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Implication of cytoplasmic p53 expression in pulmonary neuroendocrine carcinoma using next-generation sequencing analysis

Short running title: Cytoplasmic p53 in pulmonary NEC

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Author contributions

NJ and CO contributed to the conception, study design, interpretation, and drafting of manuscript. TF and MT contributed to sample collection and genetic analysis and revised the manuscript. SM, YT, and YM provided the samples, performed the prognostic analysis, and revised the manuscript. RT and TI contributed to immunohistochemistry and revised the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

All authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Aims: Cytoplasmic p53 expression indicates a high frequency of *TP53* abnormalities in gynecologic carcinoma. However, the implication of this expression in pulmonary neuroendocrine carcinoma remains unclear. Thus, our study aimed to fill this research gap.

Methods and results: Immunohistochemistry of p53 was performed on 146 cases of resected small cell lung carcinoma and large cell neuroendocrine carcinoma, and next-generation sequencing was conducted on cases showing cytoplasmic and wild-type p53 expression.

Immunohistochemistry revealed overexpression in 57% of the cases (n=83), complete absence in 31% (n=45), cytoplasmic expression in 8% (n=12), and wild-type expression in 4% (n=6) of the cases. *TP53* mutations were identified in 9 of the 13 cases with available genetic analysis.

The *TP53* mutation rates in cases with cytoplasmic and wild-type p53 expression were 88% (7/8) and 40% (2/5), respectively. All seven cases showing cytoplasmic expression with *TP53* mutations harbored loss-of-function type mutations: four had mutations in the DNA-binding domain, two in the nuclear localization domain, and one in the tetramerization domain.

Clinically, cases with cytoplasmic p53 expression had a poor prognosis similar to that in cases with p53 overexpression or complete absence.

Conclusions: Cytoplasmic p53 expression in patients with pulmonary neuroendocrine carcinoma suggests a high *TP53* mutation rate, which is associated with a poor prognosis similar to that in patients with p53 overexpression or complete absence. This cytoplasmic expression should not be misidentified as a wild-type expression. This is the first report that demonstrates the implication of cytoplasmic p53 expression in pulmonary neuroendocrine carcinoma.

Keywords: TP53, p53, cytoplasmic, NGS, neuroendocrine carcinoma, small cell lung carcinoma, large cell neuroendocrine carcinoma

3 Key points:

1. Cytoplasmic p53 expression was found in 8% of the pulmonary neuroendocrine carcinoma cases
2. Cytoplasmic p53 expression in patients with pulmonary neuroendocrine carcinoma suggests a high mutation rate of loss-of-function type *TP53* by next-generation sequencing
3. Patients with the cytoplasmic p53 expression had a poor prognosis similar to those with p53 overexpression or complete absence.

Introduction

TP53 abnormalities are found in many cancers; in particular, they are early drivers in nearly 100% cases of ovarian high-grade serous carcinomas¹⁻³. Immunohistochemistry (IHC) of p53 provides a surrogate method for detecting *TP53* mutations. For years, the 3-tier system, overexpression, complete absence, and wild-type expression, has been widely recommended for the interpretation of p53 IHC^{3,4}. Overexpression is commonly associated with nonsynonymous *TP53* missense mutations, which lead to gain-of-function effects. By contrast, complete absence is associated with nonsense, frameshift, or splicing mutations, leading to loss-of-function (LOF) effects^{3,4}.

As a third abnormal pattern, cytoplasmic p53 expression was reported in pelvic high-grade serous carcinomas in 2016³ and in endometrial carcinoma in 2020⁵. The majority of *TP53* mutations occur in the central DNA-binding domain (DBD; amino acids 94–300), whereas cytoplasmic p53 expression is often associated with *TP53* mutations in its C-terminus of, particularly in the nuclear localization domain (NLD; amino acids 300–325). A recent report suggested that cytoplasmic p53 expression was observed in 33% of the tumors with *TP53* mutations in the tetramerization domain (TD; amino acids 325–356) and 44% of the tumors with *TP53* mutations in the nuclear exclusion sequence (NES; 340–351)⁶. Abnormalities at the

C-terminus (especially at the NLD) disrupt the nuclear localization signal, leading to p53 accumulation in the cytoplasm. Therefore, cytoplasmic p53 expression has been recognized as the third most common abnormality in p53 IHC. In gynecological tumors, a 4-tier system (overexpression, complete absence, cytoplasmic expression, and wild-type expression) has been recommended for interpreting p53 IHC⁴.

