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Clinical and genomic analyses of neuroendocrine neoplasms of the breast

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Breast neuroendocrine neoplasms (NENs) constitute a rare histologic subtype that includes both neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs). In this study, we aimed to gain insight into the clinical and molecular characteristics of NENs of the breast. NEN and paired distant normal fresh tissues and clinicopathological data were obtained from 17 patients with NENs, and clinicopathological data were collected from 755 patients with invasive breast carcinomas of no special type (IBCs-NST). We compared the clinicopathological characteristics of NENs and IBCs-NST and performed whole-exome sequencing (WES) of both NEN and paired normal tissues. Compared with the IBC-NST patients, the NEN patients had a higher mean age, lower clinical stage, and lower pathological nodal (pN) stage ($P < 0.001$, $P < 0.001$, and $P = 0.017$, respectively). The most frequently mutated gene in NENs was *KMT2C* (3/17, 17.6%). NENs had copy number variations (CNVs) of 8q, 11q, and 17q amplification and 17q and 11q deletion and harbored the following specific genes related to tumorigenesis: (i) suppressor genes with loss of heterozygosity (LOH) such as *ACE* (2/17, 11.8%); (ii) tumor driver genes such as *GATA3* (2/17, 11.8%); and (iii) susceptibility genes such as *MAP3K4* (17/17, 100%) and *PDE4DIP* (17/17, 100%). The oncogenic/likely oncogenic mutations of NETs in PI3K pathway genes (50.0%, 18.2%; $P < 0.001$) and MAPK signaling pathway genes (83.3%, 18.2%; $P = 0.035$) affected higher proportions than those of NECs. In conclusion, this study provides certain clinical and molecular evidence supporting NENs as a distinct subtype of breast cancer and provides some potential molecular features for distinguishing NETs from NECs.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) of the breast constitute an extremely rare histologic subtype that includes neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs). The latest WHO classification (2019) aimed to reduce discrepancies and contradictions in their terminology, subgroups, and criteria for histologic grading and staging by unifying the definitions of other organ systems with a uniform classification framework of NENs. The definition of breast NENs in the latest classification provides the most stringent diagnostic criteria compared to previous WHO classifications;[1–3] the term NEN was characterized by diffuse neuroendocrine (NE) marker expression and NE morphology, regardless of Nottingham histologic grade[1,4]. However, few studies have investigated NENs based on the latest version of the classification criteria, and various terminologies and diagnostic criteria for breast cancer with NE differentiation have been used in earlier studies[5–7], resulting in a poor understanding of the clinicopathological and molecular features of NENs.

Although prior studies have provided some clinicopathological features of NEN, many issues remain unsolved. Many studies using the 2003 criteria showed that NENs were more likely to be older, have larger tumors, be estrogen receptor/progesterone receptor (ER/PR)-positive and be human epidermal growth factor receptor 2 (HER2)-negative than invasive breast cancers (IBCs-NST)[2,8,9]. In addition, some published studies revealed adverse outcomes in NE breast cancers[7,10–15], and most of the above studies considered NEN as a whole entity rather than dividing it into subgroups. Thus, the clinicopathological factors, prognosis, and treatment strategies for NENs and even the subgroups of NETs and NECs require more data accumulation.

Studies focusing on molecular characteristics provide certain molecular evidence regarding NENs of the breast. Compared with mucinous breast carcinomas (MBCs), breast NECs have different copy number variation (CNV) levels as follows:[16–18] 1q increase and 16q deletion coexist in 28% of NECs; breast NETs are characterized by an enrichment of transcriptional mutations and are considered representative of a spectrum of entities histologically and genetically similar to MBCs. In addition, the majority of breast NETs are distinct from ER-positive/HER2-negative IBCs-NST[19]. Moreover, although a recent study conducted in 2021 demonstrated that NETs had better disease-specific survival (DSS) and overall survival (OS) than NECs[12], the differences between NETs and NECs have not been confirmed at the molecular level.

Due to the use of diverse nomenclature and diagnostic criteria for NENs of the breast in different studies, the clinicopathological features, molecular characteristics and therapeutic strategies of NEN remain largely known. Thus, our study aimed to (i) compare the differences in the clinicopathological characteristics and prognosis between NENs and IBCs-NST, (ii) identify the molecular characteristics of NENs, and (iii) find the differences between NETs and NECs.

