Published in final edited form as:

J Pathol. 2017 February ; 241(3): 405–419. doi:10.1002/path.4837.

The Genetic Landscape of Breast Carcinomas with Neuroendocrine Differentiation

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Abstract

Neuroendocrine breast carcinomas (NBCs) account for 2–5% of all invasive breast cancers and are histologically similar to neuroendocrine tumours from other sites. They typically express oestrogen receptor (ER), are HER2-negative and of luminal 'intrinsic' subtype. Here we sought to define the mutational profile of NBCs, and to investigate whether NBCs and common forms of luminal (ER+/HER2-) breast cancer display distinct repertoires of somatic mutations. Eighteen ER+/HER2- NBCs, defined as harbouring >50% of tumour cells expressing chromogranin A and/or synaptophysin, and matched normal tissue were microdissected and subjected to massively parallel sequencing targeting all exons of 254 genes most frequently mutated in breast cancer and/or related to DNA repair. Their mutational repertoire was compared to that of ER+/HER2- (n=240), PAM50-defined luminal breast cancers (n=209 luminal A; n=111 luminal B) and invasive lobular carcinomas (n=127) from The Cancer Genome Atlas. NBCs were found to harbour a

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Conflict of interest: The authors have no conflicts of interest to declare.

Author contributions: BW and JSR-F conceived the study; CM, BW and JSR-F supervised the work; CM, MP, AS provided samples; CM, FCG, AS and JSR-F reviewed the cases; MC performed the immunohistochemistry; CM performed the tissue microdissection; SP performed Sanger sequencing; massively parallel sequencing analysis was performed by CKYN, MRDF, KAB, RSL; CKYN performed statistical analyses; CM, FCG, CKYN, EGR, AMS, LN, BW and JSR-F analysed and interpreted the data; CM and FCG wrote the first draft of the manuscript, which was initially reviewed by BW and JSR-F. All authors edited and approved the final draft of the manuscript.

median of 4.5 (range 1-11) somatic mutations, similar to that of luminal B breast cancers (median=3, range 0-17) but significantly higher than that of luminal A breast cancers (median=3, range 0-18, $p=0.02$). The most frequently mutated genes were *GATA3*, *FOXA1*, *TBX3*, *ARID1A* (3/18, 17%), and *PIK3CA*, *AKT1*, *CDH1* (2/18, 11%). NBCs less frequently harboured *PIK3CA* mutations than common forms of ER+/HER2, luminal A and invasive lobular carcinomas ($p<0.05$) and displayed a significantly higher frequency of somatic mutations affecting *ARID1A* (17% versus 2%, $p<0.05$) and the transcription factors *FOXA1* (17% versus 2%, $p=0.01$) and *TBX3* (17% versus 3%, $p<0.05$) than common-type ER+/HER2- breast cancers. No *TP53* somatic mutations were detected in NBCs. Compared to common forms of luminal breast cancers, NBCs display a distinctive repertoire of somatic mutations featuring lower frequency of *TP53* and *PIK3CA* mutations, and enrichment for *FOXA1*, *TBX3* mutations, and akin to neuroendocrine tumours from other sites, *ARID1A* mutations.

Keywords

breast cancer; neuroendocrine differentiation; chromogranin A; synaptophysin; massively parallel sequencing; copy number alterations; somatic mutations

Introduction

Breast carcinomas with neuroendocrine differentiation (also known as neuroendocrine breast carcinomas, NBCs) account for 2–5% of all invasive breast cancers. These tumours usually affect elderly women and are defined by the World Health Organization (WHO) as invasive carcinomas exhibiting morphologic characteristics similar to those of neuroendocrine neoplasms of other organs, including the gut [1]. Tumour cells are typically arranged in solid nests and/or trabeculae separated by delicate fibrovascular stroma; rosettes and peripheral palisading may be found [1]. The diagnosis of NBCs relies on the expression of neuroendocrine markers. A pragmatic threshold of >50% of neuroendocrine marker expression in tumour cells has been suggested [2], though the latest WHO classification has clarified that this cut-off was set arbitrarily [1]. Depending on the threshold and the particular immunohistochemical markers employed, the prevalence of neuroendocrine differentiation varies between 4% and 18% of breast cancers [3-6]. Nevertheless, regardless of thresholds and markers for neuroendocrine differentiation, there is substantial inter-tumour histological heterogeneity within NBCs, as demonstrated by the fact that only a subgroup of breast carcinomas showing neuroendocrine differentiation as defined by immunohistochemistry harbours the typical neuroendocrine morphology [4, 5]. Indeed, NBCs may display features shown by non-neuroendocrine carcinomas, including apocrine differentiation [7], or architectural features consistent with other histologic special types, such as cellular mucinous carcinomas (i.e. type B) and solid papillary carcinomas [8].

NBCs are typically oestrogen receptor-positive (ER+) and HER2-negative (HER2-) and more frequently of luminal A than of luminal B 'intrinsic' subtype [9]. We have previously shown that neuroendocrine and mucinous carcinomas are transcriptionally distinct from invasive ductal carcinomas (IDCs) of no special type (also known as invasive carcinomas of no special type, IC-NSTs) and that neuroendocrine and type B mucinous carcinomas share

similar transcriptomic profiles [9]. Although NBCs are preferentially of luminal A subtype and may sometimes be classified as of other histologic type with favourable prognosis, a retrospective analysis has suggested that patients with NBCs display a worse prognosis than those with IC-NSTs [5, 10], and may benefit less from endocrine therapy [10]. Therefore, the impact of neuroendocrine differentiation on the prognosis of breast cancer patients is uncertain. Further, it remains to be determined whether neuroendocrine differentiation in breast cancer is associated with, or driven by, highly recurrent somatic genetic alterations or a distinctive repertoire of mutations.

Massively parallel sequencing (MPS) studies have revealed that breast cancers comprise a heterogeneous group of cancers, with only three genes being mutated in >10% of the tumours (i.e. *TP53*, *PIK3CA* and *GATA3*) [11, 12]. The Cancer Genome Atlas (TCGA) study has revealed that although luminal A and B, HER2-enriched and basal-like PAM50-defined molecular subtypes harbour distinct repertoires of somatic genetic alterations [12], there is no highly recurrently mutated gene or highly recurrent mutation that defines each 'intrinsic' subtype [11]. It should be noted, however, that large-scale MPS endeavours have so far focused primarily on the most common types of breast cancer (IC-NSTs [12-15] and invasive lobular carcinomas [16]).

