Significance of epidermal growth factor receptor gene mutations in squamous cell lung carcinoma

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Abstract. Epidermal growth factor receptor (*EGFR*) gene mutations have been reported to be clinically significant in non-small cell lung cancer (NSCLC). However, because most previous studies focused only on adenocarcinomas, *EGFR* mutations in other histotypes are poorly investigated. We evaluated the frequency of *EGFR* gene mutations in squamous cell carcinoma (SCC) and its clinicopathological features. In total, 89 frozen tumor specimens that had been first diagnosed as SCCs, were examined for *EGFR* mutations in exons 19 and 21 using direct sequencing, PNA-enriched sequencing and SmartAmp2. Additionally, pathological investigation, including immunostaining for p63 and TTF-1, alcian blue staining and *EGFR* mutation-specific immunohisto-chemistry in mutation-positive samples was also performed. The frequency of *EGFR* mutations was 5.6% (5/89); all mutations were deletions in *EGFR* exon 19. Immunohisto-logical investigation of these samples revealed that two of five were positive for p63 and TTF-1 staining, and showed production of mucin, as evidenced by alcian blue staining. Consequently, three of the samples were considered to be true SCC at final pathological diagnosis, while the remaining two samples were revised to adenosquamous carcinoma and adenocarcinoma. The final frequency of the *EGFR* mutations in true SCC was 3.4% (3/87). In conclusion, *EGFR* mutations were found in a small, but significant, number of SCC tumor samples and thus *EGFR* mutational analysis was useful in the accurate diagnosis of SCC. Our data demonstrate that *EGFR* mutational analysis should be performed not only in adeno-carcinoma, but also in SCC to allow accurate diagnosis and treatment.

Introduction

Lung cancer is the most common cause of cancer mortality in men, the second most common cause in women, and is responsible for 1.18 million deaths annually (1). Treatment, involving a combination of surgery, chemotherapy and radiation therapy, is determined based on histological data obtained from either biopsy or surgical specimens. Despite the greater availability of treatment and substantial research efforts, the prognosis for lung cancer remains poor. Thus, there is a continuing need for the development of more effective diagnostics and therapies.

Many studies have demonstrated that the mutational analysis of the epidermal growth factor receptor (*EGFR*) can aid physicians in deciding the course of chemotherapy in patients with NSCLC (2,3). It has been reported that NSCLC patients with a mutation in the EGFR tyrosine kinase domain, respond to tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib (4). Most reports concerning *EGFR* mutations have primarily focused on adenocarcinoma, while few studies have evaluated the frequency of *EGFR* mutations in non-adeno NSCLC, such as squamous cell carcinoma (SCC). In these studies, *EGFR* mutations in SCC were observed in 0-3% of cases (5-10). However, these mutational analyses were performed by direct sequencing, which has a detection limit of approximately 20% (low sensitivity) for the target mutant allele (8,11) and a detailed pathological study of mutation-positive samples was poorly described (5-13).

Small-cell lung cancers (SCLC), for example, rarely display *EGFR* mutations (11), while specific combined subtypes of SCLC (such as those with adenocarcinoma) have been reported to harbor *EGFR* mutations (14). Likewise, though the frequency of *EGFR* mutations in SCC is thought to be

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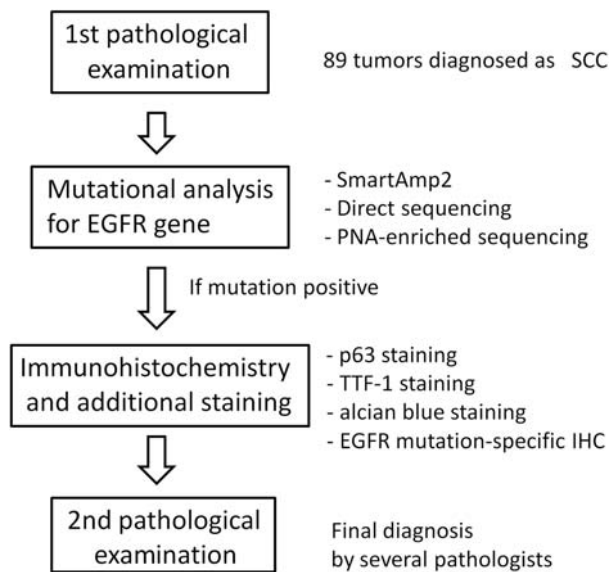
Figure 1. Schematic diagram of the study protocol.

