Novel Mutations in Neuroendocrine Carcinoma of the Breast: Possible Therapeutic Targets

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Abstract: Primary neuroendocrine carcinoma of the breast is a rare variant, accounting for only 2% to 5% of diagnosed breast cancers, and may have relatively aggressive behavior. Mutational profiling of invasive ductal breast cancers has yielded potential targets for directed cancer therapy, yet most studies have not included neuroendocrine carcinomas. In a tissue microarray screen, we found a 2.4% prevalence (9/372) of neuroendocrine breast carcinoma, including several with lobular morphology. We then screened primary or metastatic neuroendocrine breast carcinomas (excluding papillary and mucinous) for mutations in common cancer genes using polymerase chain reaction-mass spectroscopy (643 hotspot mutations across 53 genes), or semiconductor-based next-generation sequencing analysis (37 genes). Mutations were identified in 5 of 15 tumors, including 3 with *PIK3CA* exon 9 E542K mutations, 2 of which also harbored point mutations in *FGFR* family members (*FGFR1* P126S, *FGFR4* V550M). Single mutations were found in each of *KDR* (A1065T) and *HRAS* (G12A). *PIK3CA* mutations are common in other types of breast carcinoma. However, *FGFR* and *RAS* family mutations are exceedingly rare in the breast cancer literature. Likewise, activating mutations in the receptor tyrosine kinase *KDR* (VEGFR2) have been reported in angiosarcomas and non–small cell lung cancers; the *KDR* A1065T mutation is reported to be sensitive to VEGFR kinase inhibitors, and fibroblast growth factor receptor inhibitors are in trials. Our findings demonstrate the utility of broad-based genotyping in the study of rare tumors such as neuroendocrine breast cancer.

Key Words: neuroendocrine breast carcinoma, *FGFR1*, *FGFR4*, *HRAS*, *KDR*, *PIK3CA*

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Primary neuroendocrine carcinoma of the breast bears morphologic similarities to neuroendocrine tumors of the gastrointestinal tract and lung, with expression of neuroendocrine markers.[1,2] The current World Health Organization (WHO) classification subgroups them as: well-differentiated, poorly differentiated/small cell, and invasive breast carcinoma with neuroendocrine differentiation.[2] Other studies have revealed varied architectural patterns of neuroendocrine breast cancer, including papillary, mucinous, solid nesting, alveolar (diffuse nesting or lobular-like), trabecular/gyriform, micropapillary, glandular, and mixed.[3,4] The neuroendocrine subtype accounts for only 0.5% to 5% of breast cancers.[1–2,5–7] Of note, breast metastases of occult carcinoid tumors of other primary sites need to be excluded.[8,9] Literature on pathogenesis, prognosis, and options for treatment of neuroendocrine breast carcinoma is very limited. Historically, neuroendocrine breast carcinoma was thought to have prognosis similar to invasive ductal carcinoma of no special type based on small studies,[3,6,7] and was thus not necessarily considered important to recognize diagnostically. However, other studies, including a recent large Chinese single-institution study, as well as a case-control series from a referral center, have suggested that neuroendocrine breast cancer may be relatively aggressive, presenting at high stage with a relatively poor prognosis.[10–14]

Expression microarray profiling studies of small numbers of cases have demonstrated that neuroendocrine breast carcinomas comprise a discrete molecular cluster. In a cohort that included a large group of mucinous tumors with neuroendocrine differentiation, neuroendocrine tumors clustered closely with mucinous tumors.[5,14,15] This same group applied comparative genomic hybridization to study mucinous tumors, likewise including mucinous neuroendocrine tumors.[16] However, screening for mutations in common cancer genes has not been applied to the study of neuroendocrine breast carcinoma. Thus, we sought to evaluate the mutational profile of this potentially aggressive breast cancer variant.

