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ORIGINAL ARTICLE – ENDOCRINE TUMORS

Genetic and Immunohistochemical Studies Investigating the Histogenesis of Neuroendocrine and Carcinomatous Components of Combined Neuroendocrine Carcinoma

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ABSTRACT

Background. Lung combined neuroendocrine carcinomas (NECs) comprise NEC and non-NEC components, such as adenocarcinoma and squamous cell carcinoma. Mutation of epidermal growth factor receptor (EGFR) often is observed in non-NEC but is very rare in sporadic NEC, which almost always has p53 mutation. Therefore, we hypothesized the following research concept: mutation analysis of EGFR and p53 in each component of combined NEC tissues can provide important information on whether such components originate from the same tumor cells or incidentally arise as collision cancers.

Methods. We compared the mutations of EGFR and p53 in laser-microdissected NEC and non-NEC from lungs of eight cases affected by combined NEC. We examined the expression of EGFR and NEC markers in the combined NECs by immunohistochemistry.

Results. Five of eight cases of combined NEC had the same mutations of EGFR and/or *p53* in both non-NEC and NEC. One case had EGFR mutation in only the non-NEC component, and two cases did not have these mutations. Replacement transformation was observed in borderline areas between non-NEC and NEC. The signal of activated EGFR in non-NEC with the same EGFR mutation was more intense than that in NEC components.

Conclusions. Our study suggests the mechanism behind the carcinogenesis of lung combined NEC, which is partially caused by the transformation from epithelial carcinoma of non-NEC to NEC.

Primary neuroendocrine carcinomas (NECs) of the lung are categorized by the World Health Organization as small cell lung carcinoma (SCLC): 10–15%, large cell neuroendocrine carcinoma (LCNEC); 3–5%, carcinoid tumor.[1] On rare occasions (1–3%), SCLC and LCNEC contain discrete morphological components, such as adenocarcinoma (ADC) or squamous cell carcinoma (SqCC), cases of which are classified as combined NEC.[1] Rare cancer patients with combined NECs are treated according to the therapeutic strategy for SCLCs, not non-small cell lung cancer (NSCLC), but their prognosis has been poor.[2–6] It is controversial whether patients with combined NECs should be treated according to the therapeutic guidelines of SCLC or NSCLC. Further study is needed to determine the most appropriate treatment for patients with combined NECs and both NEC and non-NEC components.

Epidermal growth factor receptor tyrosine kinase inhibitors (EFGR-TKIs) have become standard and effective therapeutic agents for NSCLC patients with EGFR mutation.[7,8] Recently, Kogo et al. reported an interesting lung cancer case that showed transformation from lung ADC with EGFR mutation into LCNEC without EGFR expression as a mechanism of the acquisition of resistance to EGFR-TKI therapy.[9] Moreover, Niederst et al. reported that lung ADC transforms to SCLC without EGFR

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activation in the process of acquiring EGFR-TKI resistance and that such SCLC transformed to ADC with sensitivity to EGFR-TKI after conventional cancer therapy, including cytotoxic chemotherapy and radiation.[10] These observations suggested that NSCLCs as a non-NEC and NECs, including SCLC and LCNEC may transform into each other.

In contrast to NSCLC, the frequency of EGFR mutation in pure SCLC is approximately 1%.[11,12] Mutations are extremely rare in pure LCNEC.[13,14] In contrast, almost all SCLCs harbor p53 mutation. Some researchers have reported that combined NEC has common EGFR mutations in both NEC and non-NEC components, suggesting that these two components might originate from cells of the same origin.[15,16] From data in previous reports on the mutation frequency of EGFR and p53 in NEC and non-NEC, we hypothesized the following research concept: mutation analysis of EGFR and p53 in each component of combined NEC tissues can provide important information on whether such components originate from the same tumor cells or incidentally arise as collision cancers. From the above-mentioned findings, it was suggested that the carcinogenic mechanism of lung combined NEC may be partially caused by the replacement transformation from epithelial carcinoma of non-NEC with p-EGFR or p63 to NEC with synaptophysin or ASCL1.