Table I. Patient characteristics (n=89).

	No. of patients	%
Age		
Median ± SD	71.2±7.4	
Range	50-86	
Gender		
Male	83	93.3
Female	6	6.7
Smoking status		
Non-smoker	2	2.2
Smoker	87	97.8
Pathological TNM stage		
I	55	61.8
II	16	18.0
III	17	19.1
IV	1	1.1
Pathological differentiation		
Well-moderately	68	76.4
Poorly	21	23.6

very low (5-10), it may be possible to describe specific pathological features of SCC in *EGFR* mutation-positive samples. In the current study, thus, we sought to reveal the precise frequency of *EGFR* mutations in SCC using the highly sensitive Smart Amplification Process version 2 (SmartAmp2) combined with other conventional PCR-related methods for mutational analyses (15). Additionally, we aimed to examine the pathological features of *EGFR* mutation-positive samples in SCC. Here, we demonstrate the significance of *EGFR* mutational analysis for SCC, as well as for adenocarcinoma.

Materials and methods

Study samples and DNA extraction. The study design is depicted in Fig. 1. Tumor specimens were obtained from 89 consecutive SCC patients surgically treated at the Gunma University Hospital (Gunma, Japan) between 2003 and 2009. Institutional approval and informed consent from all patients were obtained. Specimens were classified by two experienced pulmonary pathologists according to the WHO classification system for lung carcinoma. Each pathologist classified the tumor specimens independently and unanimous agreement was obtained. Following surgical removal, a portion of each sample was immediately frozen and stored at -80˚C prior to DNA extraction. To suppress tumor heterogeneity and obtain a sufficient number of tumor cells, thin sections sliced from the frozen tumor surface (with maximum diameter) were selected and cut into small pieces. DNA was extracted from a 3-5-mm cube using a DNA mini kit (Qiagen, Hilden, Germany) and serially diluted to a concentration of 20 ng/μl.

SmartAmp2 assay. The SmartAmp2 method is the first one-step mutation detection technology in which the amplification of the target DNA is itself, the signal of the target mutation. Using a new DNA polymerase (Aac pol) and a unique primer design we carried out SmartAmp2 assays using an *EGFR* mutation detection kit (K.K. DNAFORM, Kanagawa, Japan)

according to the manufacturer's instructions. SmartAmp2 assays can detect deletions in the *EGFR* exon 19, and a mutation (L858R) in the *EGFR* exon 21. Although various deletions have been reported in the *EGFR* exon 19, PNA-clamp methods allow the detection of almost all types of known deletions in a single assay (16,17). The assay principles of SmartAmp2, including PNA-clamp methods, have been described previously (18). SmartAmp2 assay reactions were assembled on ice and incubated at 60˚C for 40 min. The Mx3000P system (Stratagene, La Jolla, CA, USA) was used to maintain isothermal conditions and monitor the transition of fluorescent intensity of intercalating SYBR-Green I (Invitrogen, Tokyo, Japan) during the reaction. We evaluated the results of SmartAmp2 assays according to the criteria of amplification versus non-amplification within 40 min. To avoid negative errors, a positive detection was recorded when both wild-type allele amplification (control) and mutant allele amplification were observed. Each DNA sample was analyzed in duplicate.

Mutant-enriched assay for EGFR exon 19 (PNA-enriched sequencing). We detected *EGFR* exon 19 deletions by PNA-enriched sequencing. The sequences of the primers and PNAs for PCR amplification were as follows: 5'-ACCAT CTCACAATTGCCAGTTAAC-3' (Ex19-F), 5'-CCAGATC ACTGGGCAGCATGTGGCACC-3' (Ex19-R), and N'-GAA TTAAGAGAAGCAACA-C' (PNA) (19,20). PNA-enriched PCR was performed in a total volume of 25 μl containing 1X PCR Gold Buffer, 1.5 mmol/l MgCl$_2$, 200 μmol/l dNTPs, 500 nmol/l of each primer (Ex19-F and Ex19-R), 1 μmol/l PNA clamp probe, 1 unit of Taq DNA Gold Polymerase (Applied Biosystems, CA, USA), and 20 ng of genomic DNA. The PNA clamp probe was designed to be homologous to the

Table II. Clinicopathological profile of patients with *EGFR* mutations.