MATERIALS AND METHODS

Cases and Immunohistochemistry

After institutional review board approval, cases of primary or recurrent/metastatic breast cancer with

neuroendocrine features were identified from the Pathology files of Oregon Health & Science University. As neuroendocrine studies were not routinely performed on breast carcinoma, tissue microarrays (TMAs) were screened immunohistochemically to identify additional cases from the Stanford University Hospital Pathology department archives.[17] Immunostaining was performed on Ventana XT instruments with Ultraview detection (both Ventana, Tucson, AZ) using primary antibodies synaptophysin (MRQ-40; Ventana, predilute) and chromogranin (LKT2H10; Ventana, predilute). The current WHO criteria require that breast carcinomas with neuroendocrine features "express neuroendocrine markers to a greater or lesser degree," without a well-defined threshold. Thus, we applied the 2003 WHO criteria requiring neuroendocrine differentiation in >50% of tumor,[1] and accordingly stained full tissue sections of TMA-positive carcinomas. Neuroendocrine carcinomas with mucinous or papillary components were excluded, as our previous studies included some of these variants.[18,19] A subset of cases was also stained for E-cadherin (36B5; Leica-Novocastra, Buffalo Grove, IL, 1:25). Information on tumor stage and hormone receptor status was compiled from pathology reports.

Molecular Analysis

Lesional tissue was isolated by punching the formalin-fixed paraffin-embedded tissue block with a coring device. DNA was extracted from paraffin using standard protocols (Qiamp Mini kit; Qiagen, Valencia, CA). The resulting DNA extracts were screened for a large panel of activating mutations using a multiplexed polymerase chain reaction (PCR)-mass spectroscopy-based technique encompassing 643 point mutations in 53 genes (Sequenom MassArray; Sequenom Inc., San Diego, CA), as previously described.[19–21] In brief, the mutation panel covers point mutations in *AKT1/2/3, ALK, BRAF, CDK4, CSF1R, CTNNB1, EGFR, ERBB2, ERCC6, FBX4, FBXW7, FES, FGFR1/2/3/4, FOXL2, GNA11, GNAQ, GNAS, HRAS, IDH1/2, IGF1R, KDR, KIT, KRAS, MAP2K1/2/7, MET, MYC, NEK9, NRAS, NTRK1/2/3, PDGFRA, PIK3CA, PIK3R1/4/5, PKHD1, PRKCB1, RAF1, RET, SMO, SOS1, STAT1, TEC,* and *TP53*. The panel includes 58 substitutions in 41 codons of the *PIK3CA* gene. Cases with >5% to 10% assay failures were excluded from further analysis (cases #12, 14, 15). Mutations identified by mass spectroscopy screening were confirmed by direct Sanger sequencing on an ABI3130 sequencer (Applied Biosystems, Carlsbad, CA) using the BigDye terminator method. For *PIK3CA* exon 9 mutations, we used a locked nucleic acid probe to partially suppress amplification of the wild-type allele, as previously described in detail.[22] Two cases (cases #17, 18) were tested by direct sequencing with a next-generation semiconductor-based platform as previously described[23] (Personal Genome Machine, Ion Torrent; Life Technologies, South San Francisco, CA) using a custom solid tumor panel covering frequently mutated exons of the following 37 genes *AKT1-3, ALK, CDK4, CDKN2A, DDR2, EGFR, ERBB2, FGFR1, FGFR3, GNA11, GNAQ, GNAS, HRAS, KIT, KDR, KRAS, MAP2K1, MET, NF1, NOTCH1, NRAS, NTRK2-3, PIK3CA, PIK3R1, PTEN, RAC1, RB1, RET, STK11, TSC1-2, TP53,* and *VHL*. The assay sensitivity is approximately 5% to 10% mutant allele for mass spectroscopy screening, 0.6% to 2.5% mutant allele for locked nucleic acid sequencing, and the allele cutoff ratio was set at 8% with semiconductor sequencing.[20,22,23]