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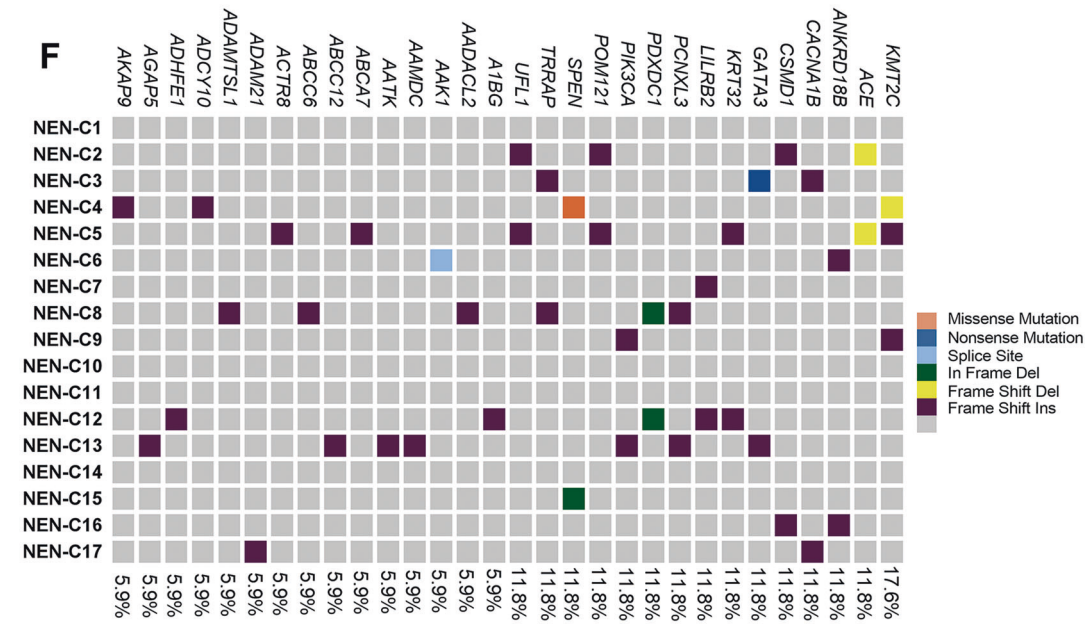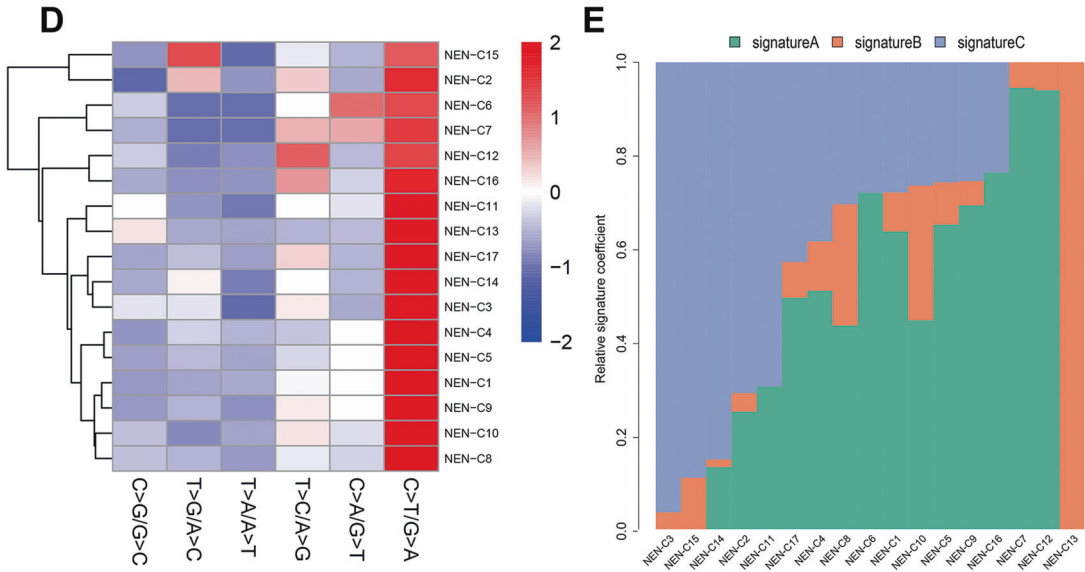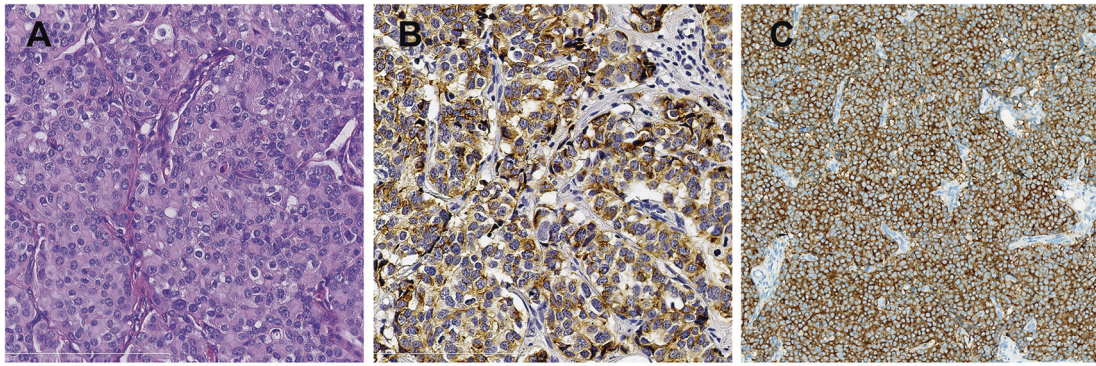
Fig. 1 Histological morphology and mutation analysis results of NENs. A Low-grade of NEN (H&E, Nottingham grade 2). IHC of NEN showing diffuse, uniform immunoreactivity of CgA **B** and Syn **C. D** Mutation spectrum analysis showing that the most frequent mutation type in the NENs was C > T/G > A, followed by T > C/A > G, and mutation type with the lowest frequency was T > A/A > T. Each row in the figure represents a sample, and each column represents a mutation type. The darker the mutation type, the higher proportion of the sample. **E** Proportion of each mutation feature in different samples. Nonnegative matrix factorization (NMF) cluster analyses revealed three different mutation signatures. The Y-axis indicates the proportion of acquired mutation signatures. Signature B was found to be identical to COSMIC mutation feature 2 with a cosine similarity greater than 0.9, and it was found in 70.6% (12/17) of the NENs in this study. **F** Frequency mutation gene analysis showing that the most frequently mutated genes in NENs were *KMT2C* (3/17, 17.6%), *ACE* (2/17, 11.8%), *ANKRD18B* (2/17, 11.8%), and *CACNA1B* (2/17, 11.8%). The X-axis on the bottom reflects frequency mutations, the X-axis on the top reflects the gene name, and the Y-axis reflects the sample name. The different colors in the map represent different types of mutations. This figure shows only some genes with high mutation frequencies, and the full list is shown in Supplementary Table 3.

MATERIALS AND METHODS
NEN and IBC-NST case collection
Patients diagnosed with NEN from 2013 to 2018 and IBC-NST from 2008 to 2019 were collected from the surgical pathology files of West China Hospital, Sichuan University. The inclusion and exclusion criteria for NEN were as follows:[1] (i) cancers with > 90% NEN pattern; (ii) tumor cells with ≥90% chromagranin A (CgA) and/or synaptophysin (Syn) expression; (iii) NENs with low Nottingham grade (grade 1–2) were classified as NETs, and high Nottingham grade NENs were classified as high grade as NECs; (iv) special breast cancers with the expression of NE markers, such as solid papillary carcinoma in situ, MBCs, were excluded.

Fresh NEN tissues and matched NEN distant normal tissues were extracted from the biobank of West China Hospital, Sichuan University, but fresh tissue from the control group was not available. Clinicopathologic data, including age, menstrual status, laterality, clinical tumor (cT) stage, clinical nodal (cN) stage, clinical (TNM) stage, pathological tumor (pT) stage, pathological node (pN) stage, and follow-up information of patients with NENs were collected from the Breast Cancer Patient Follow-up database of West China Hospital, Sichuan University.

Histological review and immunohistochemistry (IHC) for NEN and IBC-NST
Hematoxylin and eosin (H&E)-stained slides were reviewed by 3 pathologists under a multi-head microscope (BW, YW, and XK) to morphologically confirm whether each case met the diagnostic criteria of the 5th edition of the WHO classification in 2019[1].

Immunohistochemical staining was performed using the selected most representative paraffin block using the following antibodies: ER, PR, HER2, Ki67, CgA, and Syn. See Supplementary Table 1 for the details of the antibodies.