| Case no. | Age | Gender | Smoking history | First pathological diagnosis | Mutational analysis | | | IHC | | | Pathological TMN stage | Final pathological diagnosis |
					SmartAmp2 (*EGFR* exon19)	Direct sequencing	PNA-enriched sequencing	p63	TTF-1	Exon19 mutation		
1	67	M	SM	MD SCC	L747-S752 del	wt	mut	+	-	-	IB	Same
2	80	M	SM	PD SCC	L747-E749 del, A750P	wt	mut	+	-	-	IIA	Same
3	50	M	SM	MD SCC	L747-E749 del, A750P	mut	mut	+	-	-	IV	Same
4	69	M	SM	PD SCC	L746-A750 del	mut	mut	+	+	+	IIB	Adenosquamous carcinoma
5	57	F	NS	PD SCC	L746-A750 del	mut	mut	+	+	+	IIIA	Adenocarcinoma with squamous differentiation

SM, smoker; NS, non-smoker; MD, moderately differentiated; PD, poorly differentiated; wt, wild-type; mut, mutant.

wild-type allele. Thermal cycling conditions included a pre-incubation step at 94˚C for 5 min, followed by 40 cycles at 94˚C for 15 sec, 60˚C for 30 sec, 72˚C for 1 min, and a final extension at 72˚C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen). DNA sequencing was performed with the ABI PRISM 3100 DNA Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems) with the Ex19-F primer.

PCR-based direct sequencing. We performed PCR-based sequencing of the *EGFR* exon 19 using the reaction conditions as described for PNA-enriched PCR. The PCR products were then purified and sequenced as described. In direct and PNA-enriched sequencing, the sequence was determined as a mutant or wild-type sequence when an elevation over the level of non-specific background noise was observed.

Histochemistry, immunohistochemistry and second pathological examination. Tumor tissue specimens were fixed in 10% formaldehyde, and embedded in paraffin wax. The paraffin sections were cut at 3 μm, and stained with hematoxylin and eosin (H&E) and with the alcian blue method. Immunohistochemical staining was performed on the same paraffin sections using anti-p63 mouse monoclonal antibodies (1:1, Nichirei, Tokyo, Japan) and anti-TTF-1 mouse monoclonal antibodies (1:200, Thermo Fisher Scientific, Cheshire, UK). The sections were immunostained with an automated stainer, the Ventana XT system Benchmark (Roche Diagnostics K.K., Basel, Switzerland). The streptoavidin-biotin method is provided in this system. To detect the *EGFR* mutant proteins via mutation-specific antibodies, *EGFR* exon 19 mutation-specific immunohistochemistry (IHC) was performed using the same paraffin sections as described previously (21,22). Rabbit monoclonal primary antibodies with specificity for the 15-bp deletion in *EGFR* exon 19; *EGFR*eceptor (E746-A750del Specific) (6B6) XP™ Rabbit mAb (Cell Signaling

Technology, Inc., Beverly, MA) were used (1:100) and IHC was performed according to the manufacturer's recommendations. For positive samples from IHC, a section slide of the tumor was divided into several pieces (the adenocarcinomatous and squamous cell carcinomatous components) and macrodissected for DNA extraction using the QIAamp DNA FFPE tissue kit (Qiagen) as described previously (16). Mutational analyses were performed on extracted DNA samples using SmartAmp2 and PNA-enriched sequencing. Following histochemical studies and analysis of the IHC data, several pathologists reviewed the tumor specimens and a final diagnosis was agreed.

Results

Patient characteristics. The SCC patients consisted of 83 men and 6 women; their characteristics summarized in Table I. The median age of the patients was 71 years (range, 50-86 years). Most patients were heavy smokers, except for two patients, one female and one male, who were non-smokers. The pathological TNM stage according to the 6th edition of the Union Internationale Contre le Cancer (UICC) staging system was stage I in 55 patients, stage II in 16 patients, stage III in 17 patients, and stage IV in one patient.