The aims of this study were to investigate whether NBCs harbour mutations affecting the genes most frequently mutated in breast cancer, and if these cancers would display a repertoire of somatic mutations affecting the genes most frequently mutated in breast cancer and DNA repair-related genes that is distinct from that of common forms of luminal (ER+/HER2-) breast cancer.

Materials and Methods

Cohort and immunohistochemistry

From the files of the Pathology Unit of Azienda Ospedaliera Universitaria Città della Salute e della Scienza di Torino we selected 18 invasive breast carcinomas showing neuroendocrine differentiation, as defined by morphologic features reminiscent of neuroendocrine differentiation and immunohistochemical expression of chromogranin A and/or synaptophysin in >50% of tumour cells [2]. Samples were anonymized prior to analysis. This study was approved by the Institutional Review Board (IRB) of the Department of Medical Sciences of the University of Turin. Patient consent was obtained where appropriate, following the protocol approved by the IRB. Representative H&E-stained sections of each case were reviewed by four of the authors (CM, FCG, AS, JSR-F), who performed a central review of the cases, with detailed analysis of their histologic features, histologic typing [8] and histologic grading [17]. As previously recommended [18], NBCs were typed according to their main histologic features following the 2012 WHO classification [8]. For instance, NBCs fulfilling the criteria for a diagnosis of mucinous carcinoma were classified as NBC-mucinous carcinomas, whereas NBCs lacking the features of a recognized histologic special type were classified as IDCs with neuroendocrine differentiation according to the latest WHO classification.

Immunohistochemical analysis of ER, progesterone receptor (PR), HER2, Ki67, chromogranin A, synaptophysin and E-cadherin was performed as previously described [19] (Supplementary Methods and Supplementary Table S1).

Microdissection and DNA extraction

Eight- μ m-thick sections from representative formalin-fixed paraffin-embedded blocks of the tumour and normal breast tissue from each case were microdissected as previously described [20] to ensure >80% of tumour cell content and that the normal tissue was devoid of neoplastic cells (Supplementary Methods). Genomic DNA was extracted and quantified as previously described [21] (Supplementary Methods).

Targeted capture massively parallel sequencing (MPS)

DNA samples from microdissected normal and tumour components were subjected to targeted capture MPS at the Memorial Sloan Kettering Cancer Center (MSKCC) Integrated Genomics Operation (IGO) using a customized breast cancer panel targeting all exons of 254 genes recurrently mutated in breast cancer and/or DNA repair-related genes (Supplementary Table S2) as previously described [20]. Sequencing analysis was performed essentially as reported in Piscuoglio *et al* [20] (Supplementary Methods) using state-of-the-art bioinformatics tools [21-23]. The potential functional effect of each single nucleotide variant (SNV) was investigated using a combination of MutationTaster [24] and CHASM [25], as previously reported [26]. Genes affected by mutations were further annotated according to their presence in three cancer gene datasets, Kandath *et al* [27], the Cancer Gene Census [28] and Lawrence *et al* [29]. Sequencing data have been deposited in the NCBI Sequence Read Archive under accession code SRP071884

Sanger sequencing

Selected somatic mutations, including putative recurrent mutations, were further validated by Sanger sequencing as previously described [21] (Supplementary Methods, Supplementary Table S3). All analyses were performed in duplicate.

Comparisons with invasive breast cancers from the TCGA dataset

To compare the mutational frequencies of NBCs to those of ER+/HER2-, PAM50 luminal A/B invasive breast cancers and to those of invasive lobular carcinomas we retrieved the clinico-pathologic data and whole-exome sequencing-derived mutational data from the TCGA data portal (accessed in May 2016) essentially as previously described [20] (Supplementary Methods). Specific subgroup analyses are described in the Supplementary Methods. Comparisons were performed using two-tailed Fisher's exact tests and the confidence intervals for the odds ratio estimated using the minimum likelihood method [30].

Identification of transcript-defined NBCs (TD-NBCs)

As an exploratory, hypothesis-generating analysis, we sought to define TD-NBCs on the basis of mRNA expression profiles and to compare their characteristics with those of the NBCs included in this study and of ER+/HER2- breast cancers from the TCGA study. Detailed methods for the centroid generation, classification of the TCGA samples,

hierarchical cluster analysis, cluster robustness assessment and statistical considerations are described in the Supplementary Methods.

Results

NBCs display heterogeneous histologic features

As an inclusion criterion, all NBCs of the present study showed >50% of chromogranin A and/or synaptophysin expression (Table 1). Chromogranin A was expressed in a granular pattern and, in some cases, in the form of paranuclear dots, whereas synaptophysin displayed a diffuse cytoplasmic expression of varying intensity (Figure 1). The 15 carcinomas classified as IDCs with neuroendocrine differentiation displayed heterogeneous histologic features; however, as a group, these tumours displayed clearly infiltrative, large rounded solid nests and/or trabeculae of cells immersed in a hyalinised stroma. Cells were often juxtaposed to delicate fibrovascular structures, reminiscent of solid papillary differentiation. Three of those 15 cases showed focal solid papillary formations, but did not fulfil criteria to be classified as solid papillary carcinomas [8]. Of these cases, 11 (73%) were of nuclear grade 2, and, akin to neuroendocrine neoplasms of other sites, nuclei were homogeneous across the whole tumour. Three cases were classified as special histologic types; of these cases, two were classified as grade 2 mucinous type B (hypercellular) carcinomas and one as a grade 2 solid variant invasive lobular carcinoma with a minor signet ring cell component (Table 1). None of the NBCs here analysed displayed histological features of small cell carcinoma.

Immunohistochemical analysis revealed that all cases showed a luminal-like phenotype (i.e. ER+/HER2- (Table 1)). E-cadherin immunohistochemistry revealed complete lack of expression in the sole case diagnosed as an invasive lobular carcinoma. In addition, reduced and/or heterogeneous staining was observed in 6/18 (33%, Table 1, Supplementary Figure S1). The remaining 11 cases displayed strong, membranous expression of E-cadherin, similar to the pattern observed in the epithelial cells of adjacent normal breast.

