Original Study

Potential Novel Therapy Targets in Neuroendocrine Carcinomas of the Breast

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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.

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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

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primary sites (eg, DLL3 in lung)[12] or different types of breast carcinomas (eg, TROP-2, Folate Receptor 1, *NTRK* gene fusions) has been published.[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).[1,16] 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-synonymous missense mutations; common germline variants were filtered from the analysis. High TMB was reported when ≥ 17 mutations/megabase were present (more info is available here: https://www.carismolecularintelligence.com/wp-content/uploads/2016/12/TN0291-v1_Total-Mutational-Load-Immunotherapy-REVERSED-PAGES.pdf). 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.[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.[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/.

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 (overexpressed and amplified) in 1 (5%) (ER-positive [ER^+], PR-negative [PR^-]) 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+ of 20) and synaptophysin (19+ of 20). These results are in line with previous data that revealed the luminal A/B (ER^+) phenotype of the vast majority of NECs.[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),[21] prognostic (favorable in ER^+/AR-positive [AR^+] breast cancers),[22] and predictive biomarker (eg, clinical trials with antiandrogens in patients with advanced/metastatic triple-negative breast cancer).[23] AR expression has been previously described in NEC of the breast and correlated with the expression of gross cystic disease fluid protein expression.[16] Information on the potential benefits of antiandrogens in patients with NEC of the breast is not currently available.

Table 1 Overview of the Antibodies Used in the Study

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

Table 2 The Status of Novel Predictive Biomarkers for Several Classes of Drugs

Biomarker Class/Name	Function	Mechanism of Action	Targeted Drug	Common Cancers	Diagnostic Assay	Status in NEC
Drug conjugates						
Delta-like 3 (DLL3)	Notch ligand	↑	Rovalpituzumab tesirine	SCLC	IHC	2/19 low (5%-20%) 17/19 negative
TROP-2 (Tumor-associated Ca signal transducer 2)	Transmembrane glycoprotein (Ca signal transducer)	↑	Sacituzumab govitecan (IMMU-132)	TNBC SCLC NSCLC	IHC	1/19 high (> 50% cells+) 3/19 low (10%-30%) cells+ 15/19 negative (0 < 10% cells+)
Folate receptor 1 (FOLR1)	Folate antimetabolites (eg, pemetrexed therapy)	↑	Imaging probes, drug conjugates, farletuzumab, mirvetuximab	NSCLC, breast, ovarian, CRC	IHC	4/19 (high expression) 2/19 (low expression) 13/19 negative
Targeted inhibitors						
H3K36me3 (*SETD2*)	Histone H3 lysine 36 methyltransferase	↓ gene function/ protein expression	Histone deacetylase (HDAC) inhibitors	RCC T-cell lymphoma	IHC and NGS	Loss: 6/19 (32%) *SETD2* mutation in 1 case
Tropomyosin receptor kinase kinase pNTRK (1-3)	Nerve development and growth (activation by neurotrophins)	↑ owing to gene fusions	TRK inhibitors (eg, entrectinib)	Pediatric sarcomas, thyroid carcinoma, MASC, brain tumors	IHC and Archer Fusion assay (NGS)	18/19 (95%) negative 1/19 (5%) low positivity (5%) No gene fusions
O(6)-methylguanine-DNA methyltransferase (MGMT)	Involved in repairment of DNA	↓ owing to promoter methylation	Temozolomide	GBM	IHC and Pyro-sequencing	None methylated (n = 12)

Abbreviations: Ca = calcium; CRC = colorectal cancer; GMB = glioblastoma multiforme; IHC = immunohistochemistry; MASC = mammary analogue secretory carcinoma; NEC = neuroendocrine carcinoma; NGS = next-generation sequencing; 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.

Table 3 Genetic Alterations in Neuroendocrine Carcinoma of the Breast

Genetic Alterations (Number of Tested Cases)	Affected Genes in Neuroendocrine Carcinomas
Mutations (n = 12)	*TP53* (n = 2)[a] *SETD2* (n = 2) (one VUS) *PIK3CA, RB1, BRCA1* (VUS), *IDH1, ARID1A, MUTYH* (n = 1)
Fusions (n = 10)	None
Copy number variations (n = 12)	*FGF3, FGF4* (n = 3) *FGFR1, FGF19, CCND1, ZNF703, WHSC1L1* (n = 2) *FGF1, CDX2, MDM2, HMGA2, SPECC1, GPR124* (n = 1)

Abbreviation: VUS = variant of unknown significance.
[a]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.[24] 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.[24] 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 cancers.[24] 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,[12] 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.[13,25] High FOLR1 expression in non–small-cell lung cancer has been associated with a more favorable outcome and a better response to pemetrexed therapy.[26] A number of FOLR-targeted agents have been developed, including monoclonal antibodies and ADCs.[27,28] 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.[29]

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.[14] It is a biomarker for sacituzumab govitecan, an antibody-drug conjugate.[14,30] 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.[14,30] Of note, topoisomerase-1 overexpression has been previously reported in approximately 40% of breast NECs.[3] 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.[31] 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-TRK 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.[15]

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[32,33] and may be targeted by HDAC inhibitors.[34] SETD2-dependent histone H3K36 trimethylation is also essential for homologous recombination and mismatch repair and has been implicated in resistance to DNA damaging agents.[35-37] Recent studies, however, have suggested that H3K36me3-deficient cancers are sensitive to WEE1 inhibition[38]; 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 temozolamide in a patient with metastatic small-cell lung cancer[39]; a study from Kulke et al also demonstrated sensitivity to temozolamide-based therapy in > 30% pancreatic neuroendocrine tumors.[40]

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 metaplastic 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, FGF3, FGF4, FGF19*) and cyclin D1 (*CCND1*) (Table 3). The *CCND1*, *FGF3*, *FGF4*, and *FGF19* genes are localized together on chromosome 11 and often co-amplify.[45] 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,[47] 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 subpopulations 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.

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