Almost all cases of small cell lung cancer (SCLC) harbor *TP53* mutations⁷. However, the implication of cytoplasmic p53 expression in SCLC remains unclear. Therefore, we aimed to examine p53 expression in resected samples of SCLC as well as large cell neuroendocrine carcinoma (LCNEC), which also has a high *TP53* abnormality rate. In addition, whole-genome sequencing of *TP53* was performed in cytoplasmic and wild-type p53 samples, for which next-generation sequencing (NGS) analysis was available. This is the first report suggesting that abnormal cytoplasmic p53 expression in pulmonary neuroendocrine carcinoma (NEC) may significantly improve the predictive sensitivity of *TP53* mutations, and also has valuable prognostic implications.

Materials and methods

Samples

In total, 146 consecutive cases of pulmonary SCLC (n=95) and LCNEC (n=51) from Kobe University and Nara Medical University were included in this study. All cases were surgically resected, without preoperative chemoradiotherapy. The 146 patients included 126 men (86%) and 20 women (14%), with a median age of 73 years (range 40–89 years). For both SCLC and LCNEC, 49% of the patients (n=47, 25, respectively) were in pathological stage I and 51% of the patients (n=48, 26, respectively) were in pathological stages II–IV. Combined NEC was present in 48 cases (33%). This study was approved by the Ethics Committee of Kobe University Hospital. (No. B220045).

Immunohistochemistry (IHC) of p53

Immunostaining was performed using a Ventana BenchMark GX (Roche, Basel, Switzerland) with prediluted antibodies against clone DO-7 (DAKO), while employing an incubation time of 32 min and antigen retrieval time of 60 min. p53 expression was examined using whole slides in all cases. The expression of p53 was divided into four, based on previous reports⁶: overexpression, 80% or above indicated strong and diffused nuclear positivity (regardless of the presence of cytoplasmic expression); complete absence, complete absence of p53; cytoplasmic

expression, cytoplasmic staining even in small amounts that did not fit overexpression; and wild-type expression, other than the above. In cases showing overexpression and wild-type expression, the percentage of tumor cells showing strong nuclear expression was also measured. In cases with cytoplasmic p53 expression, the staining intensity (1,2,3) and percentage of the stained area (0–100%) were calculated.

NGS analysis of *TP53*

DNA was extracted from a 8 µm-thick formalin-fixed paraffin-embedded (FFPE) block of surgical samples using a DNA isolation kit for FFPE tissue samples (CELLDATE, CA, USA). The quality and concentration of the DNA samples were evaluated using a NanoDrop (Thermo Scientific, MA, USA), a 4200 TapeStation, and Genomic DNA ScreenTape Assay (Agilent Technologies, CA, USA). *TP53* whole exon sequences were determined using AmpliSeq for the Illumina Customs DNA Panel (Illumina, Inc., CA, USA).

Statistical analysis

Recurrence-free survival (RFS) and overall survival (OS) were evaluated using the Kaplan–Meier method. Differences in the survival curves were evaluated using the log-rank test. Statistical analyses were performed using EZR version 1.55 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) and R software (R Foundation for Statistical Computing, Vienna, Austria). A p-value of < 0.05 was considered statistically significant.

Results

p53 expression

As shown in Table 1, p53 IHC showed overexpression in 57% (n=83), complete absence in 31% (n=45), cytoplasmic expression in 8% (n=12), and wild-type expression in 4% (n=6) of cases. The rate of cases with wild-type p53 expression was lower in SCLC than that in LCNEC (1% vs. 10%), while the rates of cases with p53 overexpression, complete absence, and cytoplasmic expression were similar between SCLC and LCNEC (58% vs. 55%, 33% vs. 27%, and 8% vs. 8%, respectively).

Figure 1 includes a summary of the p53 IHC results. Figure 2 shows immunostaining of the 12 cases showing cytoplasmic p53 expression. The intensity and area of cytoplasmic staining

varied among cases. The median cytoplasmic-positive area was 95%, with a range of 30–100%.