Detection of EGFR gene mutations in clinical samples. We examined the *EGFR* gene mutations in 89 SCC tumor samples and compared the results of SmartAmp2, direct, and PNA-enriched sequencing. The results from mutation-positive samples are summarized in Table II. Typical data obtained from the various mutational analysis methods are shown in Fig. 2. In case 1, we detected mutations in *EGFR* exon 19 by SmartAmp2 and PNA-enriched sequencing, but not by direct sequencing. Among the 89 SCC tumor samples examined in this study, five (5.6%) samples were found to possess mutations in the *EGFR*, as assessed by SmartAmp2 (cases 1-5). All mutations were deletions in exon 19; no mutation was
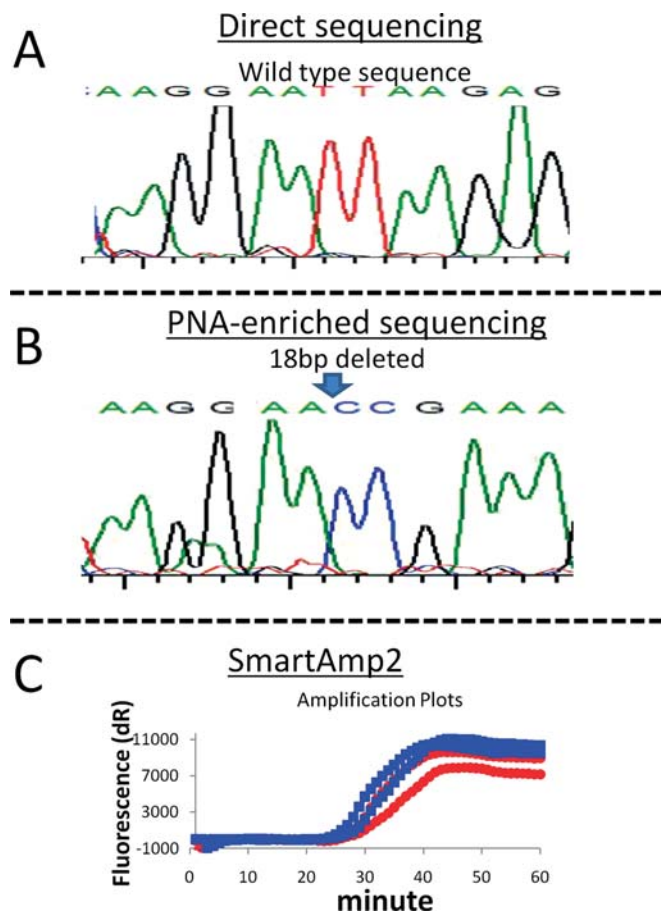
Figure 2. Typical results obtained from various methods of detection for *EGFR* exon 19 deletions (case 1). The wild-type allele is shown by direct sequencing (A), however, the 18-bp deletion was detected by PNA-enriched sequencing (B). In SmartAmp2 assays (C), wild-type (red curve) and mutant allele (blue curve) amplification are indicated. As the amplifications began within 40 min, it is interpreted as a mutation-positive result.

detected in exon 21. Identical data were obtained from PNA-enriched sequencing; however, only three of five samples were detected by direct sequencing.

Immunohistochemistry and second pathological examination. All results are summarized in Table II. P63 staining, TTF-1 staining, and *EGFR* exon 19 mutation-specific IHC were performed for all samples that harbored *EGFR* mutations.

Cases 1-3. Cases 1-3 were positive for p63 and negative for TTF-1 staining. Case 1 is shown as an example in Fig. 3. Regarding cases 1-3, *EGFR* exon 19 mutation-specific IHC was performed; however, no positive sample was found. A SCC showed positive p63 staining and negative TTF-1, consistent with these cases. The cases displayed the typical morphological features of SCC (Fig. 3) and no suggestion of any other pathological type. We thus concluded that these cases were SCC at final diagnosis.

Case 4. At first pathological examination, this case was diagnosed as SCC, according to the pathological morphology of the H&E staining (Fig. 4A and B). However, in IHC, this case was positive for both p63 and TTF-1 staining (Fig. 4C),

which was atypical for SCC. We thus performed alcian blue staining and observed that areas which had been suggested as SCC tissue, were, in fact, rich in mucin production (Fig. 4D). Taking these findings into account, a second pathological examination revealed that these areas displayed an adeno-carcinoma component, occupying >10% of the tumor section. We thus concluded that this case was an adenosquamous carcinoma at a final diagnosis. In *EGFR* exon 19 mutation-specific IHC, all areas displayed positive staining regardless of the adenocarcinoma or squamous carcinoma component (Fig. 5). Moreover, when DNA was extracted from each area via macrodissection, each area displayed *EGFR* mutations by SmartAmp2 and PNA-enriched sequencing (data not shown).