ER and PR expression was assessed based on the 2010 American society of clinical oncology/college of American pathologists (ASCO/CAP) guideline recommendations[20]. HER2 status was assessed according to the ASCO/CAP clinical practice guideline focused update[21]. A cutoff of 20% was used for the evaluation of Ki67 expression[22]. Tumor cells expressing ≥90% CgA and/or Syn were considered positive[1].

Comparison of clinicopathological features in NEN and IBC-NST patients
SPSS 25.0 (SPSS, Chicago, IL, USA) statistical analysis software was used to compare the differences in the clinicopathological characteristics among the groups (NETs vs. NECs and NENs vs. IBCs-NST). The descriptive statistics of the demographic and clinicopathologic factors were calculated, and the differences among the groups were evaluated by the chi-square or Fisher exact test, as appropriate. Kaplan-Meier curves and statistics (log-rank) were used for survival data representation and analysis[23]. Propensity score matching (PSM)[24] was used to balance the important characteristics of the NEN and IBC-NST patients (ratio 1:5). Two-tailed *P* values less than 0.05 were considered to indicate statistical significance.

DNA extraction and quality assessment of NEN fresh tissues
DNA was purified from fresh NEN tissues and matched normal tissues using a Qiagen kit (QIAamp System, Germany) and amplified by polymerase chain reaction (PCR). DNA quality control included the assessment of degradation and contamination by 0.1% agarose gel electrophoresis. In addition, the DNA concentration was measured (Qubit® DNA Assay Kit, Invitrogen, USA), and only qualified fresh samples were used for whole-exome sequencing (WES).

Whole-exome sequencing of 17 paired NEN and normal fresh tissues
According to the above DNA evaluation results, samples with DNA concentrations ≥20 ng/μL and total amounts >0.6 μg were used to generate a library (Agilent Technologies, CA, USA). Then, the genomic DNA was randomly broken into fragments of 180–280 bp in length (Covaris, Massachusetts, USA). The remaining short DNA fragments were converted into blunt ends by exonuclease/polymerase activities. When a poly(A) tail was added to the short DNA fragments, they were linked by adapter oligonucleotides. The ligated DNA fragments were enriched by PCR. The library with a specific index was subjected to liquid phase hybridization with up to 543,872 biotin-labeled probes. Then, 334,378 exons of 20,965 genes were captured by magnetic beads with streptomycin, which was linearly enriched by PCR. Finally, the DNA was purified (Beckman Coulter, Beverly, USA) and quantified (Agilent Bioanalyzer 2100 system), and then, the DNA was prepared for sequencing.

The DNA samples were clustered according to the manufacturer's instructions (HiSeq PE Cluster Kit, Illumina). Then, DNA libraries of appropriate sizes were sequenced on the Illumina HiSeq PE150 platform.

Bioinformatics analyses
The analyses mainly included the following three aspects: data quality evaluation, NEN molecular characteristics analyses, and comparative analyses of NET and NEC characteristics. The quality evaluation of the raw data included sequencing data filtering, sequencing error rate distribution checking, sequencing data quality distribution, and reference sequence alignment analysis (sequencing depth and coverage distribution). The NEN molecular characteristics analyses included DNA mutation analyses, loss of heterozygosity (LOH) analysis, driver gene, and susceptibility gene identification. See Supplementary Materials and methods for the details of the mutation signature analysis. The differences in the gene pathways enriched by somatic mutations after excluding germline mutations between the NETs and NECs were also compared. In the comparative pathway analysis, the genes were obtained from PathCards (https://pathcards.genecards.org/).

The details of raw sequencing data cleaning are shown in the Supplementary Materials and methods.

RESULTS
Comparison of the characteristics in NEN and IBC-NST patients
In total, 17 patients with NEN and 755 patients with IBC-NST were assessed in this study. Among the NEN cases, 35.3% (6/17) were NETs, and 64.7% (11/17) were NECs. Representative cases of NEN are shown in Fig. 1A–C. The clinicopathological characteristics, treatment strategies, and prognostic information of the 17 NEN patients are shown in Table 1.

The baseline characteristics and comparison results of the NENs and IBCs-NST are shown in Table 2. The mean age of the NEN patients (50.0 years) was higher than that of the IBC-NST patients (47.6 years) (*P* < 0.001). Low clinical tumor stage (cT1 and cT2), lymph node stage (cN0), and clinical stage (stage I and stage II) were more common in the NEN group than in the IBC-NST group (*P* < 0.001, *P* < 0.001, *P* = 0.001). More NET patients had pN0 stage than stage-matched IBC-NST patients (83.3% vs.26.1%, *P* = 0.017). NEC patients exhibited higher PR expression than that in IBC-NST patients (90.9% vs. 68.2%; *P* = 0.030).

Table 1. Summary of available clinicopathologic features of NENs (N = 17).

Features	NET (N = 6)						NEC (N = 11)										
Case no.	NEN-1	NEN-4	NEN-11	NEN-13	NEN-14	NEN-17	NEN-2	NEN-3	NEN-5	NEN-6	NEN-7	NEN-8	NEN-9	NEN-10	NEN-12	NEN-15	NEN-16
Gender/Age	F/47	F/60	F/32	F/54	F/73	F/48	F/75	F/52	F/35	F/41	F/52	F/59	F/46	F/49	F/53	F/49	F/25
Laterality	L	R	R	R	R	R	R	R	L	L	L	R	R	L	L	R	R
Menopausal status	Pre	Post	Pre	Pre	Post	Pre	Post	Post	Pre	Pre	Post	Post	Pre	Pre	Post	Pre	Pre
cTNM stage	I	IIA	IIIC	IIA	I	I	IIA	IIA	IIB	IIB	IIA	IIA	IIIC	IIIC	IIIC	IIIA	IIA
pT	1	2	2	2	1	1	2	2	2	2	2	2	2	2	2	2	1
pN	0	0	3	0	0	0	0	0	1	1	0	0	3	3	2	2	0
ER status	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
PR status	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
HER2 status	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	-
Ki67 (%)	30	35	3	20	10	5	30	30	80	50	75	75	30	50	45	35	30
CgA (%)	5	0	60	0	30	90	5	60	95	5	90	0	5	0	10	10	80
Syn (%)	90	95	90	90	90	90	95	100	95	95	0	95	90	90	90	95	90
LVI	N	N	N	N	N	N	N	Y	Y	N	N	N	Y	Y	N	Y	N
Surgery type	Ma	Ma	Ma	Ma	Ma	Lu	Ma	Ma	Ma	Ma	Ma	Lu	Ma	Ma	Ma	Ma	Ma
Chemotherapy	Y	N	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Radiotherapy	N	N	Y	N	N	N	N	N	Y	Y	N	N	Y	Y	Y	Y	Y
Endocrine therapy	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y
Anti-HER2 therapy	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N
Follow-up (months)	NED (44)	NED (29)	NED (55)	NED (11)	NED (1)	NED (18)	NED (61)	NED (59)	NED (43)	CM/AM (39)	NED (63)	NED (3)	NED (37)	BM (4) DOD (32)	NED (46)	NED (67)	NED (5)

