2019

Potential novel therapy targets in neuroendocrine carcinomas of the breast

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Recommended Citation
Vranic, Semir; Palazzo, Juan; Sanati, Souzan; Florento, Elena; Contreras, Elma; Xiu, Joanne; Swensen, Jeffrey; and Gatalica, Zoran, "Potential novel therapy targets in neuroendocrine carcinomas of the breast." Clinical Breast Cancer.19,2. 131-136. (2019).
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Potential Novel Therapy Targets in Neuroendocrine Carcinomas of the Breast

Semir Vranic,1 Juan Palazzo,2 Souzan Sanati,3 Elena Florento,4 Elma Contreras,4 Joanne Xiu,4 Jeffrey Swensen,4 Zoran Gatalica4

Abstract

Neuroendocrine breast cancer lacks specific therapy, but similar common neuroendocrine carcinomas may offer guidance for therapy development. This study, for the first time, identified several biomarkers for targeted therapy approaches in patients with breast neuroendocrine carcinoma.

Introduction: Neuroendocrine carcinoma (NEC) of the breast is a rare, special type of breast cancer, reportedly constituting 2% to 5% of all breast cancers. Although breast NEC does not have a specific targeted therapy, several new targeted therapies based on specific biomarkers were recently investigated in the NEC of lung and in other types of breast carcinoma, which may provide guidance to their feasibility in breast NEC. Materials and Methods: Twenty breast NECs were profiled for biomarkers of therapy including antibody-drug conjugates (DLL3, TROP-2, and FOLR1), histone deacetylase (H3K36Me3) inhibitors, tropomyosin receptor kinases (NTRK1/2/3 gene fusions) targeted inhibitors, alkylating agents (MGMT), and immune checkpoint inhibitors (PD-L1, TMB, and MSI) using immunohistochemistry and DNA/RNA next-generation sequencing assays. Results: Predictive expression of TROP-2, FOLR1, and H3K36Me3 were detected in different subsets of tumors and may pave the way for development of novel targeted therapies in some patients with breast NECs. There was no evidence of DLL3 expression, NTRK gene fusions, or MGMT hypermethylation. No biomarkers predictive of immune checkpoint inhibitor efficacy (programmed death-ligand 1 expression, tumor mutational burden, microsatellite instability) were identified. FGFR and CCND1 gene amplifications were detected in isolated cases. Conclusions: This study identified several potential targets for novel therapies in breast NEC, including farletuzumab and mirvetuximab soravtansine (FOLR1), sacituzumab govitecan (TROP-2), and HDAC inhibitors (H3K36Me3). In some cases, CCND1 gene amplification may indicate the usefulness of investigational therapies. The reported results should serve as an early indication of potential clinical relevance in selected patients with breast NEC.

Introduction

Breast neuroendocrine carcinoma (NEC) is a rare, special type of cancer, reportedly constituting 2% to 5% of all breast cancers.1 Breast NECs are typically positive for estrogen (ER) and progesterone (PR) receptors and negative for ERBB2 (human epidermal growth factor receptor 2 [Her-2/neu]).2,4 Despite its luminal (A or B) phenotype,4,5 most studies have reported an aggressive clinical course and poor outcome for patients with NEC.2,4,6,7

The mutational profile and molecular characteristics of breast NEC have been the focus of several recent studies.3,4,8,9 In contrast to gastroenterohepatic NECs,10 these studies revealed inconsistent mutational profiles of NEC with limited targetable options (eg, PIK3CA mutations in 7%-33% of the cases). The role of programmed death-ligand 1 (PD-L1) as a predictor of the response to immune checkpoint inhibitors was explored in 2 studies involving only 6 cases of NEC.3,11 No systematic attempt to analyze breast NECs for biomarkers of therapy used in NECs of other
primary sites (eg, DLL3 in lung)\textsuperscript{12} or different types of breast carcinomas (eg, TROP-2, Folate Receptor 1, NTRK gene fusions) has been published.\textsuperscript{13-15} Thus, we investigated this topic in a cohort of invasive breast NECs.