Mutational repertoire of NBCs

Targeted capture MPS was performed at a median depth of 568× (448×-754×) and resulted in the identification of a median of 4.5 somatic mutations affecting the 254 genes tested (range 1 - 11). A re-analysis of the 240 ER+/HER2-, 209 luminal A and 111 luminal B from the TCGA breast cancer study revealed a median of three somatic mutations (range 0 - 18 for ER+/HER2- and luminal A, range 0 - 17 for luminal B) affecting the exons of the 254 genes included in our targeted panel [12]. A median of three somatic mutations was also found for invasive lobular carcinomas (range 0 - 56). Whilst the frequency of somatic mutations was comparable between NBCs and ER+/HER2-, luminal B breast cancers and invasive lobular carcinomas from the TCGA, as also highlighted by the interquartile range and highest and lowest value of number of mutations excluding outliers (Figure 2), there was a significantly higher number of somatic mutations affecting these 254 genes in NBCs than in the luminal A breast cancers from TCGA ($p=0.02$, Mann-Whitney U test; Figure 2). Although these differences may reflect the biology of NBCs, it is also plausible that they

may stem from the fact that in our study cases were microdissected and sequencing depth was substantially higher than that of breast cancers analysed by TCGA.

The most frequently mutated cancer genes in NBCs were *GATA3*, *FOXA1*, *TBX3* and *ARID1A* (all mutated in three of 18 cases, 17%), followed by *PIK3CA*, *AKT1* and *CDH1* (all mutated in two of 18 cases, 11%, Figure 3, Supplementary Table S4). As expected, the lobular carcinoma (CMNE24T) was one of the two cases harbouring a *CDH1* mutation; in this case, the E806K missense *CDH1* mutation was coupled with LOH of the wild-type allele and predicted to be clonal (i.e. present in 100% of the cancer cells). Immunohistochemical analysis also revealed lack of E-cadherin expression. The other case harbouring a *CDH1* somatic mutation (CMNE22T) was classified as an IDC with neuroendocrine differentiation. A thorough review of this case failed to reveal any unequivocal areas consistent with an invasive lobular carcinoma and confirmed membranous expression of E-cadherin. In this case, the R598Q missense *CDH1* mutation was also coupled with LOH of the wild-type allele, however was predicted to be subclonal (present in 16% of cancer cells; Supplementary Table S4), potentially explaining the lack of overt lobular differentiation. None of the NBCs analysed harboured *TP53* somatic mutations. Sanger sequencing analysis of selected mutations resulted in a validation rate of 100% (26/26) and confirmed the presence of the *FOXA1*, *ARID1A*, *GATA3*, *CDH1*, *AKT1*, *PIK3CA*, *KIT*, *HUWE1*, *TBX3*, *KMT2C* and *SPEN* somatic mutations (Supplementary Figure S2).

When compared with immunohistochemically defined ER+/HER2- breast carcinomas from TCGA (n=240), NBCs less frequently harboured *PIK3CA* mutations than common type ER+/HER2- breast cancers (11% versus 42%, $p=0.01$, Fisher's exact test, Table 2), and displayed a significantly higher frequency of somatic mutations affecting the chromatin remodelling gene *ARID1A* (17% versus 2%, $p=0.01$, Fisher's exact test), akin to pulmonary carcinoids [31], and the transcription factors *FOXA1* and *TBX3* (17% versus 2% and 17% versus 3%, $p=0.01$ and $p=0.03$, respectively, Fisher's exact tests). *TP53* was significantly less frequently mutated in NBCs than in ER+/HER2- breast carcinomas from TCGA (0% versus 22%, $p=0.01$, Fisher's exact test).

As an exploratory, hypothesis-generating analysis, we compared the repertoires of somatic mutations found in NBCs with those of PAM50-defined luminal A (n=209) and luminal B (n=111) breast cancers from TCGA. NBCs displayed a repertoire of somatic mutations that was intermediate between those of luminal A and luminal B breast cancers, with a significantly higher frequency of *FOXA1*, *ARID1A* and *DCHS2* mutations in NBCs (all mutated in 17% of cases) than in both luminal A (2%, 2%, 1%, respectively, $p<0.05$, Fisher's exact tests) and luminal B breast cancers (2%, 3% and 2%, respectively, $p<0.05$, Fisher's exact tests; Table 2, Figure 4). In addition, *PIK3CA* mutations were significantly less frequent in NBCs (11%) than in luminal A cancers (46% $p<0.01$, Fisher's exact test), whereas none of the NBCs displayed *TP53* mutations as compared to 32% of luminal B cancers ($p<0.01$, Fisher's exact test) and 11% of luminal A cancers ($p=0.23$, Fisher's exact test; Table 2, Figure 4). Conversely, *TBX3* and *HUWE1* were more frequently found to be mutated in NBCs (17% and 11%, respectively) than in luminal A breast cancers (2% and 1%, $p<0.05$, Fisher's exact tests, Table 2, Figure 4).

Given that lobular and mucinous carcinomas have been shown to differ from IC-NSTs at the molecular level [9, 16, 32-34], we sought to investigate whether IDCs with neuroendocrine differentiation would constitute a more homogeneous group at the genetic level and harbour a mutational landscape different from that observed in unselected breast carcinomas. We therefore performed an exploratory comparative analysis of the set of 15 IDCs with neuroendocrine differentiation with ER+/HER2- (n=210) and PAM50-defined luminal A (n=179) and luminal B (n=105) carcinomas from TCGA, from which lobular, mucinous and neuroendocrine carcinomas were excluded (Table 3). These subgroup analyses highlighted that *FOXA1*, *TBX3* and *DCHS2* were significantly more frequently mutated in IDCs with neuroendocrine differentiation *versus* any of the three subgroups from TCGA (all 20% in NBCs *versus* 2% in ER+/HER2- and luminal A and 2%, 4%, 2% in luminal B breast cancers, respectively, $p < 0.05$, Fisher's exact tests). In addition, the lower frequencies of *PIK3CA* mutations (13% *versus* 42% in ER+/HER2-, $p = 0.03$, and 47% in luminal A breast cancers from TCGA, $p = 0.01$, Fisher's exact test) and the lack of *TP53* mutations (0% *versus* 25% in ER+/HER2-, $p = 0.02$, and 33% in luminal B, $p < 0.01$, Fisher's exact test) in IDCs with neuroendocrine differentiation remained statistically significant.