Case no., Case number; F, female; L, left; R, right; Pre, premenopausal; Post, postmenopausal; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; NA, not available; CgA, Chromogranin A; Syn, Synaptophysin; LVI, lymphovascular invasion; NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; Ma, mastectomy; Lu, Lumpectomy; Y, yes; N, no; NED, no evidence of disease; CM, chest metastasis; AM, abdomen metastasis; BM, bone metastasis.

Table 2. Comparison of the clinicopathological features in NEN and IBC-NST patients.

Features	NEN (N = 17)	NET (N = 6)	NEC (N = 11)	IBC-NST (N = 755)	P_1 NET vs. NEC	P_2 NEN vs. IBC-NST
Age						
Mean	50 ± 12.9	52.3 ± 13.8	48.7 ± 12.9	47.6 ± 9.9	0.256	**<0.001**
≤50 y	9 (52.9)	3 (50.0)	6 (54.5)	453 (60.0)	1.000	0.620
>50 y	8 (47.1)	3 (50.0)	5 (45.5)	302 (40.0)		
Gender						
Female	17 (100)	6 (100.0)	11 (100.0)	753 (99.7)	-	1.000
Male	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.3)		
Menstruation						
Prem	10 (58.8)	4 (66.7)	6 (54.5)	474 (62.3)	1.000	0.802
Postm	7 (41.2)	2 (33.3)	5 (45.5)	281 (37.2)		
Laterality						
Left	6 (35.3)	1 (16.7)	5 (45.5)	394 (52.2)	0.333	0.221
Right	11 (64.7)	5 (83.3)	6 (54.5)	361 (47.8)		
cT						
1	4 (23.5)	3 (50.0)	1 (9.1)	49 (6.5)		
2	13 (76.5)	3 (50.0)	10 (90.9)	322 (42.6)		
3	0 (0.0)	0 (0.0)	0 (0.0)	121 (16.0)	0.099	**<0.001**
4	0 (0.0)	0 (0.0)	0 (0.0)	250 (33.1)		
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	13 (1.7)		
cN						
0	9 (52.9)	5 (83.3)	4 (36.4)	61 (8.1)		
1	2 (11.8)	0 (0.0)	2 (18.2)	281 (37.2)		
2	2 (11.8)	0 (0.0)	2 (18.2)	148 (19.6)	0.263	**<0.001**
3	4 (23.5)	1 (16.7)	3 (27.3)	200 (26.5)		
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	65 (8.6)		
TNM stage						
I + II	12 (70.6)	5 (83.3)	7 (63.6)	212 (28.1)		
III + IV	5 (29.4)	1 (16.7)	4 (36.4)	522 (69.1)	0.600	**0.001**
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	21 (2.8)		
pT						
1	4 (23.5)	3 (50.0)	1 (9.1)	327 (43.3)		
2	13 (76.5)	3 (50.0)	10 (90.9)	320 (42.4)		
3	0 (0.0)	0 (0.0)	0 (0.0)	53 (7.0)	0.099	0.069
4	0 (0.0)	0 (0.0)	0 (0.0)	35 (4.6)		
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	20 (2.6)		
pN						
0	10 (58.8)	5 (83.3)	5 (45.5)	197 (26.1)		
1	2 (11.8)	0 (0.0)	2 (18.2)	275 (36.4)		
2	2 (11.8)	0 (0.0)	2 (18.2)	161 (21.3)	0.371	**0.017**
3	3 (17.6)	1 (16.7)	2 (18.2)	122 (16.2)		
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	10 (1.3)		
NG						
1	0 (0.0)	0 (0.0)	0 (0.0)	21 (2.8)	**<0.001**	0.175
2	6 (35.3)	6 (100.0)	0 (0.0)	382 (50.6)		
3	11 (64.7)	0 (0.0)	11 (100.0)	300 (39.7)		
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	52 (6.9)		
LVI						
Yes	12 (70.6)	0 (0.0)	5 (45.5)	138 (18.3)	0.102	0.348
No	5 (29.4)	6 (100.0)	6 (54.5)	578 (76.6)		
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	39 (5.2)		

Table 2 continued

Features	NEN (N = 17)	NET (N = 6)	NEC (N = 11)	IBC-NST (N = 755)	P₁ NET vs. NEC	P₂ NEN vs. IBC-NST
ER						
Negative	2 (11.8)	1 (16.7)	1 (9.1)	201 (26.6)	1.000	0.264
Positive	15 (88.2)	5 (83.3)	10 (90.9)	554 (73.4)		
PR						
Negative	1 (5.9)	0 (0.0)	1 (9.1)	239 (31.7)		
Positive	16 (94.1)	6 (100.0)	10 (90.9)	515 (68.2)	1.000	**0.030**
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)		
HER2						
Negative	12 (70.6)	6 (100.0)	6 (54.5)	596 (78.9)	0.102	0.378
Positive	5 (29.4)	0 (0.0)	5 (45.5)	159 (21.1)		
Ki67						
≤20%	3 (17.6)	4 (66.7)	0 (0.0)	220 (29.1)		
>20%	14 (82.4)	2 (33.3)	11 (100.0)	496 (65.7)	**0.029**	0.776
Unknown	0 (0.0)	0 (0.0)	0 (0.0)	39 (5.2)		
Subtypes						
HR + HER2-	12 (70.6)	6 (100.0)	6 (54.5)	479 (63.4)	0.145	0.330
HR + HER2 +	4 (23.5)	0 (0.0)	4 (36.4)	118 (15.6)		
HR-HER2 +	1 (5.9)	0 (0.0)	1 (9.1)	41 (5.4)		
HR-HER2-	0 (0.0)	0 (0.0)	0 (0.0)	117 (15.5)		
Pathways						
PI3K	4 (23.5)	3 (50.0%)	2 (18.2)	–	**<0.001**	–
Chrom remod	8 (47.1)	2 (33.3)	6 (54.5)	–	0.620	–
MAPK	7 (41.2)	5 (83.3)	2 (18.2)	–	**0.035**	–
RB	5 (29.4)	2 (33.3)	3 (27.3)	–	1.000	–
p53	2 (11.8)	0 (0.0)	2 (18.2)	–	0.515	–
RTK	5 (29.4)	3 (50.0)	2 (18.2)	–	0.280	–
MYC	0 (0.0)	0 (0.0)	0 (0.0)	–	-	–

Prem, premenopausal; Postm, postmenopausal; NG, Nottingham grade; NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; IBC-NST, invasive breast carcinomas of no special type; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PI3K, PI3K/AKT activation pathway; Chrom remod, Chromatin remodelers; MAPK, MAPK signaling pathway; RB, Retinoblastoma (RB) pathway; p53, p53 pathway; RTK, RTK/Ras signaling pathway; MYC, MYC family genes.