Four cases (cases #1, 7, 8, and 10) showed strong and diffused cytoplasmic expression, while case #9 and #11 showed weak and partial cytoplasmic expression. Figure 3 shows immunostaining of six cases showing wild-type expression. In the six cases showing wild-type p53 expression, nuclear strong p53 staining of tumor cells ranged from 1 to 40% (average 16%), with varying intensity. In 83 cases of overexpression, nuclear strong p53 staining of tumor cells ranged from 80 to 100% (average 93%).

NGS of *TP53*

Twelve cases showing cytoplasmic expression and six cases showing wild-type expression were subjected to NGS. In 13 (8 SCLC and 5 LCNEC) of these 18 cases, sufficient DNA for NGS analysis was obtained and analyzed for *TP53* whole-exon sequences. As shown in Figure 1, 9 (7 cytoplasmic and 2 wild-type expression) of the 13 cases harbored *TP53* abnormalities. In the group with cytoplasmic p53 expression, six of seven cases had nonsense mutations, such as p.Q331*, P306*, P301*, E298*, G279*, and P250*. The remaining cases harbored the mutation p.N247GfsTer97, which is a frameshift mutation that eventually becomes a stop codon. Notably, all seven cases harbored loss-of-function (LOF) mutations. Three cases had mutations within

the TD (p.Q331*) or NLD (p.P306* and P301*), whereas the four other cases had mutations within the DBD (p.E298*, G279*, P250*, and N247GfsTer97). In one case of combined SCLC and adenocarcinoma, the same mutation (p.Q331*) was detected in each component.

Conversely, in the group with wild-type expression, two cases had *TP53* missense mutations within the DBD (p.P190S) or transactivating domain (p.N29I). Among the 13 cases for which NGS was possible, the accuracy of cytoplasmic p53 expression in predicting *TP53* mutations was 77%.

Prognosis

As shown in Figure 4, no significant differences in RFS and OS were found among the four groups ($p=0.381$ and $p=0.057$, respectively). In the LCNEC group, a significant difference in RFS was found among the four groups ($p=0.016$). No significant differences in the OS of cases with LCNEC and RFS and OS of cases with SCLC were found among the four groups ($p=0.298$, $p=0.319$, and $p=0.193$, respectively). Importantly, the survival curves of the cytoplasmic expression were closer to those of the overexpression and complete absence, but not to those of the wild-type expression. No significant difference was found between the

cytoplasmic and wild-type expression ($p=0.12$ for RFS, $p=0.084$ for OS), but this finding may be due to the small number of cases.

Discussion

This study is the first to examine the significance of cytoplasmic p53 expression in pulmonary NEC. The novel findings are as follows: (1) the prevalence of cytoplasmic p53 expression in pulmonary NEC was not negligible (8%); (2) among cases with cytoplasmic p53 expression, *TP53* mutations were observed at a high rate (88%), including not only the TD or NLD, but also DBD; (3) non-specific cytoplasmic p53 staining may occur in pulmonary NEC; and (4) cases with cytoplasmic p53 expression showed a poor prognosis similar to that in cases with overexpression or complete absence.

p53 IHC is widely used as a surrogate marker for *TP53* mutations in many organs with abundant evidence, especially in gynecological organs. In this field, p53 IHC is routinely used for various applications, including subclassification and benign and malignant classification. In gynecologic carcinoma, cytoplasmic p53 expression is associated with *TP53* mutations, and has

been recognized as a surrogate for the prediction of *TP53* mutations ³⁻⁶. Pulmonary NEC (especially SCLC) is a representative malignancy with a high rate of *TP53* mutations, and an ideal tumor in which to investigate the significance of p53 IHC, but there is little evidence for the significance of cytoplasmic p53 expression. In pulmonary oncology, p53 IHC is useful in determining whether a lesion is malignant or not, and has become reliable evidence to distinguish NEC from neuroendocrine tumor ⁸. In this study, cytoplasmic p53 expression was observed in 8% (12/146) of the NEC cases. Of these 12 cases, *TP53* mutations were observed in seven of the eight cases (88%), for which NGS was available.