The purpose of this study was to clarify the genetic and immunohistological status of each of the NEC and non-NEC components of combined NEC separately using a laser microdissection technique. For this purpose, the mutation status of EGFR and p53 in both NEC and non-NEC tissues was analyzed to determine whether combined NEC tissues have multiple origins and represent a subtype of collision cancer. Moreover, morphological findings and expression of the NEC markers synaptophysin and achaete-scute family BHLH transcription factor 1 (ASCL1) were evaluated to determine the acquisition of NEC phenotypes in both non-NEC and NEC components of eight patients with combined NEC of the lung. Finally, the expression of phosphorylated EGFR was examined to evaluate the EGFR signal addiction in NEC and non-NEC components of four cases of combined NECs with EGFR mutation.

MATERIALS AND METHODS

Sample Selection

We analyzed tumor specimens from eight patients with combined NECs who underwent surgical resection between April 1999 and December 2015 at Gunma University Hospital. Three patients had combined SqCC of the lung and LCNEC; two had lung ADC and SCLC; two had SqCC and SCLC; and one had ADC and LCNEC. Clinical samples were used in accordance with institutional guidelines and the Helsinki Declaration, after obtaining informed consent from all participants.

Immunohistochemical Analysis

Immunohistochemistry was performed on 3-μm-thick, formalin-fixed, paraffin-embedded tissue sections. For antigen retrieval, the sections were boiled with Immunosaver (NJ15T; Nisshin EM Co., Tokyo, Japan) at 98 °C for 45 min. After rinsing in phosphate-buffered saline (PBS), sections were incubated in Protein Block Serum-Free Reagent (Dako, Carpinteria, CA) for 30 min to block nonspecific binding sites. They were then incubated overnight at 4 °C with rabbit monoclonal anti-synaptophysin (ab32127; Abcam, Cambridge, UK), mouse monoclonal anti-ASCL1 (2D9; Abnova, Taipei, Taiwan), rabbit monoclonal anti-p63 (EPR5701; Abcam), and rabbit monoclonal anti-phosphorylated-EGFR (Tyr1068; Cell Signaling Technology, Danvers, MA) antibodies diluted 1:300 in PBS containing 0.1% bovine serum albumin. Then, the sections were incubated with Histofine Simple Stain MAX-PO (Multi) kit (Nichirei, Tokyo, Japan) as secondary antibodies for 30 min at room temperature. The chromogen 3,3′-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution in 50 mM of ammonium acetate-citrate acid buffer (pH 6.0), containing 0.005% hydrogen peroxide. Sections were lightly counterstained with hematoxylin. The immunoreactivity level of synaptophysin, EGFR, and ASCL1 was defined as follows: (1) negative expression = no staining, weak staining or strong complete cytoplasm/membrane/nuclear staining in $< 10\%$ of tumor cells, and (2) positive expression = strong complete cytoplasm/membrane/nuclear staining in $\geq 10\%$ of tumor cells, respectively. Negative controls were established by incubation without a primary antibody, which resulted in no immunoreactivity being detected. Specimens were considered to be positive for synaptophysin if its cytoplasmic staining was present, positive for ASCL1 if its nuclear staining was present, and positive for p-EGFR if its cellular membrane and cytoplasmic staining was present. Evaluation was performed in a blinded fashion by two of the authors.

Mutation Analysis

For the former, tissue was collected from two 10-μm-thick, formalin-fixed, paraffin-embedded sections, and laser microdissection (Axio Observer D1/PALM; Zeiss, Wetzlar, Germany) was used to obtain NEC and non-NEC tissues, in accordance with the manufacturer's protocol. Genomic DNA was purified using the QIAamp DNA FFPE