Given that some genes found to be recurrently mutated in NBCs, such as *FOXA1* and *TBX3*, have been recently described as part of the molecular determinants of invasive lobular carcinomas [16], we endeavoured to assess whether similarities could be found between our cohort of NBCs and lobular carcinomas from TCGA in terms of mutations affecting the exons of the 254 genes included in the targeted panel employed in this study. In addition to the statistically significant higher frequency of *CDH1* mutations in invasive lobular carcinomas than in NBCs (63% *versus* 11%, $p < 0.01$, Fisher's exact test, Table 2, Figure 4), we observed that NBCs less frequently harboured *PIK3CA* mutations (11% *versus* 48%, $p < 0.01$, Fisher's exact test, Table 2) and more frequently displayed *DCHS2*, *MACF1* and *CTCF* mutations (17%, 11%, 11%) than invasive lobular carcinomas (1%, 1%, 0% Fisher's exact tests, Table 2, Figure 4). Overlapping results were obtained when the comparison was restricted to the 15 IDCs with neuroendocrine differentiation (Table 3).

Patterns of gene copy number alterations in NBCs

NBCs harboured recurrent copy number alterations including gains of 1q, 8q, 12q, 14p, 16p and losses of 1p, 6p, 11q, 14q and 16q (Figure 5). Concurrent 1q gains and 16q losses, which represent the hallmark genetic aberrations found in 75% of grade 1/2 luminal breast cancers [35], were observed in 5/18 (28%) cases and in none of the mucinous carcinomas with neuroendocrine differentiation, in agreement with our previous findings [32, 36]. Amplifications affecting loci reported to be recurrently amplified in breast cancer were observed in NBCs, including 8p11.23-11.21 encompassing *ZNF703*, *FGFR1* and *POLB* (two cases), 8q24.12 encompassing the *DEPTOR* gene locus (three cases), and of 8q24.3, a region encompassing the *PTK2*, *EPPK1* and *PLEC* genes (three cases, Table 4). In addition, *FOXA1* and *ERBB4* gene amplifications were found in one case each (Table 4). No homozygous deletions were detected.

A comparison of ER+/HER2- NBCs and luminal A/B breast carcinomas from TCGA revealed that the only statistically significant differences were gains of 1q, 8q, 17q and 20q,

which were more frequently found in luminal B breast cancers from TCGA than in NBCs ($p < 0.05$, Fisher's exact tests; Figure 5). Of note, when the comparison was performed between IDCs with neuroendocrine differentiation and breast carcinomas from TCGA from which we excluded lobular, mucinous and neuroendocrine carcinomas, no significant differences were detected in terms of copy number changes between NBCs and any of the groups analysed (Supplementary Figure S3).

Finally, the comparison between NBCs and invasive lobular carcinomas from TCGA revealed that 1q gains and 16q losses were significantly more frequently found in invasive lobular carcinomas than in NBCs (also after exclusion of mucinous and lobular carcinomas, $p < 0.05$, Fisher's exact tests, Figure 5 and Supplementary Figure S3). This is not surprising given that these genetic alterations represent hallmark features of invasive lobular carcinomas [16, 33] and that we observed concurrent 1q gains and 16q losses only in 28% of NBC, as reported above.

Unsupervised clustering analysis of NBCs and ER+/HER2- breast cancers from the TCGA dataset

As an exploratory, hypothesis-generating analysis, we sought to define whether the genetic differences between NBCs and IC-NSTs identified in this study could be generalized in a larger dataset. We first employed a centroid derived from the reanalysis of the NBCs and mucinous carcinomas from Weigelt *et al* [9], and used this centroid to identify the ER+/HER2- breast cancers from the TCGA breast cancer study that would display neuroendocrine differentiation based on transcriptomic data (i.e. transcript-defined or TD-NBCs, see Supplementary Methods). Of the 241 ER+/HER2- cases of the TCGA dataset, 53 (22%) were classified as TD-NBCs. An unsupervised clustering analysis based on the repertoire of somatic mutations of the NBCs included in this study and all ER+/HER2- breast cancers from the TCGA study demonstrated that 17/18 (94%) NBCs of our cohort belonged to a single stable cluster ('NBC-enriched cluster', Figure 6 and Supplementary Figure S4). Importantly, this NBC-enriched cluster was also significantly enriched for TD-NBCs (29/53, 55%, $p = 0.01$, Fisher's exact test, Figure 6), suggesting that TD-NBCs display a repertoire of somatic mutations similar to that of histologically-defined NBCs. Of note, the same cluster was also significantly enriched for mucinous carcinomas (6/7, 87%, $p = 0.044$, Fisher's exact test, Figure 6). Consistent with the results of the comparisons between NBCs and common forms of ER+/HER2- carcinomas (Figure 4), when the mutational repertoires of carcinomas from the NBC-enriched cluster were compared with those of the remaining ER+/HER2- breast cancers from TCGA, *PIK3CA*, *TP53*, *ARID1A*, *FOXA1* and *SPEN* were found to be mutated at significantly different frequencies (all p -values < 0.05 , Fisher's exact tests, Table 5).

Discussion

Here we have demonstrated that NBCs harbour a repertoire of somatic mutations distinct from that of common types of ER+/HER2- breast cancer, and that the constellation of somatic mutations in NBCs appears to be intermediate between that reported for PAM50-defined luminal A and luminal B breast cancers from TCGA. Furthermore, our study

provides yet another line of evidence demonstrating that breast carcinomas with neuroendocrine differentiation comprise a rather heterogeneous group of invasive breast cancers.

Whether NBCs constitute a special histologic type of breast cancer remains a matter of controversy. Although evidence of neuroendocrine differentiation in breast tumours has been investigated and acknowledged for decades, there is little consensus on its clinical and biological significance [18]. Part of this uncertainty stems from differing definitions of what constitutes an NBC, leading to inconsistencies in their classification [18]. In this study we investigated a cohort of invasive breast carcinomas displaying neuroendocrine morphological features as well as chromogranin A and/or synaptophysin expression in >50% of tumour cells, which were further classified according to the recognizable histologic subtype, as previously recommended [18]. The genetic profiles of the two mucinous and one lobular carcinomas with neuroendocrine differentiation analysed here were consistent with those expected for their histologic subtypes regardless of neuroendocrine differentiation. Indeed, akin to mucinous carcinomas, which have been shown to lack concurrent 1q gains and 16q losses [32, 36] and *PIK3CA* somatic mutations [37], the two mucinous NBCs did not harbour these genetic alterations. In addition, the lobular NBC harboured a *CDH1* mutation and concurrent 1q gain and 16q loss, akin to classic invasive lobular carcinomas [16, 33]. Our data therefore support the current WHO recommendation of typing invasive carcinomas according to their special cytological and architectural features rather than the presence of neuroendocrine differentiation [8, 18].