RESULTS

Neuroendocrine Breast Carcinoma Cases

Cases of neuroendocrine carcinoma, with diffuse synaptophysin and/or chromogranin immunohistochemical staining (>50% of the tumor), were identified from a database search of the Oregon Health & Science University pathology archives. We included metastatic lesions, but excluded neuroendocrine carcinomas with mucinous or papillary components. As neuroendocrine differentiation markers are not routinely employed in breast cancer diagnosis, we also stained TMAs with synaptophysin and chromogranin to identify additional cases,[17] and to the determine prevalence of neuroendocrine differentiation. In the invasive breast carcinoma TMAs, there were 372 scoreable cores, and 20 (5.4%) cores with any staining for synaptophysin or chromogranin. Chromogranin stained less than half of the synaptophysin-positive cases and did not identify additional synaptophysin-negative cases. Full sections of the moderate-strongly positive cores were then stained with synaptophysin and chromogranin, to identify cases with >50% neuroendocrine differentiation, in accordance with the 2003 WHO criteria. Overall, at least 9/372 (2.4%) of invasive carcinomas from the TMA had >50% neuroendocrine reactivity on full sections. We also stained a TMA containing ductal carcinoma in situ (DCIS) with or without accompanying invasive carcinoma, and found a slightly greater percentage of DCIS with neuroendocrine reactivity: 19 of 193 (9.8%) had any staining and 13/193 (6.7%) had strong staining on the TMA.

Combining the TMA screening and database searches, we identified 18 cases of invasive or recurrent/metastatic breast neuroendocrine breast carcinoma for further study. Most of these were estrogen receptor–positive, and many were high stage (Table 1). Surprisingly, several of these neuroendocrine carcinomas were originally diagnosed as lobular carcinoma. Excluding the metastases, 5 of 14 (35%) neuroendocrine carcinomas had either lobular morphology (discohesive single cells), were E-cadherin negative, or both (Fig. 1, Table 1, cases 12 to 16).

Molecular Analysis

Eighteen cases of neuroendocrine breast carcinoma (14 primary, 4 metastatic) were screened for a large panel of point mutations using a PCR/mass spectroscopy or semiconductor-based sequencing strategy[19–21,23]; 3 primary carcinomas were subsequently excluded based on poor DNA quality, leaving 15 cases. One or more activating mutations were identified in 5 tumors (33%), all sequence confirmed. One metastatic carcinoma had a *PIK3CA* exon 9