TABLE 1 Clinical background and tumor status in eight combined NEC patients

Combined NEC sample number	Age	Gender	Smoking (years)	Stage	Histology	Neuroendocrine marker		Mutation status	
						Synaptophysin	ASCLI	*ECFR*	*p*53
Case 1	63	Male	60 × 43	IIB	ADC	−	−	Exon19 del	WT
					SCLC	+	+	Exon19 del	WT
Case 2	67	Male	50 × 22	IIB	ADC	+	−	Exon19 del	P134L
					LCNEC	+	+	Exon19 del	P134L
Case 3	76	Male	20 × 39	IA	SqCC	−	−	Exon19 del	P134L
					LCNEC	+	+	Exon19 del	P134L
Case 4	74	Male	40 × 44	IB	SqCC	−	−	WT	A175H
					LCNEC	+	+	WT	A175H
Case 5	64	Female	40 × 39	IIIA	SqCC	−	−	Exon19 del	V216M
					SCLC	+	+	Exon19 del	V216M
Case 6	47	Male	30 × 27	IIIA	SqCC	−	−	WT	WT
					SCLC	+	+	WT	WT
Case 7	75	Male	10 × 55	IB	SqCC	−	−	WT	WT
					LCNEC	+	+	WT	WT
Case 8	76	Male	20 × 50	IIIA	ADC	−	−	Exon19 del	WT
					SCLC	+	+	WT	WT

Tissue kit (Qiagen, Hilden, Germany). p53 exons 5–8 and EGFR exons 19 and 21 were amplified by PCR using the following forward and reverse primers: p53 exon 5, 5′-CAACTCTGTCTCCTTCCT-3′ and 5′-TGTCGTCTCT CCAGCCCC-3′; p53 exon 6, 5′-AGAGACGACAGGGC TGGTTG-3′ and 5′-CTTAACCCCTCCTCCCAGAG-3′; p53 exon 7, 5′-CCTCATCTTGGGCCTGTGTT-3′ and 5′-AGTGTGCAGGGTGGCAAGTG-3′; p53 exon 8, 5′-CC TTACTGCCTCTTGCTTCT-3′ and 5′-ATAACTGCAC CCTTGGTCTC-3′; EGFR exon 19, 5′-GGATCCCAGA AGGTGAGAAAGTT-3′ and 5′-GAGAAAAGGTG GGCCTGAGGT-3′; and EGFR exon 21, 5′-GGGCA TGAACTACTTGGAGG-3′ and 5′-TCCCTGGTGTC AGGAAAATG-3′. Each PCR mixture contained the forward and reverse primers (1 pmol each), 10 ng of template DNA, 10 µl of GoldTaq, and water (up to 20 µl). The reaction conditions were as follows: 98 °C for 5 min; 40 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 15 s; and 72 °C for 5 min. p53 and EGFR PCR products were subjected to Sanger sequencing.

RESULTS

Histological and Mutation Analyses of EGFR and p53 in Laser-microdissected NEC and non-NEC from Combined NEC Samples

First, we validated the histological features of combined NECs. The expression of synaptophysin and ASCL1 as NEC markers was detected in all NEC components but not in non-NEC (Table 1). The non-NEC components were located surrounding the central necrotic parts of combined NEC; on the other hand, the NEC components in almost all cases were in contact with background lung at the surface of the main tumor, including non-NEC and necrotic tissues (Fig. 1).

We collected NEC and non-NEC tissues separately from eight combined NEC cases using laser microdissection. Among these eight cases, we found EGFR exon 19 mutation in both NEC and non-NEC of four cases (50%, Cases 1–3 and 5 in Table 1) and p53 mutation in both NEC and non-NEC in four cases (50%, Cases 2–5 in Table 1). Three cases had these mutations of EGFR and p53 in both NEC and non-NEC (37.5%, Cases 2, 3, and 5 in Table 1). There were five cases of combined NECs with shared mutation of p53 and/or EGFR between NEC and non-NEC components (Fig. 1a) (62.5%, Cases 1–5 in Table 1), suggesting that NEC and non-NEC components of combined NEC originated from the same cells. On the other hand, two cases did not have mutation of EGFR and p53 in both NEC and non-NEC (Fig. 1b) (25%, Cases 6 and 7 in Table 1). One case had an EGFR exon 19 deletion only in the non-NEC component (Fig. 1c) (12.5%, Case 8 in Table 1). Thus, the mutation status of EGFR and/or p53 was consistent between the NEC and non-NEC components in seven of eight cases (87.5%; Cases 1–7 in Table 1).