Despite the histologic heterogeneity of NBCs, our data suggest that a subset of NBCs may constitute a distinct subgroup of invasive breast cancer. Histologically, the morphology of 15 'IDCs with neuroendocrine differentiation' was rather homogenous and sufficiently distinctive to prompt an investigation of neuroendocrine differentiation. This subset of NBCs harboured a relatively simple constellation of copy number alterations and a low prevalence of concurrent 1q gain and 16q losses, lacked *TP53* mutations, and less frequently harboured *PIK3CA* mutations than common forms of ER+/HER2- and PAM50-defined luminal A breast cancers. Notably, these genomic data highlight the genetic similarities between IDCs with neuroendocrine differentiation and mucinous carcinomas. These observations are further supported by the comparison of the repertoire of somatic genetic alterations found in NBCs with that of mucinous carcinomas from the METABRIC study [38, 39] (Supplementary Figures S5 and S6). Taken together, these results are consistent with the data of our previous transcriptomic analysis demonstrating that NBCs and mucinous carcinomas are transcriptionally distinct from IC-NSTs and that NBCs and type B mucinous carcinomas share similar transcriptomic profiles [9].

Interestingly, our genomic data also suggest some similarities between NBCs and invasive lobular carcinomas. Although evidence of association between lobular carcinomas and neuroendocrine differentiation has been previously reported [3, 40, 41], here we provide the novel observation that some somatic genetic alterations are shared by NBCs and lobular carcinomas. Indeed, NBCs were enriched for mutations affecting the transcription factors *FOXA1* and *TBX3*, which have been reported to be more frequently mutated in lobular than ductal carcinomas of no special type [16]. Although one of the *CDH1* mutations found in the

NBCs analysed here was found in an invasive lobular carcinoma with neuroendocrine differentiation, the *TBX3* and *FOXA1* somatic mutations were detected in NBCs of subtypes other than invasive lobular carcinoma.

We also investigated whether NBCs would display a distinct pattern of E-cadherin expression. Reduced E-cadherin expression was observed not only in the invasive lobular carcinoma, but also in 33% of the NBCs here analysed, some of which showed negative areas at the front of invasion. Neuroendocrine tumours (NETs) of the gastrointestinal tract have been reported to lack E-cadherin expression [42], whereas reduced E-cadherin expression has been reported in a prostate adenocarcinoma with neuroendocrine differentiation [43]. In addition, reduction of E-cadherin expression has been observed in mucinous carcinomas of the breast associated with foci of lobular intraepithelial neoplasia [44]. Further studies to define the mechanisms of the reduction of the expression of E-cadherin in neuroendocrine tumours are warranted.

NBCs, albeit displaying some similarities in their repertoire of somatic genetic alterations with mucinous and lobular carcinomas, were also found to share some recurrently mutated genes with NETs of other anatomical sites. Interestingly, NETs harbour recurrent mutations affecting chromatin remodelling genes, such as *ARID1A* (17% in NBCs and 6.8% in lung carcinoids) [31] and *ATRX* (5% in NBCs and 40% in pancreatic NETs) [45]. Furthermore, the lack of *TP53* mutations in the NBCs analysed here is consistent with previous observations in regards to the prevalence of *TP53* mutations in non-small cell neuroendocrine carcinomas from other anatomical sites [31, 46]. Further studies to dissect similarities and differences in the repertoire of somatic mutations in NBCs and NETs from other anatomical sites employing more comprehensive sequencing methods (e.g. whole-exome sequencing and whole-genome sequencing) are warranted.

This study has several limitations including the small sample size, which precluded subgroup analyses between IDCs with neuroendocrine differentiation and special histologic types displaying neuroendocrine differentiation. It should be noted, however, that NBCs are rather rare. Secondly, the exploratory, hypothesis-generating analyses comparing NBCs stratified according to 'intrinsic' gene subtypes were performed using an immunohistochemical surrogate for the classification of NBCs, whereas the TCGA samples were classified using PAM50. Importantly, however, the differences observed remained significant even when NBCs were compared to ER+/HER2- breast cancers from TCGA. Third, the lack of follow-up information precluded comparisons between the genomic findings and the outcome of NBC patients. Fourth, the definitions of the TD-NBCs and NBCs are not interchangeable, and the former constitutes only an approximation of the latter. In addition, the repertoire of somatic mutations found in TD-NBCs appears to be more heterogeneous than that of NBCs defined based on morphology and immunohistochemically-assessed expression of neuroendocrine markers. Despite these limitations, tumours included in the 'NBC-enriched cluster' were found to differ from the remaining ER+/HER2- breast cancers on the basis of mutations affecting *PIK3CA*, *TP53*, *ARID1A* and *FOXA1*, consistent with the results obtained through the analysis of NBCs. Finally, this study did not result in the identification of a pathognomonic or highly recurrent genetic alteration in NBCs. It should be noted, however, that we have employed a targeted

capture sequencing approach with baits targeting all coding regions of 254 genes recurrently mutated in breast cancer and/or related to DNA repair. It is plausible that more comprehensive analyses of NBCs using whole-genome, whole-exome and RNA-sequencing approaches may reveal somatic genetic alterations that define neuroendocrine differentiation. Importantly, however, whole-exome analyses of NETs from other sites failed to reveal pathognomonic mutations in those tumours [31, 45, 47, 48].

