases (14.7%), and c.2748C>T (p.N916N) occurring in one case. Mutational analysis of ESR1 identified one synonymous variant c.975G>C
Table 1: Summary of results from PIK3CA, EGFR and ESR1 mutational analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK3CA</td>
<td>9</td>
<td>c.1633G&gt;A</td>
<td>p.E545K&lt;sup&gt;a&lt;/sup&gt;</td>
<td>primary tumor</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>9</td>
<td>c.1634A&gt;C; c.1658_1659delGTinsC</td>
<td>p.E545A&lt;sup&gt;a&lt;/sup&gt;; p.S553Tfs*7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>primary tumor</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>20</td>
<td>c.3140A&gt;G</td>
<td>p.H1047R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>metastatic lymph node</td>
</tr>
<tr>
<td>EGFR</td>
<td>20</td>
<td>c.2361G&gt;A</td>
<td>p.Q787Q&lt;sup&gt;b&lt;/sup&gt;</td>
<td>primary tumor</td>
</tr>
<tr>
<td>EGFR</td>
<td>23</td>
<td>c.2748C&gt;T</td>
<td>p.N916N&lt;sup&gt;b&lt;/sup&gt;</td>
<td>primary tumor</td>
</tr>
<tr>
<td>ESR1</td>
<td>4</td>
<td>c.975G&gt;C</td>
<td>p.P325P&lt;sup&gt;b&lt;/sup&gt;</td>
<td>primary tumor</td>
</tr>
</tbody>
</table>

<sup>a</sup> pathogenic mutation;  
<sup>b</sup> synonymous variant.

All mutations and variants identified are recorded on COSMIC database (http://cancer.sanger.ac.uk/cosmic).

Figure 1: PIK3CA mutations identified in MBCs. Partial electrophorograms from direct sequencing of PIK3CA exon 9 in a case harboring the c.1633G>A (p.E545K) mutation A. and in a case harboring c.1634A>C (p.E545A) and 1658delGTinsC (p.S553Tfs*7) mutations B.

Figure 2: Specific sequencing of PIK3CA exon 9 and pseudogene. Partial electrophorograms from direct sequencing of DNA region using primers specific for PIK3CA exon 9 A. and for pseudogene B.
(p.P325P) in 15/70 cases (21.4%). No pathogenic mutations were identified in $ESR1$ and $EGFR$ genes (Table 1).

**Gene copy number variation analysis**

Overall, gene CNV analysis showed amplification of $PIK3CA$, $EGFR$ and $CCND1$, and deletion of $ESR1$. Specifically, amplification of $PIK3CA$ was detected in 6/74 (8.1%), $EGFR$ in 5/74 (6.8%) and $CCND1$ in 10/62 (16%) of the MBC cases analyzed. Overall, gene amplifications were detected in MBCs from $BRCA1/2$ mutation negative patients. Deletion of $ESR1$ was shown in 11/73 (15%) of the cases, including one case with $BRCA2$ germ-line mutation. Co-amplification of $PIK3CA$, $EGFR$ and $CCND1$ was observed in two MBC cases and co-amplification of $EGFR$ and $CCND1$ in one case. Furthermore, one case showed both $PIK3CA$ mutation and $CCND1$ amplification.

The overall CNV profile was investigated by unsupervised hierarchical clustering analysis performed on 41 MBC cases, for which data on CNVs of all genes examined were available. Hierarchical cluster analysis revealed two groups of cases (Figure 3). One group characterized by no CNV or CNV in only one gene, mainly $CCND1$ amplification, and a second group characterized by CNVs in multiple genes. Notably, this second group included cases characterized by aggressive pathologic features, including high tumor grade (G3), large tumor size (T4) and lymph node involvement.

**Associations between somatic alterations and clinical-pathologic features**

As shown in Table 2, statistically significant differences emerged in the distribution of $EGFR$, $CCND1$ and $ESR1$ CNVs according to ER, HER2, MIB1 and tumor size (T) status. In particular, CNVs of $EGFR$ were associated to ER, HER2, MIB1 and T status, of $CCND1$ to HER2 and MIB1 status and of $ESR1$ to ER status.

By linear logistic regression analysis a statistically significant association was confirmed between $EGFR$ amplification and ER-negative status ($p=0.01$), HER2-positive status ($p=0.03$) and T4 ($p=0.01$). By using a stepwise logistic regression, in which ER, HER2, MIB1 and T status were included, the dependency of these four characteristics in relation to $EGFR$ amplification was analyzed, and only ER was selected as a significant effect ($p=0.044$). Furthermore, by linear logistic regression analysis significant association was confirmed between $CCND1$ amplification and HER2-positive ($p=0.0005$) and MIB1-positive ($p=0.04$) status, and between $ESR1$ deletion and ER-negative status ($p=0.01$).