The patients with NEN were matched 1:5 to IBC-NST, and 10 NEN and 42 IBC-NST patients were included in the Kaplan-Meier analysis. The Kaplan-Meier analysis showed that there were no significant differences in the OS or disease-free survival (DFS) between the NEN and IBC-NST patients, as shown in Supplementary Fig. 1A, B.

Sequencing data quality assessment
The ratio of the high-quality data (clean reads) of the 17 paired samples to the original test data (raw data) was greater than 90%; the error rate was less than 0.1%; and the quality of the sequencing data was distributed at Q30 (≥80%). The data quality output details of the NENs are shown in Supplementary Table 2. The analyses of the sequencing depth and coverage distribution of the NENs showed that the sequencing depth was mainly concentrated at 100×; the coverage depth of each chromosome was mainly distributed at 100× (Supplementary Fig. 2).

Mutation spectrum analyses related to the characteristics of NENs
The genetic mutation spectrum analysis of the NENs showed that the mutation type with the highest frequency was C > T/G > A (100%, 17/17), followed by T > C/A > G. The nonnegative matrix factorization (NMF) cluster analyses revealed three different

mutation signatures: Signature A (near signature 1, filter with cosine similarity = 0.82), Signature. B (near signature 2, filter with cosine similarity = 0.91), and Signature C (near signature 6, filter with cosine similarity = 0.70). If a filter has a cosine similarity > 0.9, the mutation feature of this sample and the known mutation feature are the same (Fig. 1D, E).

Frequency mutation gene analysis of the NENs showed that *KMT2C* was the most frequently mutated gene (17.6%, 3/17), followed by *ACE* (2/17, 11.8%), *ANKRD18B* (2/17, 11.8%), and *CACNA1B* (2/17, 11.8%) (Fig. 1F). This figure only partially shows some genes with mutation frequencies; the results of all mutated genes are shown in Supplementary Table 3.

The high-frequency CNV analysis showed that the NENs had amplification of 8q, 11q,17q, and 19q and deletion of 17q and 11q. The CNV distribution in each NEN sample and the high-frequency CNVs of all NENs are shown in Fig. 2A, B, respectively. The tumor clonal structure analysis showed that a high proportion of the NENs (9/17, 52.9%) were heterogeneous tumors. NENs with and without heterogeneity are shown in Supplementary Fig. 3.

Loss of heterozygosity and driver gene analyses in NEN tissues
The LOH analysis results showed that 88.2% (15/17) of the NEN patients exhibited LOH in tumor suppressor genes. Both NEN-4 and NEN-14 (2/17, 11.8%) showed LOH in the suppressor gene *ACE*
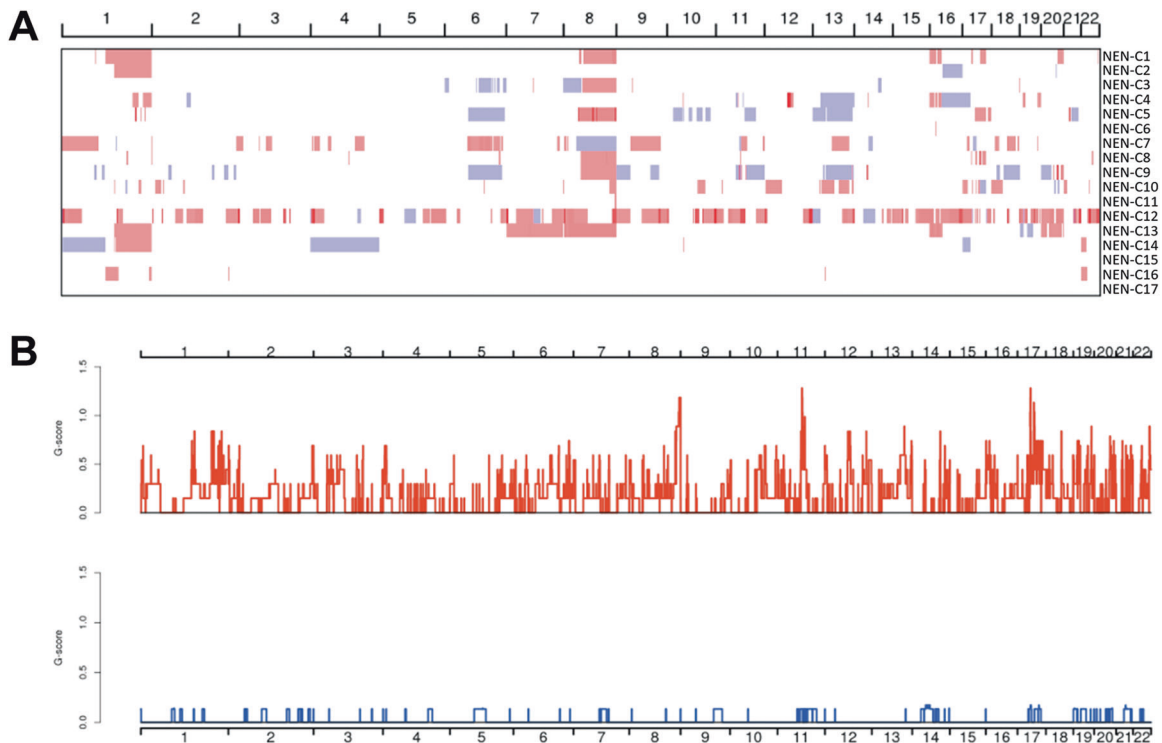
Fig. 2 CNV analysis results of NENs. A CNV distribution in each NEN sample. The X-axis is chromosomes 1-22, and the Y-axis is the NEN sample names. In the figure, red and blue represent somatic CNVs as follows: red represents an increase in the copy number, and blue represents a decrease in the copy number. The darker the color, the greater the change in the copy number. **B** High-frequency CNV analysis showed amplification of 8q, 11q, 17q, and 19q and deletion of 11q and 17q in all NEN cases. The X-axis is chromosomes 1–22; the Y-axis is the score of the high-frequency CNV segment by GISTIC software. The higher the score is, the higher the frequency of CNV in this segment. AMP (red) indicates an increase in the copy number, and DEL (blue) indicates a decrease in the copy number.

on chromosome 17. Most suppressor genes with LOH in NENs were located on chromosome 11 (11/16, 94.1%), chromosome 3 (14/17, 82.4%), the X chromosome (12/17, 70.6%), chromosome 6 (10/17, 58.8%), and chromosome 8 (9/17, 52.9%).