TABLE 1. Neuroendocrine Breast Carcinoma Cases and Mutations

Case	Mutation Status	Morphology	Stage and Clinical Follow-up	Hormone Status
1 (nodal metastasis)	Wild-type*	Nests, sheets	rTX N3a M1 breast cancer 12 y prior; chemotherapy; also bone metastasis	$ER^+PR^+Her2^+$
2 (bone metastasis)	*PIK3CA* E545K*	Nests, cords, rare acini	rTX NX M1 breast cancer 15 y prior; XRT, tamoxifen × 5 y	$ER^+PR^-Her2^-$
3 (adrenal metastasis)	*PIK3CA* E542K and *FGFR4* V550M*	Large nests, rare acini, focally spindled cytology	rTX NX M1 breast cancer 8 y prior; unknown therapy; also brain, ovary metastases	ER^+PR^+Her2eq
4 (adrenal metastasis)	Wild-type*	Small and large nests	rTX $N1^+$ M1 breast cancer 13 y prior; XRT and chemotherapy	ER^+
5 (primary)	*KDR* A1065T*	Small and large nests, cords, rare acini; ductal grade 2	TX $N1^+$	$ER^+PR^+Her2^-$
6 (primary)	Wild-type*	Large nests, focally spindled cytology; ductal grade 2	T1c N1mi	$ER^+PR^+Her2^-$
7 (primary)	Wild-type (SNP: *FGFR3* F384L) *	Small nests; ductal grade 2	Not available	$ER^+PR^-Her2^-$
8 (primary)	Wild-type*	Large nests, sheets; ductal grade 2	Not available	Not available
9 (primary)	Wild-type*	Large nests; ductal grade 2 E-cadherin positive	T1c N0 concurrent ipsilateral mixed ductal-mucinous carcinoma	$ER^+PR^+Her2^-$
10 (primary)	Wild-type*	Cords, single cells, rare acini; ductal grade 2	T2 N0	$ER^+PR^+Her2^-$
11 (primary)	Wild-type*	Small nests, cords; ductal grade 3	T2 $N1^+$	$ER^+PR^+Her2^-$
12 (primary)	Not available (poor-quality DNA)	Cords; lobular; E-cadherin positive	T3 N3 pericardial carcinoma year after diagnosis	$ER^+PR^+Her2^-$
13 (primary)	Wild-type*	Single cells; lobular grade 1 E-cadherin negative	T3 N1	$ER^+PR^+Her2^-$
14 (primary)	Not available* (poor-quality DNA)	Small clusters, single cells; grade 2 E-cadherin negative	T1 N0	$ER^+PR^+Her2^-$
15 (primary)	Not available (poor-quality DNA)	Cords; lobular	T2 N3a liver metastasis same year	$ER^+PR^+Her2^-$
16 (primary and metastasis/recurrence)	*HRAS* G12A* (both tumor samples)	Highly pleomorphic/anaplastic single cells; grade 3; E-cadherin negative	T2 N1 recurrent/metastatic within 1 y without chemotherapy	$ER^-PR^-Her2^-$
17 (primary)	Wild-type†	Small nests and cords; ductal grade 3	T2 N1a	$ER^+PR^+Her2^+$
18 (primary)	*PIK3CA* N345K and *FGFR1* P126S†	Large nests; ductal grade 1	T1b N0	$ER^+PR^+Her2^+$

*Screened with PCR-mass spectroscopy (Sequenom).
†Screened with next-generation semiconductor platform (Ion Torrent).
ER indicates estrogen receptor; eq, equivocal; Her2, Her-2/Neu; PR, progesterone receptor; SNP, single nucleotide polymorphism; XRT, radiation therapy; y, year.

E542K mutation, whereas another metastatic carcinoma harbored a *PIK3CA* exon 9 E542K mutation in addition to a *FGFR4* exon 13 V550M mutation. A primary breast carcinoma (stage pT1b N0) contained 2 mutations: *PIK3CA* exon 4 N345K, as well as *FGFR1* exon 3 P126S. A chemotherapy-naive primary invasive carcinoma presenting with lymph node metastases had a *KDR* exon 24 A1065T mutation. Another high-grade triple-negative neuroendocrine tumor with lymph node metastasis had an *HRAS* G12A substitution; the corresponding recurrent/metastatic tumor 8 months later demonstrated the same mutation.

DISCUSSION

In a TMA screen, we found 5.4% of invasive breast cancers with evidence of neuroendocrine differentiation, of which 2.4% of carcinomas were confirmed to have diffuse (>50%) immunohistochemical staining for neuroendocrine markers. In a prior TMA study, Makretsov et al[6] found 19.5% of breast carcinomas positive for neuroendocrine markers, with most staining for only synaptophysin; however, only 3% of their cases had "diffuse" neuroendocrine differentiation. These neuroendocrine carcinomas were mostly estrogen and progesterone receptor–positive, Her2 negative, as is typical of luminal A tumors, and similar to other neuroendocrine series in the literature.[3,5–7,11–13,20,24] Makretsov et al[6] identified at least 3 neuroendocrine cases with some degree of lobular morphology; likewise, 35% of our cohort had lobular morphology or E-cadherin negativity. Importantly, we also encountered many high-stage neuroendocrine breast cancers, as has been recently elucidated in other series.[3–4,7,11–13,24] Even if only the TMA-screen cases are considered, 5 of 9 cases had lymph nodes metastases at presentation, and 2 of 9 patients were diagnosed with distant metastases within 1 year (Table 1).