**DISCUSSION**

In this study we investigated somatic mutations and CNVs of $PIK3CA$, $EGFR$, $CCND1$ and $ESR1$ in a large series of MBCs characterized for germ-line $BRCA1$ and $BRCA2$ mutations. We found that gene CNVs are frequent

![Figure 3: Unsupervised hierarchical clustering analysis of CNVs in EGFR, PIK3CA, ESR1 and CCND1 genes in male breast tumors.](image-url)
Table 2: Frequencies of clinical-pathologic features considered in 103 primary male breast tumors crossed with gene copy number variations (CNVs)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total cases (%)</th>
<th>Gene CNVs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PIK3CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>amp/N</td>
</tr>
<tr>
<td>FH(^c) of breast/ovarian cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>75 (72.8)</td>
<td>6/53</td>
</tr>
<tr>
<td>Positive</td>
<td>28 (27.2)</td>
<td>0/21</td>
</tr>
<tr>
<td>Personal history of cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>77 (74.8)</td>
<td>5/56</td>
</tr>
<tr>
<td>Positive</td>
<td>26 (25.2)</td>
<td>1/18</td>
</tr>
<tr>
<td>BRCA1/2 status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA1 mutated</td>
<td>2 (2)</td>
<td>0/1</td>
</tr>
<tr>
<td>BRCA2 mutated</td>
<td>9 (8.7)</td>
<td>0/7</td>
</tr>
<tr>
<td>BRCA1/2 wild-type</td>
<td>92 (89.3)</td>
<td>6/66</td>
</tr>
<tr>
<td>Hystological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltrating ductal carcinoma</td>
<td>82 (83.7)</td>
<td>5/59</td>
</tr>
<tr>
<td>Other</td>
<td>16 (6.3)</td>
<td>0/13</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15 (14.8)</td>
<td>2/11</td>
</tr>
<tr>
<td>Positive</td>
<td>86 (85.2)</td>
<td>4/62</td>
</tr>
<tr>
<td>PR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24 (23.8)</td>
<td>1/20</td>
</tr>
<tr>
<td>Positive</td>
<td>77 (76.2)</td>
<td>5/53</td>
</tr>
<tr>
<td>HER2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>69 (74.2)</td>
<td>2/46</td>
</tr>
<tr>
<td>Positive</td>
<td>24 (25.8)</td>
<td>4/21</td>
</tr>
<tr>
<td>MIB1 (Ki67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>60 (61.2)</td>
<td>1/38</td>
</tr>
<tr>
<td>Positive</td>
<td>38 (38.8)</td>
<td>5/34</td>
</tr>
<tr>
<td>Hystological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1+G2</td>
<td>61 (64.2)</td>
<td>4/43</td>
</tr>
<tr>
<td>G3</td>
<td>34 (35.8)</td>
<td>2/26</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not involved</td>
<td>43 (55.8)</td>
<td>1/29</td>
</tr>
<tr>
<td>Involved</td>
<td>34 (44.2)</td>
<td>3/26</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>67 (83.6)</td>
<td>3/49</td>
</tr>
<tr>
<td>T4</td>
<td>13 (16.4)</td>
<td>2/10</td>
</tr>
</tbody>
</table>

\(^a\) amp: amplified; del: deleted; N: number of tumors that were successfully analyzed;  
\(^b\) \(p\) value from Chi-square test;  
\(^c\) FH: Family history of breast and/or ovarian cancer.  
In bold \(p\)-value <0.05, considered statistically significant.
in BRCA1/2 mutation negative MBCs and allow for the identification of MBC subsets characterized by pathologic characteristics suggestive of aggressive phenotype. By contrast, somatic mutations were rarely detected and were identified only in PIK3CA gene. We did not detect somatic mutations in ESR1 and EGFR, indicating that EGFR and ESR1 mutations are infrequent in MBC and confirming recent data showing no deleterious somatic mutations of these genes in MBC [6].

In our series PIK3CA mutations were identified in 2% of MBCs. Our data indicate a lower PIK3CA mutation frequency compared with previous data on MBC. PIK3CA mutations have been reported in 18–20% of MBCs, not characterized for BRCA1/2 mutation status, and in about 10% of familial BRCA1/2 mutation negative MBCs [6, 24–26]. Though differences in PIK3CA mutation frequency may be due to different screening methods and sample selection criteria, it is worth noting that our results refer to a MBC series that represents the twice of those previously reported and included hereditary, familial and sporadic cases, all characterized for BRCA1/2 mutation status. Overall, in our series PIK3CA mutations were identified in BRCA1/2 mutation negative MBCs, thus confirming the hypothesis that PIK3CA mutation status is dependent on germ-line genotypes of MBC [25–26].