In gynecologic tumors, LOF-type *TP53* mutations, mainly in the region of NLD, TD, and NES, were common in cases with cytoplasmic p53 expression ^{3,5,6}. However, missense *TP53* mutations in DBD were also reported in four of the six high-grade endometrial carcinomas with cytoplasmic p53 expression ⁹. In our study, LOF-type *TP53* mutations were found in seven cases with cytoplasmic expression. However, *TP53* sites were found in NLD in two cases, TD in one case, and DBD in four cases. These results suggest that LOF-type mutations on the C-terminal side were common in case with cytoplasmic p53 expression, while some cases deviate from these characteristics.

The median cytoplasmic-positive area in 12 cytoplasmic expression cases was 95%, with a range of 30–100%, and the intensity of expression was also variable. *TP53* mutations were found in cases with 30% cytoplasmic positivity for p53 (case #9), whereas no *TP53* mutations were found in cases with 100% cytoplasmic positivity for p53 (case #5). In this mutation-negative case, a single nucleotide variant, which appears to be a single nucleotide polymorphism, was found, as in other cases, suggesting that NGS was successfully performed and non-specific cytoplasmic p53 staining may occur. The cut-off values of cytoplasmic p53 expression in predicting *TP53* mutations may be difficult to establish. Rabban et al. also concluded that a cutoff value is difficult to establish for cytoplasmic staining because *TP53* mutations can be found in a minimum of 25% of cytoplasmic p53 staining cases ⁶.

TP53 mutations occur with a certain probability, even in the cases with wild-type expression of p53 IHC. In the present study, the frequency of *TP53* mutations in the cases with wild-type expression was 40% (2/5). In our study, the prognosis of cases with cytoplasmic p53 expression was similar to that of cases with p53 overexpression and complete absence, but different from that of cases with wild-type p53 expression. This result suggests a rationale for recognizing

cytoplasmic expression as an abnormal pattern equivalent to overexpression and complete absence.

Cytoplasmic p53 expression with *TP53* mutation has been reported in colorectal cancer¹⁰, prior to the report of gynecologic cancers, and may be found in any organ. In non-gynecologic oncology including pulmonary oncology, whether the staining criteria can be followed directly or whether new criteria are required should be verified.

A limitation of this study is that NGS was restricted to cytoplasmic and wild-type expression, but not to overexpression or complete absence. Further studies with a larger number of cases, including cases with overexpression and complete absence, are warranted to verify the implication of cytoplasmic p53 expression in pulmonary NEC. Moreover, future studies on reproducibility in small specimens are needed.

In conclusion, 8% of the pulmonary NEC showed cytoplasmic p53 expression. Seven of the eight cases of cytoplasmic staining for which NGS analysis was available had LOF-type p53 mutations on the C-terminal side above the amino acid 247, but included DBD. Patients with

cytoplasmic p53 expression had a poor prognosis similar to those with p53 overexpression or complete absence.

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Figure legends

Table 1: Number of cases in each pattern of p53 IHC by histotype

The rate of cases with the wild-type pattern in SCLC were lower than those in LCNEC (1% vs. 10%), whereas the rates of cases with the other patterns were similar between SCLC and LCNEC.

Figure 1: Tile plots showing *TP53* mutation characteristics and p53 IHC pattern for each case.

In 13 of the 18 cases showing a cytoplasmic or wild-type pattern, *TP53* whole exon sequences were analyzed; 9 of the 13 cases harbored *TP53* abnormalities. In the group showing a cytoplasmic pattern, seven of eight cases harbored a loss-of-function mutation. Three cases had mutations at the C-terminus (>300 amino acids), whereas the four other cases had mutations within the DNA-binding domain. In the group showing a wild-type expression, two of the five cases had missense mutations in the DNA-binding or transactivating domain.

Figure 2: Immunostaining representing cytoplasmic p53 expression

Here, 12 of the 146 cases (eight SCLC and four LCNEC) showed cytoplasmic p53 expression. The intensity and area of cytoplasmic staining varied among cases. Four cases (cases #1, 7, 8, and 10) showed strong and diffused cytoplasmic expression, while case #9 and #11 showed weak and partial cytoplasmic expression. Each scale bar indicates 100 μm .

Figure 3: Immunostaining representing wild-type p53 expression

In the six cases showing wild-type p53 expression, nuclear strong p53 staining of tumor cells ranged from 1 to 40% (average 16%), with varying intensity. Each scale bar indicates 100 μm .