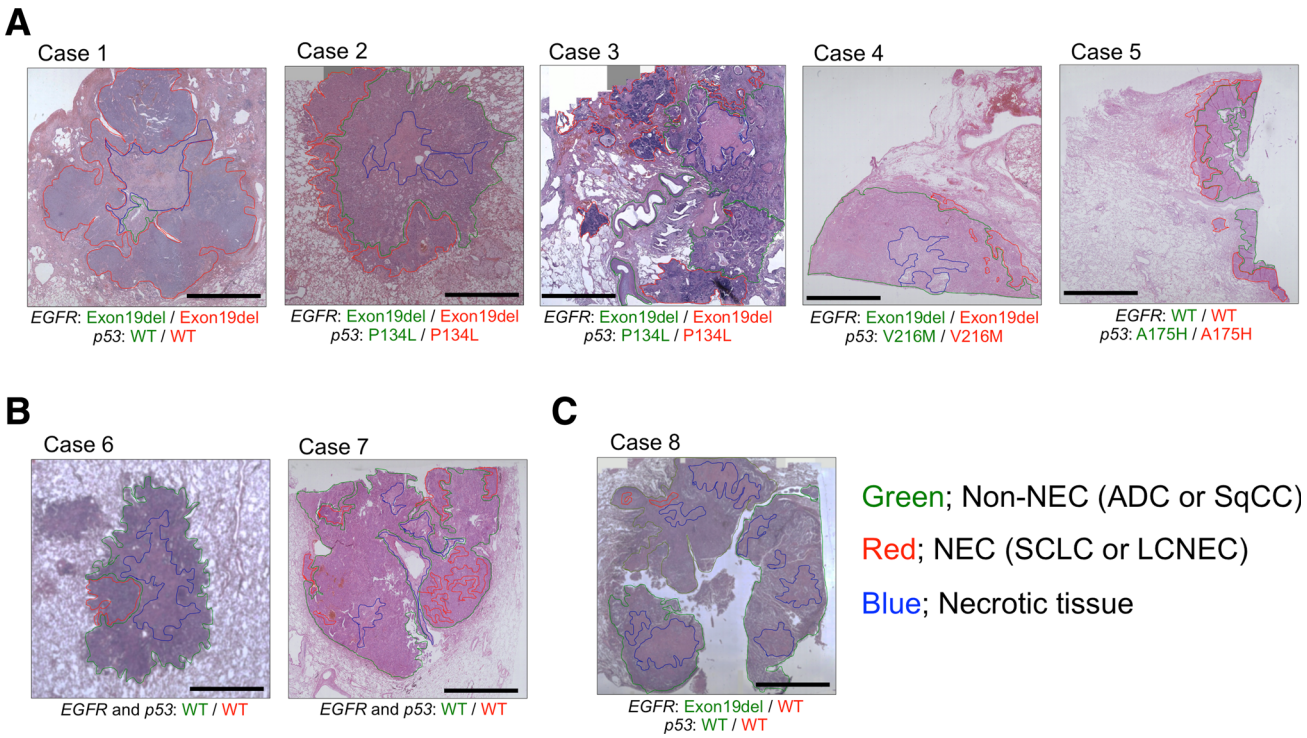

FIG. 1 Distribution of tumor histological types in both components of non-NEC and NEC in combined NEC samples, according to the EGFR and p53 mutation status. **a** Tumor distributions of combined NECs in both components with EGFR or p53 mutation. **b** Tumor distributions of combined NECs in both components without EGFR or p53 mutation. **c** Tumor distributions of combined NECs in non-NEC with EGFR mutation and NEC without mutation. Black bar indicates 5000 μm. *NEC* neuroendocrine carcinoma; *WT* wild type; *ADC* adenocarcinoma; *SqCC* squamous cell carcinoma; *SCLC* small cell lung cancer; *LCNEC* large cell neuroendocrine carcinoma