In conclusion, here we demonstrate that NBCs are a heterogeneous group of tumours that, as a group, differ from common forms of ER+/HER2- breast based on their lack of *TP53* mutations, low frequency of *PIK3CA* mutations (similar to mucinous carcinomas) and enrichment of *FOXA1* and *TBX3* mutations (similar to lobular carcinomas). In a way akin to NETs of other sites, NBCs also harbour mutations in chromatin remodelling genes, including *ARID1A* and *ATRX*. Finally, we confirmed that neuroendocrine features may occur in breast cancers displaying the hallmark features of WHO-defined histologic special types, including mucinous and invasive lobular carcinomas. Such cases harbour somatic genetic alterations consistent with those reported for the special histologic type of breast cancer analysed, suggesting that neuroendocrine differentiation in these tumours may have developed after the acquisition of the genetic/epigenetic features that define the special histologic phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported in part by a Breast Cancer Research Foundation grant (JSR-F). CM was funded in part by AIRC (MFAG13310), SP in part by a Susan G Komen Postdoctoral Fellowship Grant (PDF14298348), AMS by a stipend from the German Cancer Aid (Dr. Mildred Scheel Stiftung). Research reported in this paper was supported in part by a Cancer Center Support Grant of the National Institutes of Health/National Cancer Institute (grant No P30CA008748). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1. Breast carcinomas with neuroendocrine differentiation (neuroendocrine breast carcinomas, NBCs)

Representative micrographs of an invasive ductal carcinoma with neuroendocrine differentiation (A; haematoxylin and eosin (H&E); B: chromogranin A), a mucinous type B breast cancer (C: H&E, D: synaptophysin) and an invasive lobular carcinoma of solid variant with signet ring cell component (E: H&E, F: synaptophysin). Original magnification 100×.

Figure 2. Box-and-whisker plot illustrating the number of somatic mutations in NBCs and in common forms of luminal breast carcinomas and invasive lobular carcinomas from TCGA
 The median number of synonymous and non-synonymous somatic mutations in NBCs *versus* immunohistochemically defined ER+/HER2-, PAM50-defined luminal A and luminal B carcinomas and invasive lobular carcinomas from TCGA is illustrated by the horizontal line within each box. Boxes show the interquartile range; lines extending vertically from the boxes (whiskers) indicate variability outside the upper and lower quartiles. ILCs: invasive lobular carcinomas; N: number; NBCs: neuroendocrine breast carcinomas.

Figure 4. Non-synonymous somatic mutations in cancer genes in NBCs and in common forms of luminal breast cancers as well as invasive lobular carcinomas

Cancer genes included in any of the three cancer gene sets (Kandoth *et al* [27], Cancer Gene Census [28] and Lawrence *et al* [29]) mutated in NBCs as well as *TP53* are included in the figure. Non-synonymous somatic mutations in NBCs, common forms of ER+/HER2-, luminal A/B breast carcinomas, invasive lobular carcinomas (ILCs) from TCGA ordered from top to bottom in decreasing order of mutational frequency in NBCs. *PIK3CA* mutations were significantly less frequent in NBCs (11%) than in ER+/HER2- (42%), luminal A (46%) breast cancers and ILCs ($p < 0.05$, Fisher's exact tests). *ARID1A* and *FOXA1* mutations were significantly more frequently found in NBCs (both 17%) than in ER+/HER2- (both 2%), luminal A (both 2%) and luminal B (3% and 2%) ($p < 0.05$, Fisher's exact tests). *TP53* somatic mutations were found in 22% of ER+/HER2-, 11% of luminal A and in 32% of luminal B breast cancers but in none of the NBCs.

ILCs: invasive lobular carcinomas; NBCs: neuroendocrine breast carcinomas.

Figure 5. Constellation of copy number alterations in NBCs and comparison with common forms of ER+/HER2-, luminal A and luminal B breast carcinomas as well as invasive lobular carcinomas from TCGA

Frequency plots depicting copy number gains (purple) and losses (orange) in NBCs (A, B, C, D, top) and in common forms of ER+/HER2- (A, middle), luminal A (B, middle), luminal B (C, middle) breast cancers and invasive lobular carcinomas (D, middle) from TCGA. Significant differences (Fishers' exact test $p < 0.05$) are plotted in the bottom panel (A, B, C, D). Smoothed Log_2 ratios are plotted on the y-axis according to their genomic

positions (chromosomes) indicated on the x-axis. ILCs: invasive lobular carcinomas; NBCs: neuroendocrine breast carcinomas.

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Figure 6. Unsupervised clustering analysis of NBCs and ER+/HER2- breast cancers from the TCGA dataset

(A) Unsupervised clustering analysis based on the somatic mutations detected in the NBCs analysed in this study (N=18), and TD-NBCs (N=53) and the remaining ER+/HER2- breast cancers (N=188) from TCGA. The NBC-enriched cluster is highlighted in red. The molecular and histologic characteristics of the cases in the clusters are color-coded according to the legends. (B) Non-synonymous somatic mutations in cancer genes in carcinomas included in the ‘NBC-enriched cluster’ compared to the remaining ER-positive/HER2-negative breast carcinomas from TCGA. Cancer genes included in any of the three

cancer gene sets (Kandoth *et al* [27], Cancer Gene Census [28] and Lawrence *et al* [29]) are presented. Non-synonymous somatic mutations ordered from top to bottom in decreasing order of mutational frequency in carcinomas of the 'NBC-enriched cluster'. NBCs: neuroendocrine breast carcinomas. TD-NBCs: transcript-defined neuroendocrine breast carcinomas.

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Table 1
Clinical, morphological and immunophenotypic characteristics of the 18 NBCs included in the study

Case ID	Diagnosis	ER (%)	HER2 status	Ki67 (%)	Histological grade	Chromogranin A	Synaptophysin	E-cadherin	Number of mutations**
CMNE01T	IDC-NE	98	Score 0	4	2	Positive	Positive	Reduced	2
CMNE03T	IDC-NE	99	Score 0	13	2	Positive	Positive	Positive	2
CMNE04T	IDC-NE	99	Score 0	15	2	Positive	Positive	Positive	4
CMNE05T	IDC-NE with focal mucinous and solid-papillary components	95	Score 0	16	2	Positive	Positive	Positive	2
CMNE06T	IDC-NE	99	Score 0	36	3	<50%	Positive	Reduced	5
CMNE08T	IDC-NE	100	Score 0	12	2	Positive	Positive	Positive	6
CMNE09T	IDC-NE	95	Score 0	10	2	Positive	Positive	Reduced	5
CMNE10T	Mucinous type B carcinoma	100	Score 0	28	2	Positive	Positive	Heterogeneous	1
CMNE11T	IDC-NE with focal solid-papillary component	100	Score 0	23	2	Positive	Positive	Positive	7
CMNE12T	IDC-NE	95	Score 0	33	2	Negative	Positive	Positive	4
CMNE13T	Mucinous type B carcinoma	95	Score 0	13	2	Positive	Positive	Positive	4
CMNE14T	IDC-NE	70	Score 0	60	3	Positive	Positive	Positive	4
CMNE15T	IDC-NE	95	Score 0	29	2	Positive	Positive	Positive	9
CMNE16T	IDC-NE	100	Score 0	7	2	Positive	<50%	Positive	1
CMNE19T	IDC-NE	98	Score 0	3	2	Negative	Positive	Reduced	10
CMNE21T	IDC-NE with focal solid-papillary component	99	Score 2+ (<i>HER2</i> not amp*)	14	1	Negative	Positive	Reduced	11
CMNE22T	IDC-NE	95	Score 0	25	3	Negative	Positive	Positive (faint)***	5
CMNE24T	ILC (solid and signet ring cell growth pattern)	90	Score 0	25	2	Positive	Positive	Negative***	7