We detected the co-existence of two PIK3CA mutations, c.1634G>A (p.E545A) and c.1658_1659delGTinsC (p.S553Tfs*7) both located in exon 9, in two independent MBC cases. The concurrence of these two somatic mutations has been previously reported in large B cell lymphoma and non-small cell lung cancer [46–47]. Moreover, the concurrence of these mutations as germ-line mutations has been suggested to predispose to Cowden and Cowden like syndromes [48]. Double mutations in PIK3CA are rare phenomena [49]. To exclude that a pseudogene of PIK3CA located on chromosome 22q11.2 and that retains about 95% homology to the pseudogene, we performed true-specific PIK3CA and pseudogene sequencing analysis. These analyses showed that both c.1634A>C (p.E545A) and c.1658_1659delGTinsC (p.S553Tfs*7) were due to the pseudogene amplification. Similar observations have been reported in previous studies [50–52]. Therefore, when interpreting data corresponding to PIK3CA mutations, attention should be given to the possible co-amplification of PIK3CA gene and its pseudogene.

Interestingly, we detected PIK3CA c.3140A>G (p.H1047R) hotspot activating mutation in a metastatic lymph node of a MBC case that did not harbor PIK3CA mutation in the corresponding primary breast tumor. A different PIK3CA mutation status between primary tumors and corresponding metastatic lesions has been previously reported in FBC [53–54], indicating that PIK3CA mutations may be acquired during tumor progression. This finding may be relevant for clinical management, suggesting the need of assessing PIK3CA mutation status in metastatic lesions for the selection of PIK3CA inhibitor therapy.

We reported the occurrence of PIK3CA amplification at higher frequency than that previously reported in MBC [26]. The frequency of PIK3CA amplification found in our study is consistent with that reported in FBC [27–29]. As for PIK3CA mutations, PIK3CA amplification was found in BRCA1/2 mutation negative MBCs.

In our MBC series EGFR amplification was found at a higher frequency compared to previous data on MBC [26, 39] and FBC [32–33]. Furthermore, we showed that EGFR amplification was associated with ER-negative status, HER2-positive status and large tumor size (T4). These findings suggest that EGFR amplification could represent a prognostic marker in MBC and could identify a subset of MBC patients at high risk for adverse prognosis. Our data also suggest that this subset of MBCs (i.e. ER-negative and HER2-positive) might benefit from target therapy. Interestingly, there is evidence showing that therapies combining HER2 inhibitors with EGFR inhibitors, such as trastuzumab with lapatinib, may produce promising results in MBC treatment [55]. Overall, the association between EGFR amplification and ER-negative status suggest that EGFR signaling pathway may play a role in male breast carcinogenesis, mainly in estrogen-insensitive breast tumors. Interestingly, association between EGFR amplification and ER-negative status was also reported in FBC [32].

CCND1 amplification was the most frequent CNV observed in our MBC series, thus supporting the hypothesis that this gene may play a relevant role in male breast carcinogenesis, particularly in BRCA1/2 mutation negative cases. Our results are in agreement with previous data showing that CCND1 was one of the genes that most frequently showed copy number gain in MBC [39]. In our series, CCND1 amplification identified a subset of MBCs characterized by HER2-positive and MIB1-positive status, pathologic features suggestive of aggressive phenotype. These findings are in line with the function of CCND1 protein product and are consistent with previous data showing that MBCs with CCND1 copy number gain tended to have a higher mean mitotic count compared to tumors without CCND1 amplification [39]. Notably, CCND1 amplification seems to be an independent prognostic factor in MBC correlating with poor survival [39]. Amplification of CCND1 has also been associated with poor prognosis in ER-positive FBC [12, 37]. As CCND1 is an estrogen-responsive gene, one possible mechanism by which its amplification could lead to a poor prognosis is by conferring resistance to hormone therapy [13, 56]. These findings may acquire great importance in MBC, for which endocrine therapy is the most common treatment [3].


*ESR1* deletion was reported at high frequency in our MBC series. Our results are consistent with data reporting gene deletion in MBC [39] and confirm that *ESR1* amplification is not a characteristic alteration of MBC. By contrast, *ESR1* amplification has been reported, although with controversial results, in FBC in which *ESR1* deletion does not appear to be a significant event [45]. In our series, a significant association emerged between *ESR1* gene deletion and ER-negative status. Interestingly several data suggest that low-level of *ESR1* expression may be one of the mechanisms responsible for tamoxifen resistance in ER-positive FBC [57]. These observations confirm the utility of our results in the identification of MBC subsets that may need specific therapeutic approach.

In agreement with the hypothesis that tumors rising on gene amplification are more likely to develop multiple amplifications as they have greater genetic instability [15], in this study we identified co-amplification of genes analyzed. By cluster analysis, we identified a group of cases characterized by co-amplification of two or more genes, suggesting the need to develop new therapeutic strategies involving multiple targets.