Figure 4: Prognosis

No significant differences in RFS and OS were found between the four groups ($p=0.381$, $p=0.057$, respectively). In LCNEC, a significant difference in RFS was found among the four groups ($p=0.016$). No significant differences in the OS of LCNEC and RFS and OS of SCLC were found among the four groups ($p=0.298$, $p=0.319$, $p=0.193$, respectively). Importantly, the survival curves of p53 cytoplasmic expression were closer to those of p53 overexpression and complete absence, but not to those of wild-type p53 expression.

Table 1

Histology	p53 IHC			
	Overexpression	Complete absence	Cytoplasmic expression	Wild-type
SCLC (n=95)	55 (58%)	31 (33%)	8 (8%)	1 (1%)
LCNEC (n=51)	28 (55%)	14 (27%)	4 (8%)	5 (10%)
Total (n=146)	83 (57%)	45 (31%)	12 (8%)	6 (4%)

Table 1: Number of cases in each pattern of p53 IHC by histotype
The rate of cases with the wild-type pattern in SCLC was lower than that in LCNEC (1% vs. 10%), whereas the rates of cases with the other patterns were similar between SCLC and LCNEC.

Figure 1

case number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
histology	S	S	S	S	S	S	S	S	L	L	L	L	S	L	L	L	L	L
TP53 mutation type					none								none	none			none	
TP53 mutation site	p.Q331*	p.E298*	p.G279*	p.N247fs					p.R306*	p.P301*	p.P250*				p.P190S	p.N29I		
p53 IHC pattern	cytoplasmic expression														wild-type			
% p53 strong nuclear (+)													20	30	40	1	1	3
% p53 cytoplasmic (+)	100	90	90	100	100	80	100	100	30	100	50	90						
intensity of p53 cytoplasmic (+)	3	1	1	2	1	2	3	3	1	3	1	1						
histology Ssmall cell lung carcinoma Llarge cell neuroendocrine carcinoma					TP53 mutation type loss of function type (stop codon or frameshift) gain of function type (missense) no test					TP53 mutation site tetramerization domain nuclear localization domain DNA-binding domain transactivating domain					intensity of p53 cytoplasmic (+) 3strong 2moderate 1weak			

Figure 1: Tile plots showing *TP53* mutation characteristics and p53 IHC pattern for each case. In 13 of the 18 cases showing a cytoplasmic or wild-type pattern, *TP53* whole exon sequences were analyzed; 9 of the 13 cases harbored *TP53* abnormalities. In the group showing a cytoplasmic pattern, seven of eight cases harbored a loss-of-function mutation. Three cases had mutations at the C-terminus (>300 amino acids), whereas the four other cases had mutations within the DNA-binding domain. In the group showing a wild-type expression, two of the five cases had missense mutations in the DNA-binding or transactivating domain.