Case 5. Similar to case 4, this case was first diagnosed as SCC, according to the pathological morphology of the H&E staining (Fig. 6A and B). In IHC, this case was positive for p63, TTF-1 staining (Fig. 6C), and some areas of mucin production, which had been suggested to be SCC tissue (Fig. 6D). Taking these findings into account, a second pathological examination revealed that the majority of the area was an adenocarcinoma and that the squamous carcinoma component was observed as only a small portion of the tumor. The final diagnosis was thus adenocarcinoma with squamous diffe-rentiation. In *EGFR* exon 19 mutation-specific IHC, all areas, including the squamous differentiated parts, were positive. As in case 4, we confirmed that each area possessed *EGFR* mutations via SmartAmp2 and PNA-enriched sequencing (data not shown). Based on these results, cases 4 and 5 were discounted as SCC; thus, for 87 tumor samples, typical SCC was the final pathological diagnosis. The frequency of *EGFR* mutations in true SCC was 3.4% (3/87) by the SmartAmp2 assay and PNA-enriched sequencing, and 1.1% (1/83) by direct sequencing.

Discussion

In the current study, we sought to improve our understanding of the molecular and pathological status of SCC, which has remained unclear, compared to that of adenocarcinoma. We also aimed to reveal the true frequency of *EGFR* mutations in SCC. Previous reports, using low sensitivity methods, indi-cated