The predictive analysis of the driver genes indicated that GATA3 (2/17, 11.8%), KMT2C (2/17, 11.8%), TRRAP (2/17, 11.8%), and SPEN (2/17, 11.8%) were the driver genes of NENs (Fig. 3A).

Susceptibility gene analyses in tumor distant normal tissues
The following susceptibility genes were found in the NEN-matched normal tissues: MAP3K4 (17/17, 100%), PDE4DIP (17/17, 100%), NCOR2 (15/17, 88.2%), BPTF (14/17, 82.4%), MAP3K1 (14/17, 82.4%), and MUC20 (14/17, 82.4%) (Fig. 3B).

Analysis related to the treatment of NENs
This study compared somatic mutations in NENs to previously known databases such as PD FDA (KEGG-based targeted drug data), PharmGKB, DrugBank, My Cancer Genome, and therapeutic trial findings. In total, 76.5% (13/17) of NENs may harbor targetable mutations, rendering them candidates for targeted therapy. (Supplementary Fig. 4 and Supplementary Table 4).

The mutant genes that may be related to drug resistance in NENs were PIK3CA (2/17, 11.8%), CAT (1/17, 5.9%), AKT1 (1/17, 5.9%), TP53 (1/17, 5.9%), and ABCC6 (1/17, 5.9%) (Supplementary Table 5).

Comparative analyses of NETs and NECs
The comparative analysis of NETs and NECs showed that Ki67 expression and Nottingham grade in NECs were significantly higher than those in NETs (P = 0.029) (Fig. 4A, B), but there was no significant difference in OS and DFS between NETs and NECs (P = 0.540, P = 0.377, respectively; Supplementary Fig. 1C, D).

The mutated gene with the highest frequency in the NETs was ACE (2/6, 33.3%), followed by KMT2C (1/6, 16.7%) and ANKRD18B (1/6, 16.7%); the mutated gene with the highest frequency in the NECs was KMT2C (2/11, 18.2%), followed by CSMD1 (2/11, 18.2%) and KRT32 (2/11, 18.2%). The most common mutation types in NETs and NECs were nonsense mutations (3/6, 50.0%) and missense mutations (8/11, 72.7%), respectively. (Fig. 4C, D)

The analysis of the differences in gene pathways between NETs and NECs showed that the pathway with the highest gene mutation rate in NETs is MAPK (83.3%), followed by RTK/ras (50.0%), PI3K/AKT (33.3%), chromatin remodelers (33.3%), retinoblastoma (RB) (33.3%), and p53 (0.0%); the pathway with the highest gene mutation rate in NECs is chromatin remodelers (54.5%), followed by RB (27.3%), PI3K/AKT (18.2%), MAPK (18.2%), RTK/ras (18.2%) and p53 (18.2%) (Figs. 4E, F and Table 2). The genes contained in the gene pathways in this study are shown in Supplementary Table 6. Oncogenic/likely oncogenic mutations in chromatin remodelers, RB pathway genes, p53 pathway genes, and RTK/Ras signaling pathway genes affected similar proportions of the NETs and NECs (P = 0.620, P = 1.000, P = 0.515, P = 0.280, respectively); the oncogenic/likely oncogenic mutations of NETs in PI3K pathway genes (3/6, 50.0%; 2/11, 18.2%; P < 0.001) and MAPK signaling pathway genes (5/6, 83.3%; 2/11, 18.2%; P = 0.035) affected higher proportions than those of NECs.

DISCUSSION
NEN patients tend to be older, have a lower clinical stage, be hormone receptor (HR)-positive, HER2-negative and have a low Ki67 proliferation index[12]. Breast cancers are diagnosed at various ages in different parts of the world;[25–28] the peak age of breast cancer in some Asian and African countries was even over 10 years earlier than
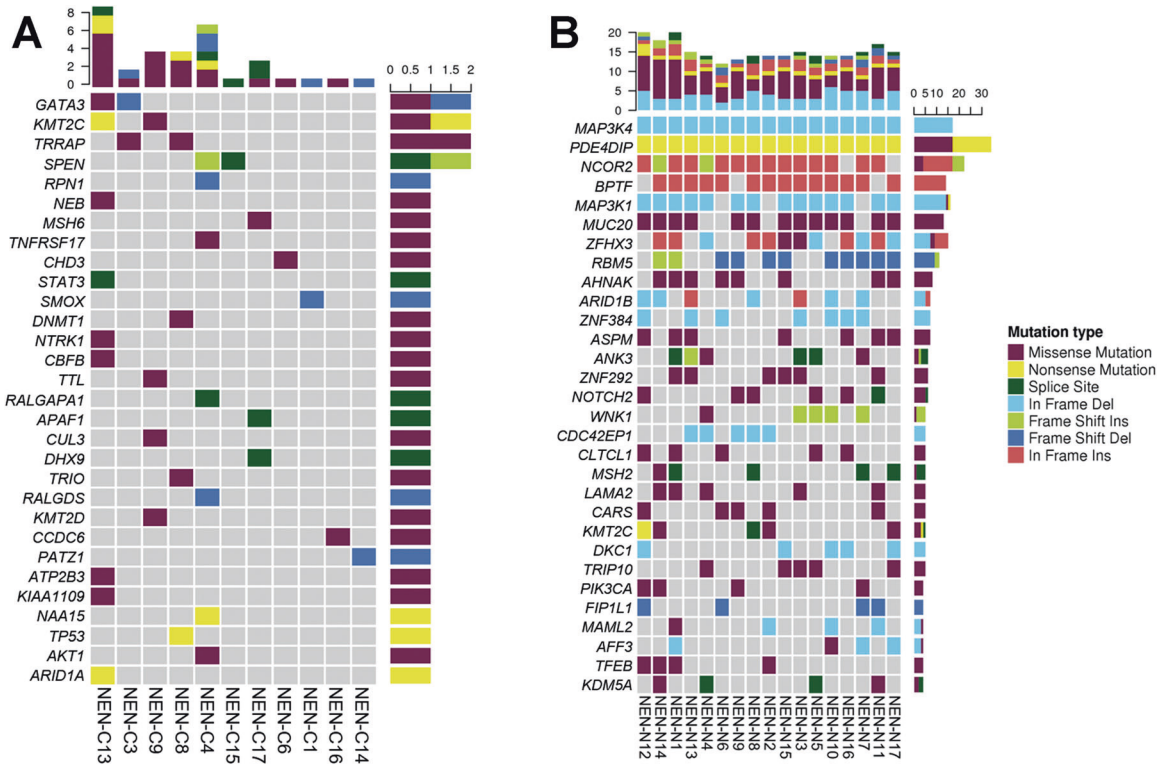
Fig. 3 Driver genes and susceptibility genes in NENs. A Landscape of driver genes in NENs. The X-axis is the sample, the Y-axis is the gene, the top is the number of gene mutations in each sample, and the right is the number of samples per mutant gene. This analysis was only based on somatic mutations in NEN tissues. **B** Landscape of susceptibility genes in NENs. Susceptibility genes were screened from germline mutations. The X-axis is the NEN-matched normal sample name, the Y-axis is the gene, the top is the number of gene mutations in each sample, and the right is the number of samples per mutant gene.