Figure 2

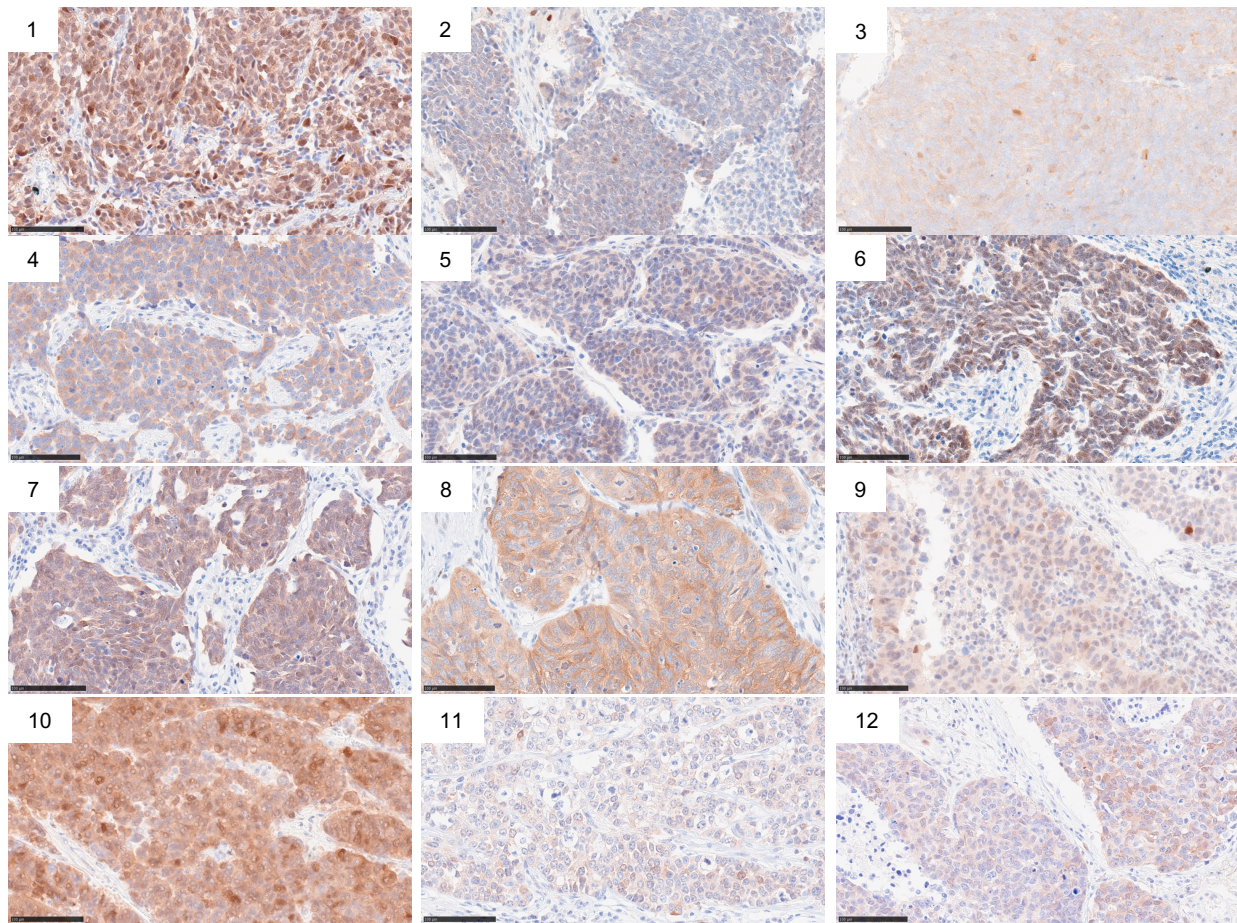


Figure 2: Immunostaining representing cytoplasmic p53 expression

Here, 12 of the 146 cases (eight SCLC and four LCNEC) showed cytoplasmic p53 expression. The intensity and area of cytoplasmic staining varied among cases. Four cases (cases #1, 7, 8, and 10) showed strong and diffused cytoplasmic expression, while case #9 and #11 showed weak and partial cytoplasmic expression. Each scale bar indicates 100 μ m.