**Materials and Methods**

**Patients and Samples**

The study included 20 surgical tumor samples previously defined by strict criteria for the diagnosis of breast NEC (> 50% of the neoplastic cells expressing neuroendocrine markers synaptophysin and/or chromogranin-A).\textsuperscript{16} Board-certified pathologists (Z.G., J.P., S.S., and S.V.) reviewed all cases to confirm the diagnoses and to select appropriate slides for molecular assays. None of the patients had a history or concurrent NEC of the lung or any other extra-mammary neuroendocrine neoplasm.

**Immunohistochemistry**

Immunohistochemical methods (IHC) were used to analyze the expression of PD-L1, delta like canonical Notch ligand 3 (DLL3), folate receptor 1 (FOLR1), trimethylated Lys-36 of histone 3 (H3K36me3), TROP-2 (or tumor-associated calcium signal transducer 2 [TACSTD2]), and neurotrophic receptor kinases 1-3 (pan-NTRK). O-6-Methylguanine-DNA Methyltransferase (MGMT) protein expression was evaluated using IHC, whereas MGMT promoter methylation was analyzed by pyrosequencing (manufacturers, clones, thresholds, and subcellular localization for each biomarker are provided in Table 1). For all IHC assays, both positive and negative controls reacted appropriately. IHC assays were performed using fully automated staining platforms (Ventana-Roche and DAKO-Agilent) in a CLIA/CAP/ISO15189-certified clinical laboratory (Caris Life Sciences, Phoenix, AZ).

**Next-generation Sequencing (NGS)**

The NGS panel included complete exon sequencing of 592 genes (the full list of genes is available at: http://www.carismolecularintelligence.com/solid_tumors_international). Tumor mutational burden (TMB) was calculated using non-synchronous missense mutations; common germline variants were filtered from the analysis. High TMB was reported when > 5% of the mean age was 60 years (range, 43-83 years). The study included 4 grade 1 NEC, 11 moderately differentiated (grade 2), and 5 poorly differentiated (grade 3) NECs.

**Results and Discussion**

**Clinicopathologic Data**

The study included 19 naive primary and 1 metastatic (axillary lymph node) patients with breast NEC. None of the patients had been treated by chemotherapy, endocrine therapy, or radiotherapy prior to the tumor sample collection. All the patients were women; the mean age was 60 years (range, 43-83 years). The study included 4 grade 1 NEC, 11 moderately differentiated (grade 2), and 5 poorly differentiated (grade 3) NECs.

ER was positive in all 20 cases (100%), whereas PR was expressed in 17 (85%) of 20 cases. ERBB2 was positive (over-expressed and amplified) in 1 (5%) (ER-positive [ER\textsuperscript{+}], PR-negative [PR\textsuperscript{-}]) case. Androgen receptor (AR) was positive (≥ 10% positive cells) in 9 (50%) of 18 tested cases. Each breast NEC stained diffusely and strongly for at least 1 neuroendocrine biomarker: chromogranin-A (17\textsuperscript{+} of 20) and synaptophysin (19\textsuperscript{+} of 20). These results are in line with previous data that revealed the luminal A/B (ER\textsuperscript{+}) phenotype of the vast majority of NECs.\textsuperscript{4,9,20} Given the positivity for ER and/or PR, most patients with NEC are eligible for endocrine therapy against ER. AR expression has been extensively studied in breast cancer, as a diagnostic (eg, apocrine breast cancer),\textsuperscript{21} prognostic (favorable in ER\textsuperscript{+/}AR-positive [AR\textsuperscript{+}] breast cancers),\textsuperscript{22} and predictive biomarker (eg, clinical trials with antiandrogens in patients with advanced/metastatic triple-negative breast cancer).\textsuperscript{23} AR expression has been previously described in NEC of the breast and correlated with the expression of gross cystic disease fluid protein expression.\textsuperscript{16} Information on the potential benefits of antiandrogens in patients with NEC of the breast is not currently available.