that in European or American countries[26–30]. As a result, factors impacting the age of onset of NENs, such as geography and ethnicity, should also be a considered in addition to the tumor subtype. In addition, most NENs and IBCs-NST with NE differentiation (IBC-NST-NE) expressed ER and PR, lacked HER2 expression, and had a low Ki67 proliferation index according to previous studies[7,15,31], which is consistent with our finding in NENs. Regarding NECs, a recent study investigating small cell neuroendocrine carcinomas (SCNECs) showed that 30–50% of cases were ER-positive, and none of the cases were HER2-positive;[32,33] some studies found PR expression in SCNECs, while the others did not[32–34].

Our study found that *KMT2C* was the most frequently mutated gene in breast NENs; the mutation appears in both breast NENs and other types of breast cancer, while the mutation rate may differ between the two types above. A study revealed that the frequency of *KMT2C* mutations in IBCs-NST-NE was higher than that in NETs[19]. In addition, another study investigating stomach NEC and mixed NEN found alterations in *KMT2C* in NE components only[35], and another recent study showed that *KMT2C* was the most frequently mutated gene in inflammatory breast cancer[36], suggesting that a mutation of *KMT2C* may play an important role in NE differentiation in breast cancer. Moreover, *KMT2C* was identified as a driver gene with relatively high mutation rates in our study, indicating that *KMT2C* may play a driving role in the progression of breast cancer. Interestingly, mutant *KMT2C* may be related to resistance to HR therapy[37], as a previous study showed that in an HR-rich environment, *KMT2C* can regulate enhancers and promote tumor proliferation[38], possibly playing an important role in ER-positive breast cancer. Two cases of NEN in our study were ER-negative, which is consistent with the results of the above.

Compared with IBCs-NST, NENs have a unique gene mutation spectrum as follows: lower *PIK3CA* and *TP53* mutation rates and higher *FOXA1* and *TBX3* mutation rates[39,40]. The mutation rate of *GATA3* in our study was slightly lower than that in Marchiò's study (16.7%) but was close to that reported by Fresia Pareja (10%)[19,40]. Noticeably, the mutation rate of *GATA3* in our study did not significantly differ from that in IBC-NST but was higher than that in IBCs-NST in the study by Marchiò[39,40]. In addition, Lavigne[10] found that the mutation rate of *PIK3CA* in primary NECs was consistent with the rate in The Cancer Genome Atlas (TCGA) database (9%) but lower than that in our study (11.8%). Although IBCs-NST-NE may account for 12.5% of all IBCs-NST[6], studies comparing NENs and this group of tumors are limited.

NENs have some intrinsic CNV features; the incidence of some CNVs may provide some evidence suggesting that NEN is an independent subtype. Both our and Marchiò's studies[40] found 8q amplification in NENs, and Bao et al[41] discovered multiple gene region CNVs, which were partially similar to our findings and related to lymph node metastasis; the variation of 8q in NENs may be a high-frequency CNV and potential target for tumor therapy. In addition, breast NETs were found to harbor a lower frequency of 1q gains and 16q losses than ER-positive/HER2-negative breast cancers[16,19]. Compared with that in luminal breast cancer and lobular carcinoma, the incidence of 1q amplification and 16q deletion in NENs was lower and differed from that in MBCs with NE differentiation[17,18], supporting that NEN is an independent breast cancer subtype. Additionally, breast tumors with 16q deletion were related to a small tumor size, low histological grade, decreased lymph node metastasis, and high ER expression, suggesting that 16q deletion is a good prognostic marker for breast cancer[42,43].