that *EGFR* mutations were rare (5-10). Here, we used highly sensitive methods to evaluate 87 SCC tumor samples. We observed that three (3.4%) of these tumors were identified as having *EGFR* mutations by SmartAmp2 (two additional samples were also identified, but they were ruled out from SCC as discussed). Despite the low frequency of *EGFR* mutations detected, this detection was more sensitive than direct sequencing. Thus, if SmartAmp2 had been used in previous reports, the reported frequency of mutations in SCC may have been higher.

We additionally examined features of *EGFR* mutation-positive samples. We hypothesized that SCC with *EGFR* mutations may be associated with adenocarcinoma (as reported *EGFR* mutations are specific for adenocarcinomas). We therefore performed p63 and TTF-1 staining on *EGFR* mutation-positive samples as a method of differentiating SCC and adenocarcinoma, respectively (23,24). As expected, cases 1-3 were positive for p63 and negative for TTF-1
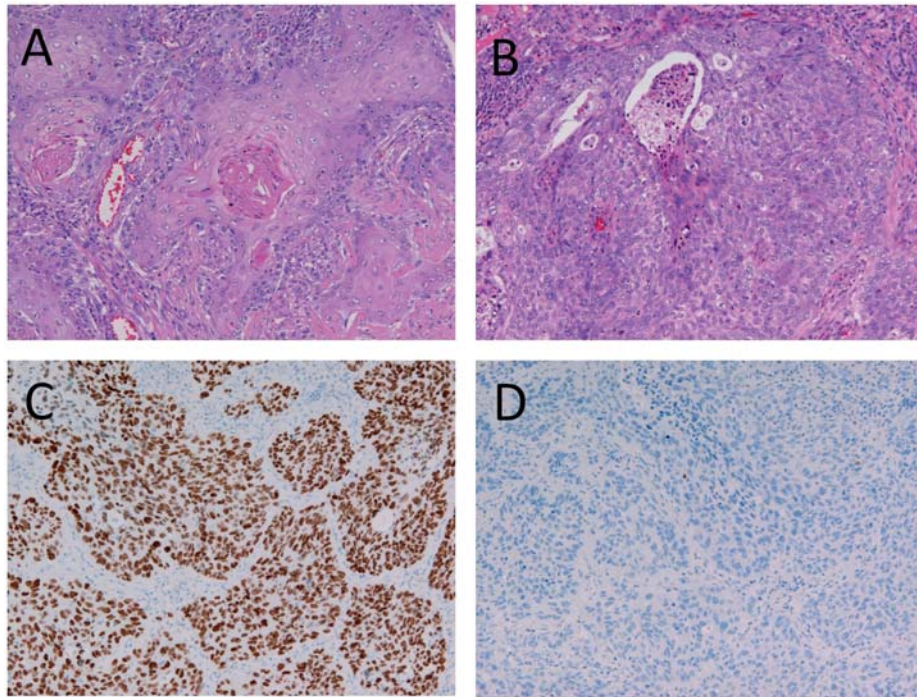
Figure 3. Typical features of squamous cell carcinoma. (A: case 1, B-D: case 3) (A) Moderately differentiated squamous cell carcinoma with keratin pearl formation (H&E stain). (B) Moderate differentiation of squamous cell carcinoma forming a sheet-like growth pattern (H&E stain). (C) Tumor samples positive for p63 staining. (D) Tumor samples negative for TTF-1 staining.
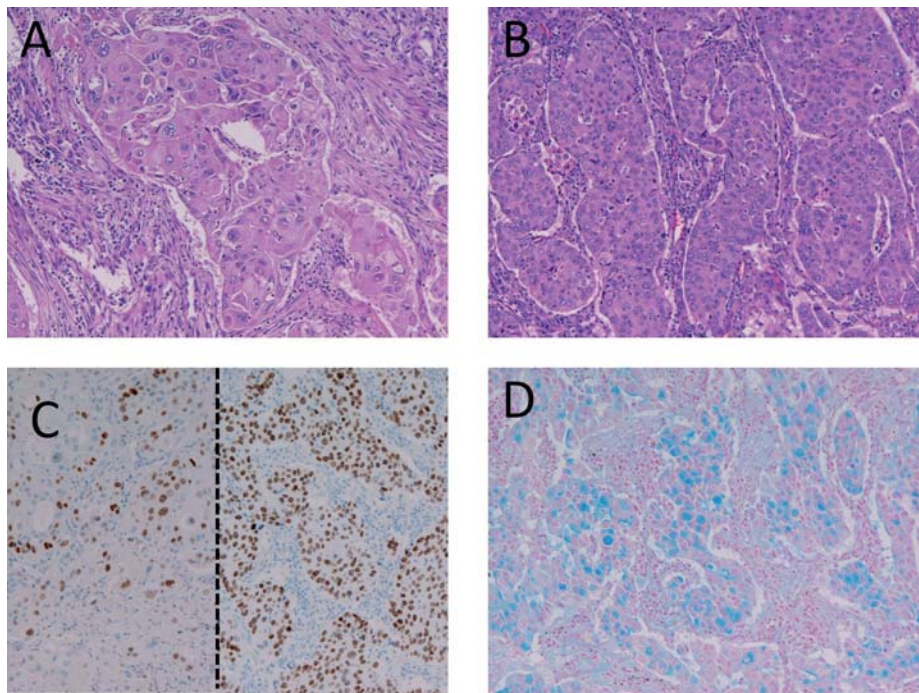