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**Table 1 Overview of the Antibodies Used in the Study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/Manufacturer</th>
<th>Threshold for Positivity</th>
<th>Subcellular Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLL3</td>
<td>SP347 clone, Ventana</td>
<td>Any positivity ≥ 50% (high expression)</td>
<td>Membranous/cytoplasmic</td>
</tr>
<tr>
<td>TROP-2</td>
<td>Anti-human Trop-2, R&amp;D Systems</td>
<td>≥ 10%, 2+ intensity ≥ 50% (high expression)</td>
<td>Membranous</td>
</tr>
<tr>
<td>FOLR1</td>
<td>Clone 26B3.F2, Biocare Medical</td>
<td>H-score ≥ 1 (≥ 20 for high)</td>
<td>Membranous</td>
</tr>
<tr>
<td>H3K36me3</td>
<td>Rabbit polyclonal, Abcam</td>
<td>Any positivity</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Pan-NTRK</td>
<td>Clone EPR17341, Abcam</td>
<td>≥ 1% of tumor cells</td>
<td>Membranous/cytoplasmic and nuclear</td>
</tr>
<tr>
<td>MGMT</td>
<td>Monoclonal antibody MT 23.2, Invitrogen</td>
<td>&gt; 35%, ≥ 1+ intensity</td>
<td>Nuclear</td>
</tr>
<tr>
<td>PD-L1</td>
<td>SP142, Ventana</td>
<td>≥ 5% tumor cells, 2+ intensity</td>
<td>Membranous/cytoplasmic</td>
</tr>
</tbody>
</table>

Microsatellite instability (MSI) status was evaluated by analysis of microsatellite loci in the target regions of the sequenced genes. High MSI was defined as ≥ 46 altered loci, as previously reported.\textsuperscript{17-19}

Gene copy number variations were identified by comparing the depth of NGS sequence reads to reads from a diploid control. Genes with ≥ 6 copies were considered amplified.\textsuperscript{17,18}

ArcherDx FusionPlex Assay (ArcherDX, Boulder, CO) was used to explore gene fusions. Fifty-three gene targets were analyzed in 12 NECs. The panel of tested gene fusions is available here: https://www.carismolecularintelligence.com/tumor-profiling-menu/mi-profile-usa-excluding-new-york/.
<table>
<thead>
<tr>
<th>Biomarker Class/Name</th>
<th>Function</th>
<th>Mechanism of Action</th>
<th>Targeted Drug</th>
<th>Common Cancers</th>
<th>Diagnostic Assay</th>
<th>Status in NEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug conjugates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delta-like 3 (DLL3)</td>
<td>Notch ligand</td>
<td>↑</td>
<td>Rovaipituzumab tesirine</td>
<td>SCLC</td>
<td>IHC</td>
<td>2/19 low (5%-20%) 17/19 negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TROP-2 (Tumor-associated Ca signal transducer 2)</td>
<td>Transmembrane glycoprotein (Ca signal transducer)</td>
<td>↑</td>
<td>Sacituzumab govitecan (IMMU-132)</td>
<td>TNBC, SCLC NSCLC</td>
<td>IHC</td>
<td>1/19 high (&gt; 50% cells+) 3/19 low (10%-30%) cells+ 15/19 negative (0 &lt; 10% cells+)</td>
</tr>
<tr>
<td>Folate receptor 1 (FOLR1)</td>
<td>Folate antimetabolites (eg, pemetrexed therapy)</td>
<td>↑</td>
<td>Imaging probes, drug conjugates, farletuzumab, mirvetuximab</td>
<td>NSCLC, breast, ovarian, CRC</td>
<td>IHC</td>
<td>4/19 (high expression) 2/19 (low expression) 13/19 negative</td>
</tr>
<tr>
<td>Targeted inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K36me3 (SETD2)</td>
<td>Histone H3 lysine 36 methyltransferase</td>
<td>↓ gene function/ protein expression</td>
<td>Histone deacetylase (HDAC) inhibitors</td>
<td>RCC T-cell lymphoma</td>
<td>IHC and NGS</td>
<td>Loss: 6/19 (32%) SETD2 mutation in 1 case</td>
</tr>
<tr>
<td>Tropomyosin receptor kinase kinase pNTRK (1-3)</td>
<td>Nerve development and growth (activation by neurotrophins)</td>
<td>↑ owing to gene fusions</td>
<td>TRK inhibitors (eg, entrectinib)</td>
<td>Pediatric sarcomas, thyroid carcinoma, MASC, brain tumors</td>
<td>IHC and Archer Fusion assay (NGS)</td>
<td>18/19 (95%) negative 1/19 (5%) low positivity (5%) No gene fusions</td>
</tr>
<tr>
<td>O(6)-methylguanine-DNA methyltransferase (MGMT)</td>
<td>Involved in repairment of DNA</td>
<td>↓ owing to promoter methylation</td>
<td>Temozolomide</td>
<td>GBM</td>
<td>IHC and Pyro-sequencing</td>
<td>None methylated (n = 12)</td>
</tr>
</tbody>
</table>