ER, oestrogen receptor; IDC-NE: invasive ductal carcinoma with neuroendocrine differentiation; ILC: invasive lobular carcinoma;

* *HER2* gene status assessed by fluorescence *in situ* hybridization;

** number of synonymous and non-synonymous somatic mutations;

case harbouring *CDH1* somatic mutation.

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Table 2
Non-synonymous somatic mutations with statistically significantly different frequencies between NBCs (whole cohort, n=18) and common forms of ER-positive carcinomas as well as invasive lobular carcinomas from TCGA

Gene	NBCs (n=18)	ER+/HER2- (n=240)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)	Lum A (n=209)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)	Lum B (n=111)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)	ILCs (n=127)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)
<i>ARID1A</i>	3 (17%)	5 (2%)	0.01	9.217 (1.766-41.63)	4 (2%)	0.01	10.02 (1.821-50.25)	3 (3%)	0.03	7.016 (1.17-42.2)	7 (5%)	0.11	3.386 (0.6882-15.09)
<i>FOXA1</i>	3 (17%)	5 (2%)	0.01	9.217 (1.766-41.63)	5 (2%)	0.01	8.003 (1.533-36.18)	2 (2%)	0.01	10.55 (1.524-91.06)	9 (7%)	0.17	2.599 (0.5482-10.97)
<i>DCHS2</i>	3 (17%)	4 (2%)	<0.01	11.53 (2.097-57.78)	3 (1%)	<0.01	13.35 (2.238-79.93)	2 (2%)	0.01	10.55 (1.524-91.06)	2 (1%)	0.01	12.09 (1.749-104.3)
<i>TBX3</i>	3 (17%)	7 (3%)	0.03	6.559 (1.34-28.9)	5 (2%)	0.01	8.003 (1.533-36.18)	5 (4%)	0.08	4.17 (0.7941-18.96)	12 (10%)	0.40	1.906 (0.4158-7.681)
<i>PIK3CA</i>	2 (11%)	100 (42%)	0.01	0.1759 (0.0283-0.7385)	96 (46%)	<0.01	0.1481 (0.0238-0.6265)	34 (31%)	0.09	0.2853 (0.0445-1.321)	61 (48%)	<0.01	0.1367 (0.0216-0.6048)
<i>SPEN</i>	2 (11%)	3 (1%)	0.01	9.672 (1.13-66.55)	7 (3%)	0.15	3.574 (0.4978-19.33)	2 (2%)	0.09	6.646 (0.6775-65.25)	7 (5%)	0.30	2.129 (0.2954-11.62)
<i>MACF1</i>	2 (11%)	4 (2%)	0.06	7.254 (0.9117-42.13)	5 (2%)	0.09	5.034 (0.6625-27.32)	0 (0%)	0.02	Inf (1.833-Inf)	1 (1%)	0.04	15.18 (1.133-459.8)
<i>HUWE1</i>	2 (11%)	4 (2%)	0.05	7.254 (0.9117-42.13)	2 (1%)	0.03	12.59 (1.289-123.2)	4 (4%)	0.19	3.299 (0.4126-19.27)	2 (1%)	0.07	7.616 (0.7773-74.71)
<i>CDH1</i>	2 (11%)	21 (9%)	0.66	1.302 (0.201-5.641)	22 (10%)	1	1.062 (0.1643-4.632)	5 (4%)	0.25	2.624 (0.3437-14.39)	80 (63%)	<0.01	0.07464 (0.0118-0.3318)
<i>CTCF</i>	2 (11%)	7 (3%)	0.12	4.121 (0.5744-22.25)	9 (4%)	0.21	2.76 (0.3972-13.13)	1 (1%)	0.05	13.25 (0.9888-401.8)	0 (0%)	0.01	Inf (2.098-Inf)
<i>TP53</i>	0 (0%)	54 (22%)	0.01	0 (0-0.8477)	24 (11%)	0.23	0 (0-1.709)	36 (32%)	<0.01	0 (0-0.4559)	10 (8%)	0.61	0 (0-3.208)

Genes are ordered in decreasing order of mutational frequency in NBCs. Statistical analyses were performed using two-tailed Fisher's exact tests and the confidence intervals for the odds ratio estimated using the minimum likelihood method. In bold, statistically significant *p* values.

CI, confidence interval; ER, oestrogen receptor; ILCs; invasive lobular carcinomas; Inf: infinite; Lum: luminal; n: number; NBCs: neuroendocrine breast carcinomas.

Table 3
Non-synonymous somatic mutations with statistically significantly different frequencies between NBCs (excluding mucinous and lobular carcinomas, n=15), and common forms of ER-positive carcinomas as well as invasive lobular carcinomas from TCGA