To our knowledge, this study represents the first systematic investigation of activating mutations in

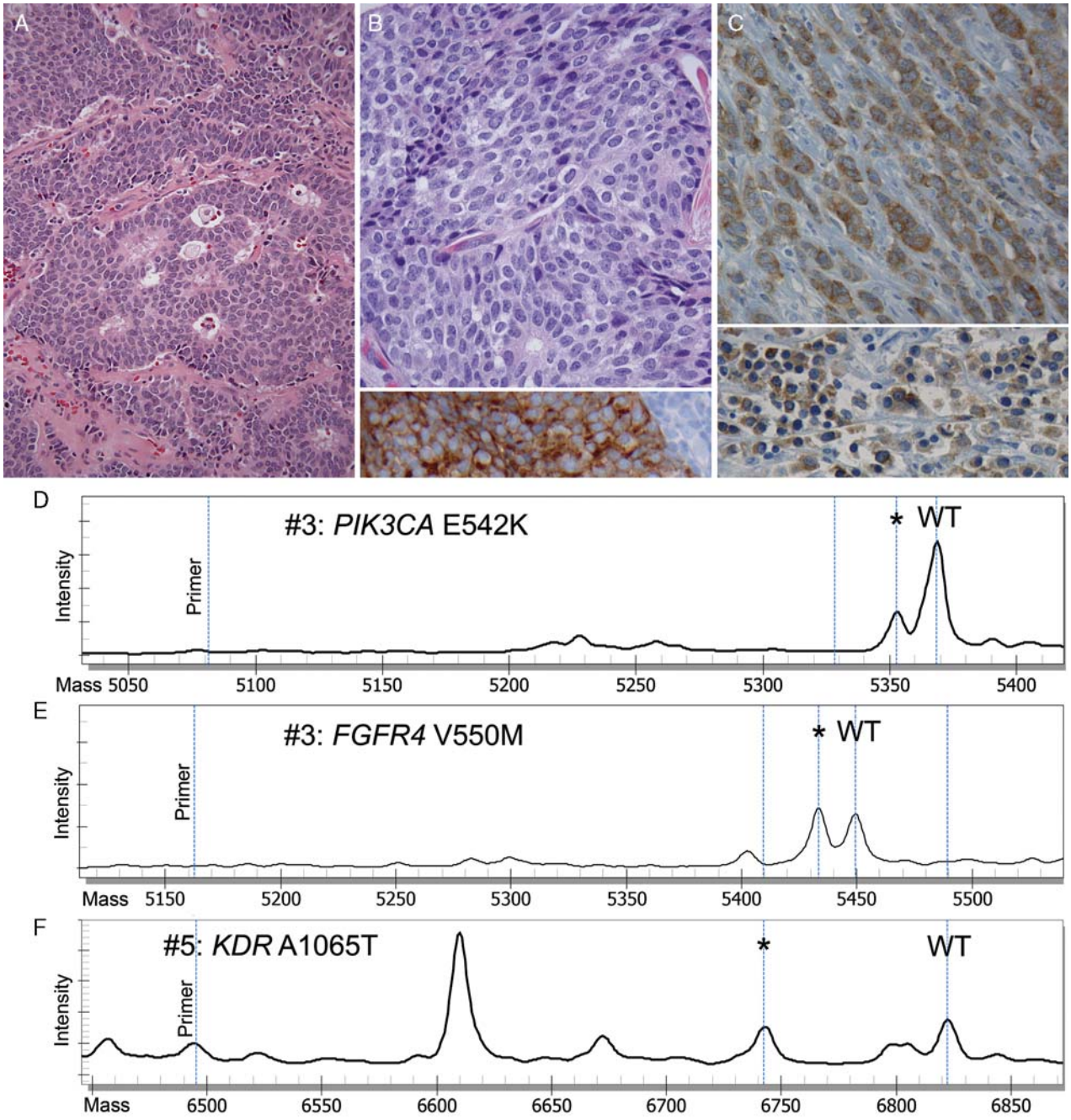

FIGURE 1. Histologic, immunohistochemical, and mutational characterization of neuroendocrine carcinoma. A, Morphologic features of case 3, metastatic to adrenal gland. Cells with neuroendocrine chromatin are arranged in nests and rosettes. Mutational analysis is shown in (D–E). B, Morphologic features of case 5, a primary breast neuroendocrine carcinoma. Positive synaptophysin staining in shown in the bottom part of the panel. Mutational analysis of this case is in (F). C, Synaptophysin staining of cases 12 and 13, both with lobular-like architecture. Case 12 at top, with cords and small nests of cells. Case 13, at bottom with discohesive cells. D, Polymerase chain reaction (PCR)-mass spectroscopy analysis of lesion 3 demonstrates a *PIK3CA* E542K mutant (*) peak, along with a larger wild-type peak (WT). The E542K mutation was confirmed by direct sequencing (data not shown). E, Lesion 3 also harbors a *FGFR4* V550M mutation (*). WT designates the wild-type peak for this assay. The V550M mutation was confirmed by direct sequencing (data not shown). F, PCR-mass spectroscopy analysis of lesion 5 demonstrates a *KDR* A1065T mutant (*) peak, along with a larger wild-type peak (WT). The A1065T mutation was confirmed by direct sequencing (data not shown). The large peak in the middle of the PCR-MS tracings represent a wild-type peak from an unrelated, multiplexed assay. full color online

neuroendocrine breast carcinoma. We identified mutations in 5 of 15 (33%) neuroendocrine carcinomas. These included mutations common to invasive ductal carcinoma, not otherwise specified (*PIK3CA*), as well as mutations that are rare or novel in breast cancer (*FGFR1* and *FGFR4, KDR, HRAS*). Our prior study of mucinous carcinomas included at least 3 with neuroendocrine differentiation, yet we found that none of the mucinous neuroendocrine carcinomas had *PIK3CA* or other activating mutations.[19] Further, these activating mutations have not been reported in neuroendocrine neoplasms of nonbreast derivation, to our knowledge.