Abbreviations: Ca = calcium; CRC = colorectal cancer; GBM = glioblastoma multiforme; IHC = immunohistochemistry; MASC = mammary analogue secretory carcinoma; NEC = neuroendocrine carcinoma; NSCLC = non–small-cell lung cancer; RCC = renal cell carcinoma; SCLC = small-cell lung cancer; TNBC = triple-negative breast cancer. 
† Denotes activation (overexpression). 
↓ Denotes loss of function/expression.

Denotes activation (overexpression).
Denotes loss of function/expression.
Novel Targets in Neuroendocrine Carcinoma of the Breast

**Table 3** Genetic Alterations in Neuroendocrine Carcinoma of the Breast

<table>
<thead>
<tr>
<th>Genetic Alterations (Number of Tested Cases)</th>
<th>Affected Genes in Neuroendocrine Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutations (n = 12)</td>
<td>TPS3 (n = 2) *</td>
</tr>
<tr>
<td></td>
<td>PIK3CA, RB1, BRCA1 (VUS), IDH1, ARID1A, MUTH1 (n = 1)</td>
</tr>
<tr>
<td>Fusions (n = 10)</td>
<td>None</td>
</tr>
<tr>
<td>Copy number variations (n = 12)</td>
<td>FGF3, FGF4 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>FGFR1, FGFR9, CCND1, ZNF703, WHSC1L1 (n = 2)</td>
</tr>
<tr>
<td></td>
<td>FGFI1, CDX2, MDM2, HMGA2, SPEC2C1, GPR124 (n = 1)</td>
</tr>
</tbody>
</table>

Abbreviation: VUS = variant of unknown significance.

*Numbers in parenthesis represent number of cases with a certain gene alteration.

**Biomarkers Expression**

**Antibody-drug Conjugate (ADC) Targets.** The targeted delivery of drug conjugated to an antibody is a novel approach in cancer treatment that has resulted in several recently approved therapies. The antibody component of the ADC is directed against an epitope enriched in the targeted cancer cell population. The number of ADCs is increasing rapidly; a recent study of Moek et al, based on a comprehensive search of PubMed and ClinicalTrials.gov, revealed 87 ADCs directed against 59 unique targets. The authors applied a functional genomic mRNA-profiling assay to predict the frequency of protein predictive biomarkers to ADC targets across various cancers. Their study revealed that ADC targets are commonly expressed in cancers such as breast (especially in triple-negative), lung, and prostate cancer. In our study, we chose to investigate expression of 3 such epitopes that have shown promising predictive values in either breast cancer or extramammary neuroendocrine neoplasms: DLL3, FOLR1, and TROP-2.