Figure 3

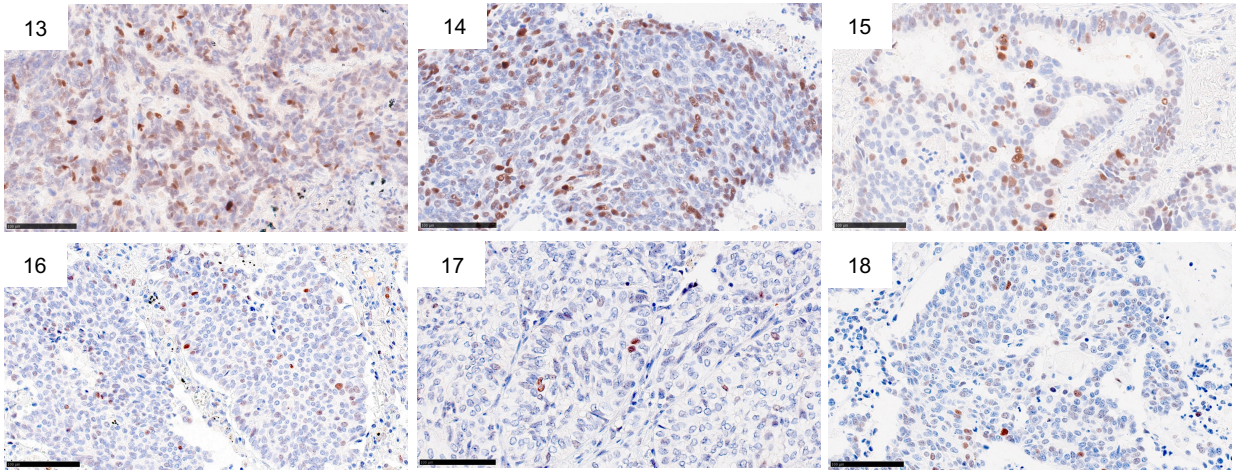
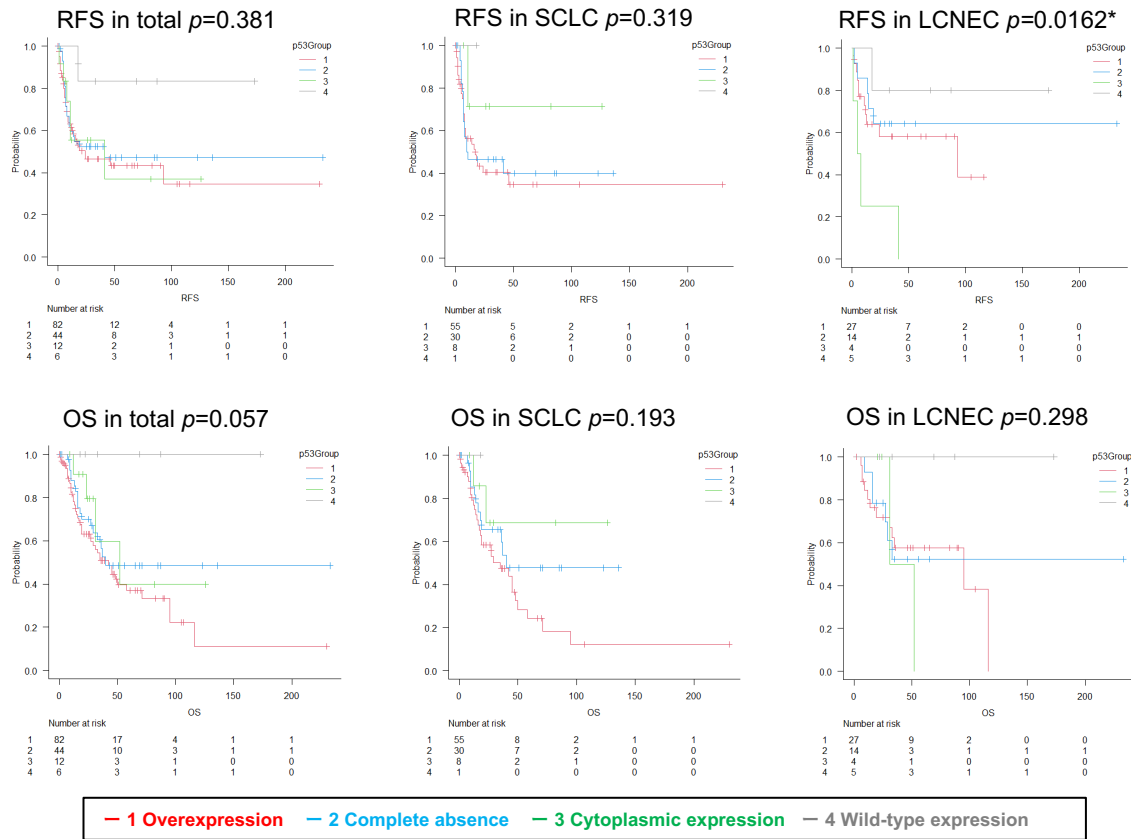


Figure 3: Immunostaining representing wild-type p53 expression
In the six cases showing wild-type p53 expression, nuclear strong p53 staining of tumor cells ranged from 1 to 40% (average 16%), with varying intensity. Each scale bar indicates 100 μ m.

Figure 4



No significant differences in RFS and OS were found between the four groups ($p=0.381$, $p=0.057$, respectively). In LCNEC, a significant difference in RFS was found among the four groups ($p=0.016$). No significant differences in the OS of LCNEC and RFS and OS of SCLC were found among the four groups ($p=0.298$, $p=0.319$, $p=0.193$, respectively). Importantly, the survival curves of p53 cytoplasmic expression were closer to those of p53 overexpression and complete absence but not to those of wild-type p53 expression.