Mutations in *PIK3CA*, the gene encoding phosphatidylinositol-3-kinase catalytic subunit, have been well described in 25% to 30% of breast cancers, and are further enriched in luminal-type breast cancers.[25,26] We found *PIK3CA* mutations in 3/15 (20%) of neuroendocrine breast cancers. This suggests that neuroendocrine and invasive ductal breast carcinoma may share some elements of molecular pathogenesis. Further, therapeutics targeting the PIK3CA-AKT1-mTOR pathway are under development, with a number in clinical trials.[25,26]

In contrast to *PIK3CA*, activating point mutations in fibroblast growth factor receptors (FGFR) are distinctly rare among the unselected series of breast carcinomas studied to date (Table 2), although the *FGFR1/ZNF703* locus is frequently amplified in breast cancer.[27–29] FGFR1-4 comprise a family of transmembrane receptor tyrosine kinases that activate downstream signaling cascades, especially RAS-MAPK, STAT, and cross-talk with phosphatidylinositol-3-kinase pathways.[27–29] Two cases in our neuroendocrine breast carcinoma series had mutations in both *PIK3CA* and a FGFR family member (*FGFR1* P126S, *FGFR4* V550M). *FGFR3* and *PIK3CA* mutations commonly coexist in bladder cancers,[28] but have not been previously reported in breast cancer.[27]

Tyrosine kinase domain mutations in *FGFR4* have been reported in a small percentage of pediatric rhabdomyosarcomas (3% to 7%), especially embryonal type.[30–32] Taylor et al[30] demonstrated that *FGFR4* tyrosine kinase V550E mutations cause upregulation of STAT3, decreased AKT phosphorylation, and are associated with increased invasion and metastasis in in vitro and xenograft models. An *FGFR4* mutation (cataloged as V510M, but equivalent to V550M) was previously discovered in a pleomorphic lobular carcinoma which harbored an unusually large number of mutations, but its neuroendocrine status was not published (Table 2).[33] As the case in our series was a metastatic tumor in a patient who had previously received chemotherapy, we cannot exclude some effect of the prior treatment in causing or selecting for mutations.