Gene	IDCs-NE (n=15)	ER+/HER2- (n=210)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)	Lum A (n=179)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)	Lum B (n=105)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)	ILCs (n=127)	<i>p</i> value	<i>Odds ratio</i> (95% <i>CI</i>)
<i>FOXAI</i>	3 (20%)	5 (2%)	0.01	10 (1.881-47)	4 (2%)	0.01	10.63 (1.897-54.54)	2 (2%)	0.01	12.35 (1.758-108.3)	9 (7%)	0.11	3.239 (0.6694-13.43)
<i>TBX3</i>	3 (20%)	5 (2%)	0.01	10 (1.881-47)	4 (2%)	0.01	10.63 (1.897-54.54)	4 (4%)	0.04	6.151 (1.092-31.71)	12 (9%)	0.19	2.377 (0.5078-10.36)
<i>DCHS2</i>	3 (20%)	4 (2%)	< 0.01	12.51 (2.234-64.11)	3 (2%)	< 0.01	14.17 (2.334-86.47)	2 (2%)	0.01	12.35 (1.758-108.3)	2 (1%)	< 0.01	14.98 (2.136-131.2)
<i>PIK3CA</i>	2 (13%)	88 (42%)	0.03	0.2144 (0.0338-0.9846)	85 (47%)	0.01	0.1714 (0.0269-0.7949)	31 (29%)	0.23	0.3698 (0.0566-1.607)	66 (48%)	0.01	0.1682 (0.0262-0.7295)
<i>HUWE1</i>	2 (13%)	4 (2%)	0.05	7.763 (0.9622-45.91)	2 (1%)	0.03	13.17 (1.332-130.5)	4 (4%)	0.16	3.819 (0.4709-22.71)	2 (1%)	0.05	9.315 (0.94-92.46)
<i>SVEP1</i>	2 (13%)	4 (2%)	0.06	7.763 (0.9622-45.91)	3 (2%)	0.04	8.804 (1.014-61.58)	3 (3%)	0.11	5.114 (0.5868-35.92)	3 (2%)	0.08	6.211 (0.7138-43.55)
<i>MACF1</i>	2 (13%)	4 (2%)	0.05	7.763 (0.9622-45.91)	4 (2%)	0.06	6.599 (0.8172-39.06)	0 (0%)	0.01	Inf (2.095-Inf)	1 (1%)	0.03	18.52 (1.37-565.7)
<i>CTCF</i>	2 (13%)	6 (3%)	0.09	5.159 (0.6914-27.92)	8 (4%)	0.17	3.257 (0.4548-16.84)	1 (1%)	0.04	15.29 (1.13-467.7)	0 (0%)	0.01	Inf (2.536-Inf)
<i>CDH1</i>	1 (11%)	6 (3%)	0.38	2.415 (0.1002-18.09)	4 (2%)	0.33	3.096 (0.1205-25.76)	2 (2%)	0.33	3.619 (0.1186-48.93)	80 (63%)	< 0.01	0.04274 (0.002-0.2785)
<i>TP53</i>	0 (0%)	53 (25%)	0.02	0 (0-0.7689)	23 (13%)	0.22	0 (0-1.888)	35 (33%)	< 0.01	0 (0-0.5474)	10 (8%)	0.59	0 (0-3.991)

Genes are ordered in decreasing order of mutational frequency in NBCs. Statistical analyses were performed using two-tailed Fisher's exact tests and the confidence intervals for the odds ratio estimated using the minimum likelihood method. In bold, statistically significant *p* values.

CI, confidence interval; ER, oestrogen receptor; IDCs-NE: invasive ductal carcinomas with neuroendocrine differentiation; ILCs: invasive lobular carcinomas; Inf: infinite; Lum: luminal; N, number; NBCs: neuroendocrine breast carcinomas.

Table 4
List of amplifications identified in the NBCs analysed (n=18)

Sample ID	Cytoband	Selected genes mapping to the region
CMNE24T	1p34.3	<i>MACF1</i>
CMNE05T	1q21.3	<i>HRNR, SHC1</i>
CMNE05T	1q21.1	<i>SPTA1</i>
CMNE05T, CMNE24T	1q25.3	<i>CACNA1E</i>
CMNE05T, CMNE06T, CMNE24T	1q25.3	<i>APOBEC4</i>
CMNE05T, CMNE06T	1q42.12	<i>PARP1</i>
CMNE05T, CMNE06T	1q42.2	<i>PCNXL2</i>
CMNE05T, CMNE06T	1q43	<i>FMN2</i>
CMNE05T	1q43	<i>AKT3</i>
CMNE19T	2q34	<i>ERBB4</i>
CMNE19T	2q36.3	<i>IRS1</i>
CMNE04T, CMNE15T	8p11.23-11.21	<i>ZNF703, FGFR1, POLB</i>
CMNE04T, CMNE12T	8q21.11-22.1	<i>ZFX4, RAD54B, NBN</i>
CMNE04T, CMNE12T, CMNE21T	8q24.12	<i>DEPTOR</i>
CMNE04T, CMNE12T, CMNE21T	8q24.3	<i>PLEC, PTK2, EPPK1</i>
CMNE12T	12q13.12	<i>KMT2D</i>
CMNE12T	12q14.1	<i>CDK4</i>
CMNE12T	12q15	<i>MDM2</i>
CMNE21T	14q12	<i>PRKD1</i>
CMNE21T	14q21.1-q21.2	<i>FOXA1, FANCM</i>

NBCs: neuroendocrine breast carcinomas.

Table 5
Non-synonymous somatic mutations with statistically significantly different frequencies
between carcinomas from the “NBC-enriched cluster” *versus* the remaining (i.e. not in the
“NBC-enriched cluster”) ER+/HER2- breast cancers from the TCGA study

Gene	NBC-enriched cluster (n=110)	Other cases (n=149)	<i>p</i> values	Odds ratio (95% CI)
<i>ARID1A</i>	7 (6%)	1 (0.6%)	0.01	9.982 (1.366-224.8)
<i>FOXA1</i>	7 (6%)	1 (0.6%)	0.01	9.982 (1.366-224.8)
<i>PIK3CA</i>	10 (9%)	92 (61%)	< 0.01	0.0627 (0.0279-0.1312)
<i>TP53</i>	5 (4%)	49 (33%)	< 0.01	0.09792 (0.0357-0.2541)
<i>SPEN</i>	5 (4%)	0 (0%)	0.01	Inf (1.361-Inf)
<i>AKT1</i>	9 (8%)	1 (0.6%)	< 0.01	13.08 (1.971-286.8)
<i>CTCF</i>	8 (7%)	1 (0.6%)	< 0.01	11.52 (1.738-255.5)
<i>NEB</i>	8 (7%)	1 (0.6%)	< 0.01	11.52 (1.738-255.5)
<i>CDH6</i>	0 (0%)	6 (4%)	0.04	0 (0-0.9448)
<i>ANK3</i>	4 (4%)	0 (0%)	0.03	Inf (1.234-Inf)
<i>SRCAP</i>	4 (4%)	0 (0%)	0.03	Inf (1.234-Inf)

NBC: neuroendocrine breast carcinoma.