Overall, our data indicate that genes known to be important prognostic biomarkers and molecular therapeutic targets in FBC are also important in MBC. For instance, adjuvant trastuzumab, a monoclonal antibody that selectively targeted HER2 and is used as therapeutic agent in HER2-positive FBCs, can be considered for high-risk HER2-positive MBCs [58]. However, differences emerged between male and female breast cancers, emphasizing the importance of identifying biomarkers based on research in MBC.

In conclusion our results showed that CNVs of *PIK3CA, EGFR, CCND1* and *ESR1* genes may play a relevant role in male breast carcinogenesis and could lead to the identification of specific molecular profiles. In particular, amplification of targetable oncogenes is frequent in *BRCA1/2* mutation negative MBCs and may identify biomarkers relevant for clinical management and targeted therapeutic approach.

MATERIALS AND METHODS

Patients and genomic DNA

A series of 103 primary male breast tumors was included in the study. A metastatic lymph node sample was available for one case and was also included in the analyses. All MBC cases were characterized for germ-line *BRCA1* and *BRCA2* mutations and for the main clinical-pathologic features, including: age at diagnosis, family and personal history of cancer, tumor histological type, grade (G), nodal status, tumor size (T), estrogen receptor (ER), progesterone receptor (PR), MIB1 (Ki67) and HER2 expression (Table 2). The expression of ER, PR, and Ki67/MIB1 was scored based on the percentages of positive nuclei (ER/PR positive if >10%; Ki67/MIB1 high if >20%) over the total number of counted cancer cell nuclei HER2 positivity was defined as a score of 3+ using immunohistochemistry (IHC) test, or amplification shown by fluorescence in situ hybridization (FISH), in equivocal cases [59–60].

Genomic DNA was isolated from microdissected formalin fixed paraffin-embedded (FFPE) and fresh frozen tumor sections. DNA from FFPE sections was extracted using DNA/RNA FFPE kit (Qiagen Inc., Venlo, Netherlands) according to the manufacturer’s instructions. DNA from fresh frozen samples was extracted by following standard phenol-chloroform extraction protocol. For some cases the amount of DNA was inadequate to carry out all molecular analyses.

The participants signed an informed consent form with a detailed description of the study protocol. The study was approved by The Local Ethical Committee (Sapienza University of Rome, Protocol 264/12).

Mutational screening

Mutational analysis of *PIK3CA, EGFR* and *ESR1* was performed mainly by direct sequencing and by SSCP (Single Strand Conformation Polymorphism). Genomic DNA from each tumor sample was amplified using primers covering exons in which is reported to cluster the great majority of mutations (Supplementary Table S1), in particular exons 9 and 20, coding the helical and kinase domains of *PIK3CA*, respectively [61]; exons from 18 to 24, coding kinase domain of *EGFR*; exons from 4 to 8, coding the ligand binding domain, of *ESR1*.

To overcome the interference of a pseudogene of *PIK3CA*, we also designed specific primers for both *PIK3CA* gene and pseudogene (Supplementary Table S1). DNA samples from cell lines (HCC15 and HCT16) with known mutations in exons 9 (c.1633G>A, p.E545K) and 20 (HCT-116 c.3140 A>G, p.H1047R) of *PIK3CA*, were used as positive control.

Gene copy number variation analysis

*PIK3CA, EGFR, ESR1* and *CCND1* gene copy variations (CNVs) were analyzed by Real-Time PCR, using TaqMan probe-based technology (Applied Biosystems, Foster City California, USA). The RNaseP as housekeeping gene and a normal male breast tissue sample as calfibrator were used. TaqMan Copy number Assays (Hs02708380_cn for *PIK3CA*, Hs00756893_cn for *EGFR*, Hs02577150_cn for *ESR1* and Hs01818912_cn for *CCND1*) and TaqMan Copy number Reference Assay human RNaseP, containing primers and probe mix for target and housekeeping genes respectively, were used (Applied Biosystems, Foster City California, USA). The analysis of relative gene expression data was performed, using the 2^{-ΔΔCt} method. The fold change in studied gene
copy number, normalized to endogenous control, was calculated using $RQ = 2^{\Delta\Delta Ct}$ [62–63]. Values of $RQ \geq 2$ were considered amplified and values of $RQ < 0.5$ were considered deleted.

**Statistical analysis**

Unsupervised hierarchical clustering was performed to analyze relevant clusters characterized by co-amplification/deletion in *EGFR, PIK3CA, ESR1* and *CCND1*.

Chi square test and logistic regression models were performed in order to evaluate the potential associations between each gene alteration (mutations and CNVs) and clinical-pathologic features. A P value $< 0.05$ was considered statistically significant. All statistical analyses were performed with the R software (www.r-project.org).

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**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

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