The specific genes identified in this study, such as suppressor genes and tumor susceptibility genes, suggest that tumorigenesis is a multistep process. First, we found that NEN had a loss of
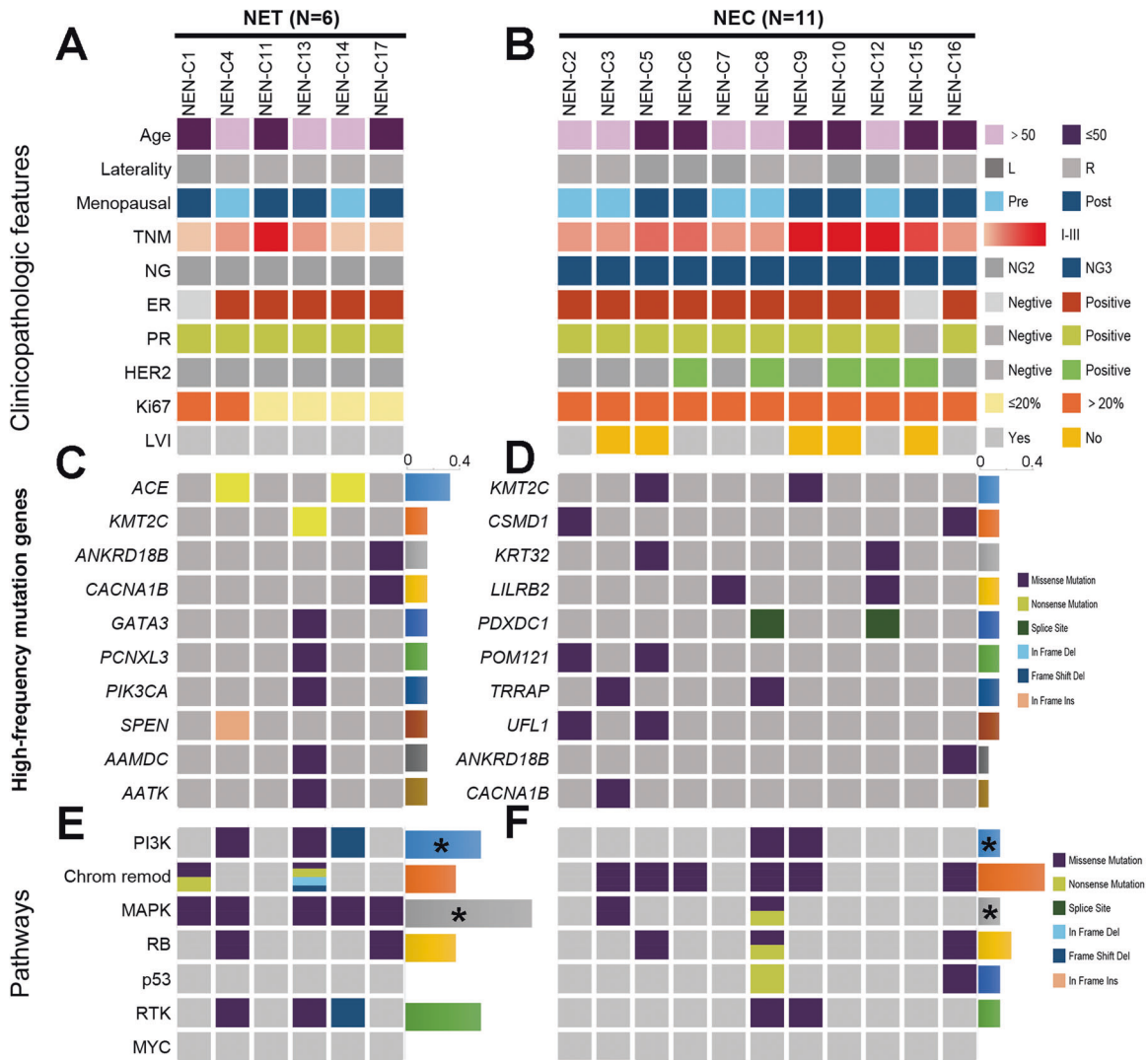
Fig. 4 Comparative analyses of NETs and NECs. A, B Comparison of the baseline characteristics between NETs and NECs. **C, D** Landscape of frequency mutated genes in NETs and NECs. **E, F** Comparative analyses of 7 pathways between NETs and NECs. Comparative analysis of NETs and NECs showed that Ki67 expression in NECs was significantly higher than that in NETs. *ACE* (33.3%, 2/6) was the most frequently mutated gene in the NETs, while *KMT2C* (18.2%, 2/11) was the most frequently mutated gene in the NECs. The oncogenic/likely oncogenic mutations of NETs in PI3K pathway genes ($P < 0.001$) and MAPK signaling pathway genes ($P = 0.035$) affected higher proportions than those of NECs. *$P < 0.05$, Fisher's exact test. The X-axis is the sample type, and the Y-axis is the percentage of NENs with genetic mutations in each genetic pathway.

heterozygosity in the suppressor gene *ACE* on chromosome 17 as previously reported by a study which may be related to more aggressive breast cancer[44]. Second, the top three mutated susceptibility genes in our study were *MAP3K4*, *PDE4DIP*, and *NCOR2*. Mutation of *MAP3K4* can regulate filamin expression and neuronal migration[45], suggesting that the neuroendocrine function in the NENs may be obtained from neurons immigrating from other sites; *MAP3K4* also plays an important role in multiple signaling pathways[46–49]. Although the significance of *PDE4DIP* in breast cancer is still unknown, recent studies have revealed that *PDE4DIP* has a high predictive value for neuroblastoma prognosis[50]. *NCOR2* mutation was found in ER-positive breast cancer with local recurrence[51] and may be related to tamoxifen resistance in primary breast cancer[52].

Studies on pancreatic carcinomas have revealed the different genetic phenotypes of NETs and NECs, which can be used clinically to distinguish these two tumors with quite distinct therapeutic strategies and outcomes[53–55]. However, to date,

research comparing the mutated genes involved in different gene pathways in breast NETs and NECs has not been reported. In this study, the percentages of NETs with gene mutations involved in the PI3K and MAPK signaling pathways were significantly higher than those of NECs, suggesting that the detection of genes in those two pathways may be a novel method to discriminate breast NETs and NECs. Interestingly, the MAPK pathway is highly ranked in the NEC component of gastric mixed NENs[56], and MYC family pathways were commonly found in prostate NECs[57] and cervical NECs[58] but not in breast NENs, suggesting that the pathogenic mechanism of breast NENs may differ from that of NE tumors of other systems.

Our study has some limitations. First, the number of samples is still limited due to the low incidence of NEN under strict diagnostic criteria. Second, the fresh tissues used for WES were more difficult to obtain than paraffin samples.

In conclusion, comprehensive clinical and genomic analyses of breast NEN revealed that NENs are an entity distinct from IBCs-NST

in clinicopathological characteristics, show that the tumorigenesis of NENs may be a multistep process resulting from multiple genetic alterations, and present some potential molecular features to distinguish breast NETs from NECs.

DATA AVAILABILITY

The sequencing data generated during the current study are available in the NCBI Sequence Read Archive (SRA) database (PRJNA741833) and can be accessed at the following link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA741833?reviewer=knskiu0jsm5h4lin58l2m7rejg.

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AUTHOR CONTRIBUTIONS
B.W. and Y.W. designed the study. B.W. and H.B. managed and supervised the project. Y.W., X.K., X.Z., Q.J. and J.Y. prepared the samples. Y.W. and B.W. reviewed the slides and confirmed the pathological diagnosis. Y.W., H.B. and B.W. analyzed and interpreted the data. Y.W. wrote the manuscript, which was reviewed by all co-authors. All the authors participated in the writing of the paper and ultimately approved the version for submission and publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This study was reviewed and approved by the clinical ethics committee of West China Hospital, Sichuan University (2021-644). The study was performed in accordance with the approval of Biomedical Ethics Review Committee.

COMPETING INTERESTS
The authors declare no competing interests.

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