Figure 4. Adenosquamous carcinoma (case 4). (A) Squamous cell carcinoma component (H&E stain). (B, D) Adenocarcinoma component. Although thought to be a part of the squamous cell carcinoma component, mucin production was positive by alcian blue staining and was determined to be an adenocarcinoma component, with a solid and acinar pattern. (B) H&E staining. (C) Tumor samples positive for both p63 (left) and TTF-1 (right) staining. (D) alcian blue staining.

staining. However, cases 4 and 5 were positive for both p63 and TTF-1, and mucin production was evident via alcian blue staining. Based on the these results, our pathological diagnosis was revised to adenosquamous carcinoma in case 4 and to adenocarcinoma with squamous differentiation for case 5, following a second pathological examination. For all the
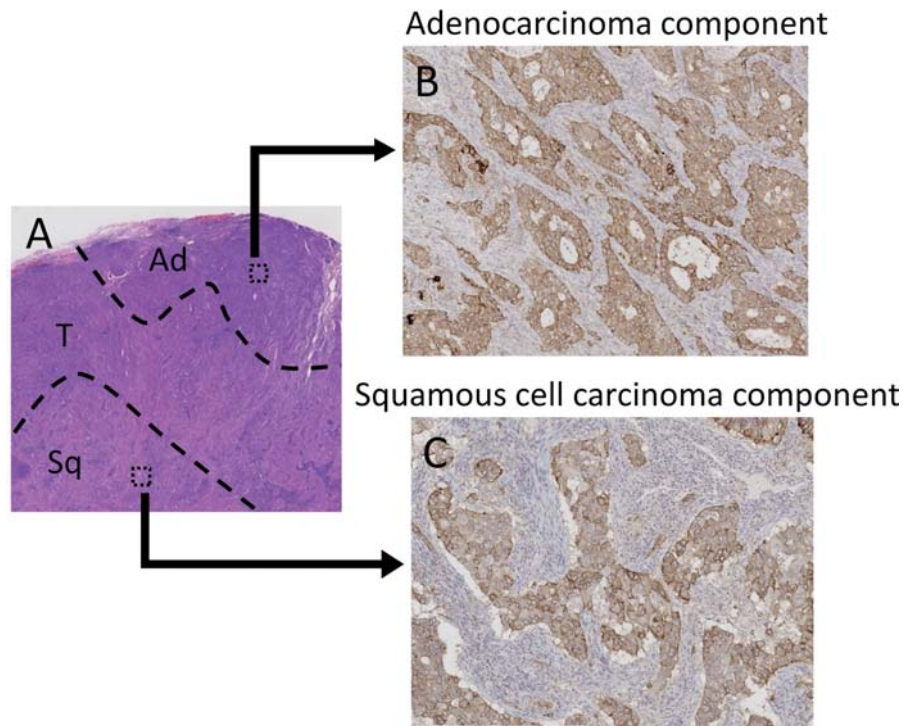
Figure 5. Adenocarcinoma and squamous cell carcinoma components. (A) Low-power field of tumor sections (case 4, H&E). Adenocarcinoma (Ad) and squamous cell carcinoma (Sq) components found in tumor sections. Most parts were transitional areas (T) displaying a mixture of both components. (B, C) *EGFR* exon 19 mutation-specific IHC observed in case 4 (B) adenocarcinoma component (C) squamous cell carcinoma component. Both components were positive for staining.
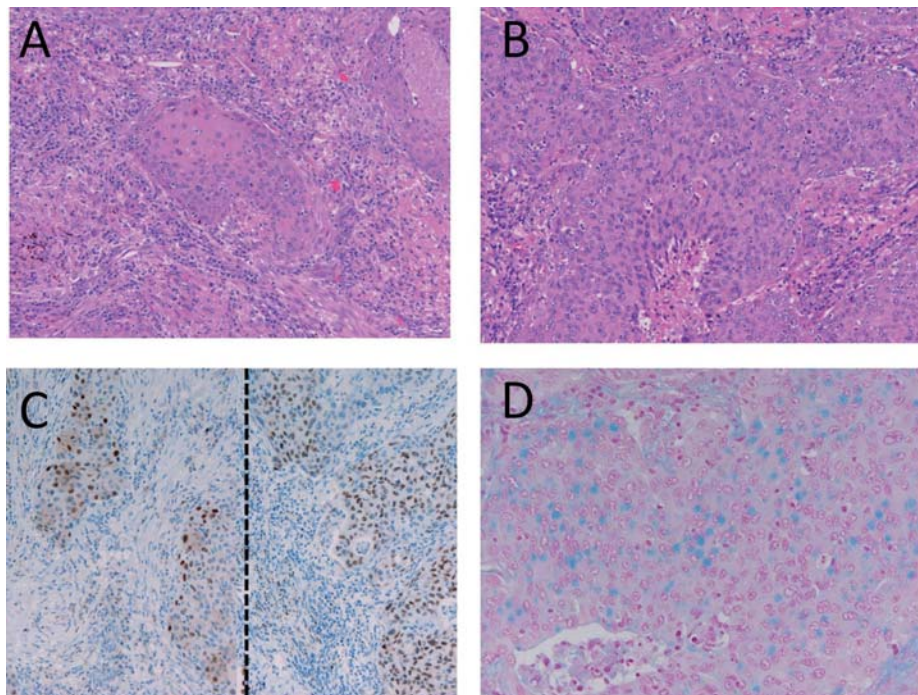

Figure 6. Adenocarcinoma with squamous differentiation (case 5). (A) Squamous cell carcinoma component (H&E stain). (B, D) Adenocarcinoma component. Although it was thought to be a part of the squamous cell carcinoma component initially, mucin was positive by alcian blue staining and was therefore determined to be an adenocarcinoma component with a solid and acinar pattern. (B) H&E stain. (C) Positive for both p63 (left) and TTF-1 (right) staining. (D) alcian blue staining.