Replacement Transformation Between Non-NEC Structure and NEC with Synaptophysin

We performed a detailed evaluation of the histological findings in the borderline area of the combined NEC cases. The expression level of the NEC marker synaptophysin in the NEC component was higher than that of non-NEC components, including ADC and SqCC (Fig. 2a, b). To examine the transition pattern in the borderline area, we evaluated the NEC marker expression and morphological characteristics in the borderline area under high magnification (Fig. 2a, b, lower panel). As a result, the combined NEC in the borderline area showed replacement transformation, which preserved the structures of both ADC and NEC with NEC marker synaptophysin (Fig. 2a). Using the SqCC marker p63, we confirmed similar replacement transformation in the borderline area of combined NEC with SqCC and NEC (Fig. 2b). The replacement transformation was observed in 4/8 cases (50%; Cases 2, 3, 5, and 7).

EGFR Activation of Combined NEC Cases with EGFR Mutation was Detected only in non-NEC Components

We examined the expression of phosphorylated EGFR, which is associated with the activation of EGFR signaling, in four combined NEC cases with EGFR mutation. EGFR phosphorylation was detected in both the ADC and the SqCC components of combined NEC cases. As expected, expression was stronger in the former than in the latter and was not detected in NEC (Fig. 3). As shown in Fig. 3, the area negative for phosphorylated EGFR as an NEC component was suggested to show replacement transformation in the borderline area between non-NEC and NEC (the same as shown in Fig. 2).

DISCUSSION

In this study, we found that, in combined NEC in the lung, the NEC often harbors *EGFR* mutation that corresponds to that in the non-NEC component, in contrast to the case in sporadic NEC tissues lacking a non-NEC component, and that replacement transformation occurs in the borderline area between non-NEC and NEC. Moreover, the signal of activated EGFR found in non-NEC components was shown to be downregulated in NEC components.

The replacement transformation of non-NEC and NEC showed typical histological features along the border of clinical combined NEC. Moreover, among eight combined NEC cases in this study, five cases had a common mutation of p53 and/or EGFR in both NEC and non-NEC