Low DLL3 expression was observed in 2 (11%) of 19 cases (5% and 20% with weak 1+ positive cells, respectively) (Table 2). High DLL3 expression (> 50% of cells) had been shown to predict therapeutic benefit from rovalpituzumab tesirine (a DLL3-targeted antibody-drug conjugate), in small-cell lung cancers, but none of the tested breast NECs exhibited high DLL3 expression. Given these results, it is unlikely that patients with breast NEC will benefit from rovalpituzumab tesirine.

FOLR1 expression was detected in 6 (31.5%) of 19 cases, with H-score ≥ 20 in 4 cases. FOLR1 expression in breast is predominantly observed in triple-negative breast cancers. High FOLR1 expression in non-small-cell lung cancer has been associated with a more favorable outcome and a better response to pemetrexed therapy. A number of FOLR-targeted agents have been developed, including monoclonal antibodies and ADCs. A recent study by Kalli et al indicates that FOLR vaccine may enhance the immune response in patients with breast and ovarian cancers that overexpress FOLR.

TROP-2 protein expression was detected in 4 (21%) of 19 cases, with 1 case exhibiting high (2+/++ > 50% cells) Trop-2 expression. TROP-2 is a cell-surface receptor that is over-expressed in various carcinomas. It is a biomarker for sacituzumab govitecan, an antibody-drug conjugate. In this ADC, the anti-Trop-2 antibody (hRS7) serves as a mode of delivery for SN-38, the active metabolite of irinotecan, an inhibitor of topoisomerase-1. Of note, topoisomerase-1 overexpression has been previously reported in approximately 40% of breast NECs. In addition, a recent phase II trial showed durable therapeutic responses induced by sacituzumab govitecan in patients with heavily pretreated and metastatic triple-negative breast cancers. Our results also suggest that a small proportion of breast NECs may be amenable to treatment with sacituzumab govitecan.

**NTRK and Histone Deacetylase (HDAC) Inhibitors.** NTRK expression using pan-TRO1 antibody was negative in all but 1 case that exhibited low (5%) positivity. NTRK gene fusions were absent in all cases (Tables 2 and 3). Based on these findings as well as the lack of ALK and ROS1 gene alterations, patients with breast NEC are unlikely to benefit from the NTRK inhibitors (eg, larotrectinib [or LOXO-101] and entrectinib [pan-Trk, ROS1, and ALK inhibitor]) in contrast to secretory breast carcinoma, characterized by consistent ETV6-NTRK3 fusions and NTRK expression. Significantly, a complete loss of H3K36me3 was seen in 6 (32%) of 19 cases. One of the cases with loss also harbored a loss of function SETD2 gene mutation by NGS, whereas another had a SETD2 variant of unknown significance. SETD2 is responsible for H3K36me3 histone modification. Loss of function mutations in the SETD2 gene are associated with impaired DNA methylation and increased genomic instability. These genetic alterations have been well-characterized in renal cell carcinoma and may be targeted by HDAC inhibitors. SETD2-dependent histone H3K36 trimethylation is also essential for homologous recombination and mismatch repair and has been implicated in resistance to DNA damaging agents. Recent studies, however, have suggested that H3K36me3-deficient cancers are sensitive to WEE1 inhibition; several clinical trials are ongoing (eg, NCT03284385 phase II clinical trial).

**O(6)-methylguanine-DNA Methyltransferase (MGMT) Status in NEC**

None of the tested cases of NEC (n = 14) exhibited a loss of MGMT protein by IHC, and the pyrosequencing assay detected no MGMT promoter hypermethylation (n = 10). These results indicate a lack of benefit for patients with NEC from temozolomide, an alkylating agent used for treatment of high-grade gliomas (eg, glioblastoma multiforme) and melanomas with an epigenetically silenced MGMT gene. A recent study by Gay et al reported a therapeutic success and durable remission induced by temozolomide in a patient with metastatic small-cell lung cancer; a study from Kulke et al also demonstrated sensitivity to temozolomide-based therapy in > 30% pancreatic neuroendocrine tumors.