EGFR mutation-positive samples (cases 1-5), *EGFR* exon 19 mutation-specific IHC was performed. Although cases 1-3 were negative for staining, this is consistent with previous studies indicating that this immunostaining was sensitive for

the 15-bp deletion, but less sensitive for other deletion types in SCC (22). Cases 4 and 5, however, displayed positive staining for both the adenocarcinomatous component and the squamous cell carcinomatous component (Fig. 5). This was confirmed by SmartAmp2 and PNA-enriched sequencing from the DNA extracted from each component. In previous reports, there was great interest as to whether the heterogeneity of *EGFR* mutations was evident in adenosquamous cell carcinoma samples (25,26). Because *EGFR* gene mutations were believed to be specific for adenocarcinoma, it was suggested that *EGFR* mutations would be located only in the adenocarcinomatous components, but would be absent in the squamous cell carcinomatous component in adenosquamous carcinoma tissue. Previous reports have demonstrated that *EGFR* mutations were detected in DNA extracted from both tissue components (25,26); however, the possibility remained that these results were artifacts, caused by the contamination of the two component cells during micro-dissection or extraction. In our IHC, contamination could not influence the results and thus our findings support the notion that *EGFR* mutations are found in both components in adenosquamous tissue. We present the first study to examine the heterogeneity of *EGFR* mutations in adenosquamous carcinoma tissue by *EGFR* exon 19 mutation-specific IHC. That no heterogeneity was observed in this assay is helpful in understanding the carcinogenesis of adenosquamous carcinoma. It seems unlikely that identical *EGFR* mutations occurred in both adenocarcinomatous and squamous cell carcinomatous components simultaneously. Thus, we suggest a monoclonal pathway whereby each component of the adenosquamous carcinoma originated from common progenitor cells, which seems more probable than a polyclonal pathway in the histogenesis of adenosquamous carcinoma.

Currently, pathological diagnosis is of great importance because it dictates the therapeutic strategy used (27-29). For example, bevacizumab or pemetrexed are not available for SCC therapy, although bevacizumab is one of the few drugs found to significantly impact lung cancer survival (30,31). Johnson *et al* reported that some SCC patients treated with bevacizumab experienced severe pulmonary hemorrhage. This led to the exclusion of SCC cases from bevacizumab therapeutic adoption (32). Pemetrexed is also expected to be effective in lung cancer chemotherapy; however, its use is permitted only in non-squamous NSCLC, because previous studies showed little benefit in SCC (33,34). Thus, distinguishing SCC from other histological types is important when making therapeutic decisions. In this regard, *EGFR* mutational analysis is helpful in differentiating the diagnosis of poorly differentiated SCC. As the frequency of *EGFR* mutations in adenocarcinoma or adenosquamous carcinoma was much higher than that of SCC (2,35-38), if *EGFR* mutation-positive samples are found in SCC, we should reconsider the pathological diagnosis. This makes the *EGFR* mutational analysis a unique differential diagnostic tool using the differences in mutational frequency.

It is also important to note that some SCC cases (such as cases 1-3) displayed *EGFR* mutations. Although TKIs, such as gefitinib, were originally indicated for the treatment of advanced or metastatic NSCLC, they have rarely been used for tumors other than adenocarcinoma, despite reports showing that TKIs may be effective for *EGFR* mutation-positive SCC or adenosquamous carcinoma. Mitsudomi *et al* reported the case of a male patient with adenosquamous carcinoma expressing *EGFR* mutations. The patient had a large, recurring chest-wall mass, which showed a dramatic response to gefitinib (36). Park *et al* reported three SCC patients with *EGFR* mutations that were responsive to gefitinib therapy, while 17 SCC patients with wild-type *EGFR* showed a response in only one patient (39). Although these reports are limited, the use of TKIs for lung cancers other than adenocarcinoma with *EGFR* mutations, might become an option for therapy as the trial numbers increase.

In clinical practice, not all cancers display a definite pathological diagnosis. For example, some samples obtained through transbronchial lung biopsy or transcutaneus needle biopsy are either too small, or have too much destruction (40-43), meaning their malignancy can barely be determined. Such samples therefore remain pathologically unclassified (so-called 'not-otherwise-specified', NOS). This is problematic for physicians because current therapeutic strategies are often selected based on the pathology. As *EGFR* mutations can be detected from even such small biopsy specimens, *EGFR* mutation-positive cancer can provide a new factor as to whether the histotype is adenocarcinoma, adenosquamous carcinoma, or SCC (44-46).

In conclusion, we demonstrate the potential use of *EGFR* mutational analysis in non-adeno NSCLC, including SCC. Although mutational analysis does not replace a pathological examination, it can provide valuable clues to the pathological/clinical diagnosis and subsequent therapeutic decisions.

Acknowledgements

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