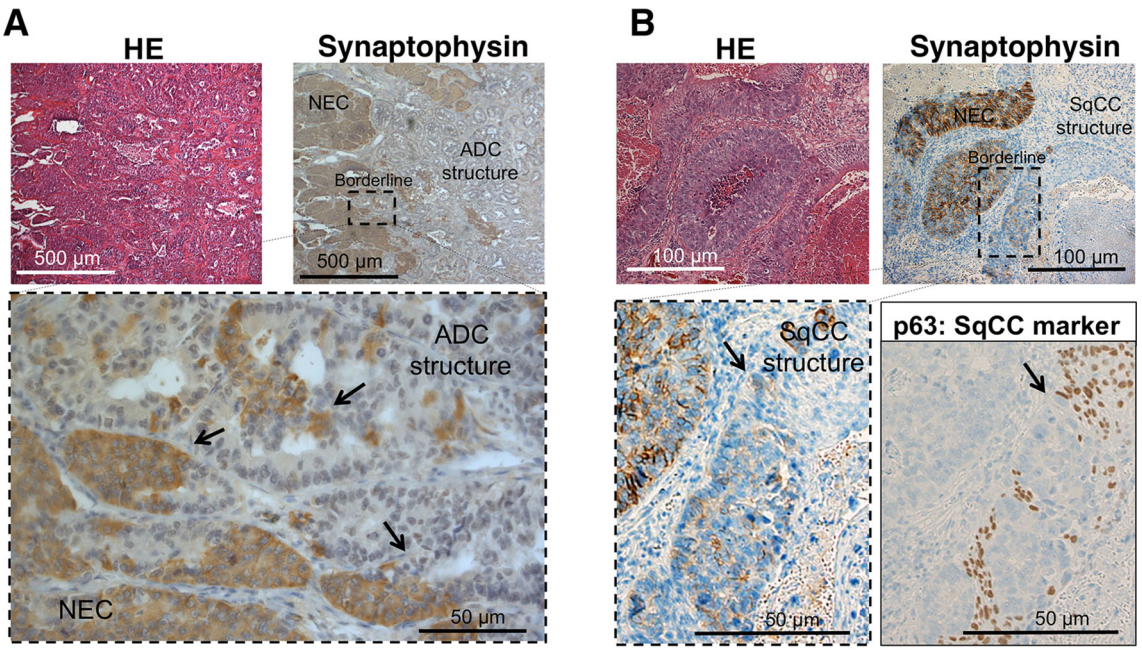

FIG. 2 Immunohistochemical detection of differentiation markers in different histological types along the border region of tumors in representative combined NEC samples. **a** Case 3: The expression pattern of the NEC marker synaptophysin in the borderline area of NEC and ADC showed structure replacement transformation between ADC and NEC with synaptophysin in a clinical sample of combined NEC. **b** Case 5: The expression pattern of synaptophysin and SqCC marker p63 in the borderline area of NEC and SqCC displayed the structure replacement transformation between SqCC and NEC in a clinical combined NEC sample. Black arrows show the borderline of marker expression between the two types of component in lung combined NECs

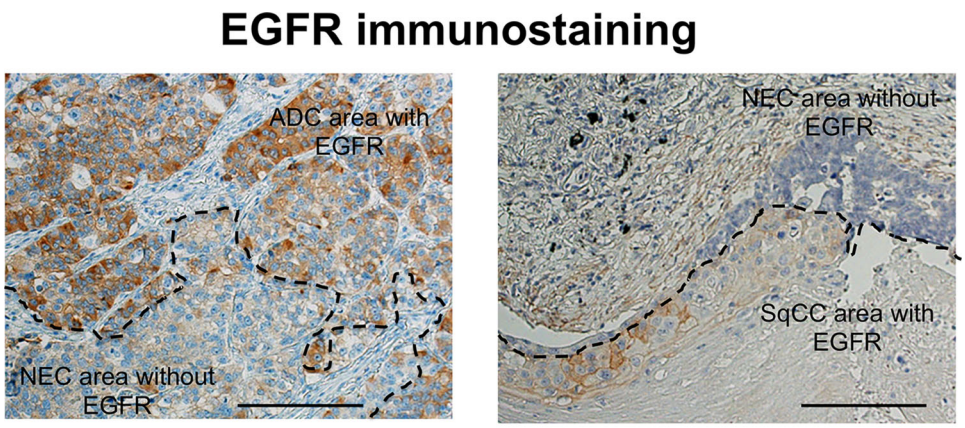

FIG. 3 Immunohistochemical detection of phosphorylated EGFR in different histological types along the borderline region of tumors in representative combined NEC samples with EGFR mutation. Replacement transformation between ADC and SqCC with activated-EGFR signal to NEC without EGFR was observed in clinical combined NEC samples with EGFR mutation (left panel; Case 3: NEC with ADC, right panel; Case 5: NEC with SqCC). Black bar indicates 50 μm. *p-EGFR* phosphorylated EGFR

components (Cases 1–5 in Table 1), which strongly suggested that each component of combined NEC has tumor cells of the same origin. Interestingly, one case had EGFR mutation in only the non-NEC component, implying that this combined NEC (Case 8 in Table 1) may have been caused by transformation from NEC to non-NEC or the collision of two different histological tumors of NEC and non-NEC. To clarify the mechanism behind the

carcinogenesis and the origins of Cases 6 and 7 without mutation of EGFR and p53 in each component of combined NECs, several mutations, including not only driver mutations but also passenger mutations, should be evaluated in detail in laser-microdissected samples using next-generation sequencing technology (Table 2).