Although the *FGFR1/ZNF703* locus is amplified in about 10% of breast carcinomas, notably estrogen receptor–positive cases,[28] *FGFR1* point mutations have been identified in breast cancer only rarely (Table 2). Dovitinib (TKI258) is a small molecule tyrosine kinase inhibitor of FGFR1-3 and VEGFR1-3 that inhibits growth of cell lines and xenografts with amplified FGFR1, and is currently in phase II clinical trials for breast cancer.[34] Other orally bioavailable FGFR inhibitors under development include Ki23057, ponatinib, and AZD4547.[35]

TABLE 2. Point Mutations of *FGFR1, FGFR4, KDR,* and *HRAS* Reported in Breast Cancers

Gene	Mutations	Comments and References
FGFR1	S125L	Cell line (HCC1395)[33]
	S125P	Neuroendocrine breast cancer, $ER^+PR^+Her2^+$ (present study #18)
	K566R	Triple-negative breast cancer[46]
FGFR4	E326K	Her2 intrinsic type[45]
	A484T	Luminal B intrinsic type[45]
	V550M	Pleomorphic lobular carcinoma[33]
	V550M	Neuroendocrine breast carcinoma metastasis ER^+PR^+Her2 equivocal (present study #3)
	E741deletion	Luminal A intrinsic type with frameshift deletion[45]
KDR	H269Y	Luminal A intrinsic type[45]
	A1065T	Neuroendocrine breast cancer $ER^+PR^+Her2^-$ (present study #5)
	S1200Y	Luminal B intrinsic type[45]
HRAS	G12A	Neuroendocrine pleomorphic lobular carcinoma $ER^-PR^-Her2^-$ (present study #16)
	G12D	Breast papilloma with atypical hyperplasia[18]
	G12D	Breast cancer cell lines (Hs578T, SUM159PT)[47]
	Q61R	Two breast papillary carcinomas[44]

ER indicates estrogen receptor; Her2, Her-2/neu; PR, progesterone receptor.

Ras family proteins (K-ras, N-ras, H-ras) are small GTP-ases of Raf-MEK-ERK signaling pathways, which cross-talk with the PI3K pathway.[39,40] Hotspot point mutations in codons 12, 13, or 61 perturb GTP hydrolysis, resulting in prolongation of the activated state.[39] Although *HRAS* mutations are found in follicular thyroid carcinoma, head and neck squamous cell carcinoma, and melanocytic Spitz lesions,[40,41] they are quite rare in other human cancers, including breast cancers (Table 2).[39–43] Our group previously identified an *HRAS* G12D mutation in a breast papilloma with atypical hyperplasia,[18] and the *HRAS* Q61R mutation was identified in 2 of 3 intracystic papillary carcinomas by Esposito et al[44] (Table 2).

KDR (VEGFR2) encodes a receptor tyrosine kinase involved in angiogenesis.[36–38] Activating *KDR* mutations have been reported in non–small cell lung cancers, and in breast angiosarcomas, including primary and radiation-induced, but only in isolated cases of breast cancer (Table 2).[36–38] Antonescu and colleagues showed that the *KDR* A1065T mutation in the intracellular tyrosine kinase domain imparts ligand-independent kinase activation, which can be inhibited by VEGFR kinase inhibitors such as sorafenib and suntinib in an in vitro model.[36,38]

In summary, our findings from this small cohort of neuroendocrine carcinomas reinforce the notion that this special subtype of carcinoma may have a propensity for lymph node and distant metastasis, and thus may be important to recognize diagnostically. Importantly, some

of these tumors harbor potentially actionable oncogene mutations. We also noted that neuroendocrine breast carcinoma may manifest a discohesive "lobular-like" growth pattern. Further studies will be necessary to determine the clinical and prognostic impact of neuroendocrine breast carcinoma, and the prevalence and implications of activating mutations and other molecular alterations.

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