**Biomarkers of Response to Immune Checkpoint Inhibitors**

PD-L1 expression in cancer cells (above 5% tumor cells’ threshold) was not observed in any case (n = 19). Additionally, all successfully tested cases (n = 10) were microsatellite stable and exhibited low TMB (5 mutations/Mb; range, 1-9 mutations). These results are in line with previous studies on luminal breast cancer that confirmed a low frequency (< 5%) or a complete lack
of MSI and/or low TMB.41 Our data indicate that patients with breast NEC would be ineligible for treatment with the current immune checkpoint inhibitors (anti-programmed cell death protein 1 [PD-1]/PD-L1 drugs) in contrast to patients with PD-L1-positive metastatic breast cancers and breast cancers with high MSI or high TMB.42-44

**NGS and Archer Fusion Results**

NGS detected amplifications of genes in the fibroblast growth factor signaling pathway (FGFR1, FGFR3, FGFR4, FGF19) and cyclin D1 (CCND1) (Table 3). The CCND1, FGFR3, FGFR4, and FGF19 genes are localized together on chromosome 11 and often co-amplify.44 Pathogenic mutations were rare and affected TP53, SETD2, PIK3CA, IDH1, and RB1 (Table 3). Gene fusions were not observed in any of the tested cases (n = 10). Observed genomic alterations of NECs in our series are comparable with the data from previous studies on NEC.4,8,9,46-47 The molecular alterations (PIK3CA, TP53, RB1, CCND1) in breast NEC are common in invasive ER+ (luminal) ductal carcinomas of no-special-type suggesting potential relevance of cell cycle (CDK4/6 inhibitors) in isolated cases of this rare cancer.48,49

There are also ongoing clinical trials with cell cycle inhibitors aimed to treat patients with lung and head/neck cancers harboring CCND1 amplification (trials: NCT03356223 and NCT02785939). Although FGFR inhibitors have been recently considered as a promising therapeutic option in breast cancer, the preliminary clinical data with FGFR inhibitors have yielded disappointing results.50 IDH1 (isocitrate dehydrogenase 1) mutations have also been described in breast cancer, including luminal breast cancers,51 and have been associated with a poor prognosis.51

**Conclusions**

This study for the first time identified several potential targets for novel therapy approaches in breast NEC. Predictive expression levels of FOLR1 and TROP-2 were detected in different, but small subpopulations of patients with breast NEC. Additional biomarker support (H3K36me3/SETD2) for the use of HDAC inhibitors may be explored in selected breast NECs. Gene alterations seen in common breast carcinomas, including those in the cell cycle control pathway, were also found in breast NEC, suggesting relevance of CDK4/6 inhibitors in isolated cases of this rare cancer. Patients with breast NEC are generally unlikely to benefit from immune checkpoint inhibitors, as all current biomarkers (PD-L1 expression, TMB, and MSI) are uniformly negative. Reported results should serve as an early indication of potential clinical relevance in selected patients with breast NEC (eg, including patients with NEC in basket trials like the NCI-MATCH trial [The National Cancer Institute-Molecular Analysis for Therapy Choice] and other precision medicine trials that may include rare cancers such as NEC of the breast).

**Clinical Practice Points**

- Predictive protein expression levels of FOLR1, TROP-2, and H3K36Me3 were detected in single individuals or small sub-populations of breast NEC samples.
- Gene alterations affecting cell-cycle control pathway suggest relevance of cell-cycle (CDK4/6) inhibitors in isolated cases.
- The patients with breast NECs are unlikely to benefit from immune checkpoint inhibitors as all currently approved biomarkers (PD-L1 expression, TMB, and MSI) are uniformly negative.

**Disclosure**

E. Florento, E. Contreras, J. Xiu, J. Swensen, and Z. Gatalica are all employees of Caris Life Sciences. The remaining authors have stated that they have no conflicts of interest.

**References**

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