It was previously reported that p53 mutation is associated with lung tumor transformation from NSCLC to SCLC, and the p53 mutation frequency in NEC is reportedly approximately 90%.[17] Therefore, we speculated that the NEC component of combined NEC might be caused by the new acquisition of p53 mutation in non-NEC. However, unexpectedly, we could not detect the new acquisition of p53 mutation in only the laser-microdissected NEC component of eight cases of combined NEC. In our study, the frequency of p53 mutation was 50% (4/8 cases), which is closer to that observed in sporadic lung cancers, such as ADC and SqCC.[11,18] On the other hand, the frequency of EGFR mutation in pure NEC is extremely low, and we confirmed the EGFR mutation status of 35 sporadic NEC cases at our institute wherein, as expected, we could not detect EGFR mutations as previously described (data not shown).[11–14] Using the same methodology, we detected EGFR mutation in both laser-microdissected non-NEC and NEC components from four cases with combined NECs (50%, Cases 1–3 and 5 in Table 1), and the frequency of EGFR mutation was significantly higher than the previously reported frequency in sporadic NEC. From previous reports and our mutation analysis of p53 and EGFR, it was suggested that NEC components might arise from non-NEC components acquiring NEC characteristics in some cases of combined NEC.

Next-generation sequencing analysis of colon mixed adenoendocrine carcinoma (MANEC) with non-NEC and NEC components revealed that the mutation of SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4 (SMARCA4), was detected only in NEC and not in the non-NEC component, implying that SMARCA4 may cause the transformation from non-NEC to NEC.[19] SMARCA4 is related to stem cell renewal and tumor suppressor function in cancer cells.[20,21] To clarify the significance of SMARCA4 mutation in our cohort of eight cases of combined NEC, we directly sequenced SMARCA4 exons 2–4, 6, 23, 27, and 30 in the NEC and non-NEC components of all cases; we found no evidence of mutations (data not shown). In contrast to colon MANEC, lung NEC transformation from non-NEC may not require the acquisition of a SMARCA4 mutation.

The EGFR phosphorylation level was high in the non-NEC component with EGFR mutation but was undetectable in the NEC component regardless of the existence of EGFR mutation. The EGFR mutation has been used as one of the most reliable markers for predicting EGFR-TKI sensitivity in pulmonary ADC.[22,23] In this study, we detected the overexpression of phosphorylated EGFR in not only the ADC component but also the SqCC component of combined NECs with EGFR mutation. These findings suggest that non-NEC components, such as ADC and SqCC, in combined NECs with EGFR mutation depend on the EGFR signal the same as pulmonary ADC treated by EGFR-TKI as standard treatment. In a clinical setting, combined NEC is treated in the same manner as sporadic SCLC, namely, with platinum-based chemotherapy, because large-scale, randomized, clinical trials have not been performed to clarify the suitable therapy for rare cancer patients with combined NEC.[5,6] Some case series showed that EGFR-TKI treatment was effective for patients with SCLC with EGFR mutation.[24,25] On the other hand, a signal of activated EGFR in non-NEC components was downregulated in NEC components in this study, which is consistent with previous reports showing that EGFR-TKI was not effective against sporadic NECs. Therefore, we propose that the combination of

TABLE 2 Phosphorylated-EGFR expression in non-NEC and NEC components of combined NEC cases with EGFR mutation

Combined NEC sample number	Age	Gender	Smoking (years)	Stage	Histology	Mutation status		Phospho-EGFR expression
						ECFR	*p*53	
Case 1	63	Male	60 × 43	IIB	ADC	Exon19 del	WT	++
					SCLC	Exon19 del	WT	−
Case 2	67	Male	50 × 22	IIB	ADC	Exon19 del	P134L	++
					LCNEC	Exon19 del	P134L	−
Case 3	76	Male	20 × 39	IA	SqCC	Exon19 del	P134L	+
					LCNEC	Exon19 del	P134L	−
Case 5	64	Female	40 × 39	IIIA	SqCC	Exon19 del	V216M	+
					SqCC	Exon19 del	V216M	−

chemotherapy and EGFR-TKIs against combined NEC with EGFR mutation may be a suitable therapeutic strategy.

CONCLUSIONS

Our study suggests the mechanism behind the carcinogenesis of lung combined NEC, which is caused partially by the transformation from epithelial carcinoma of non-NEC to NEC. This carcinogenic mechanism was suggested to be definitively different from that in sporadic NECs without non-NEC components. Further study is needed to clarify this interesting phenomenon.

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DISCLOSURE There are no conflicts of interest to declare.

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