Expression of p63 and its Isoform, deltaNp63, in Non-Small Cell Lung Carcinoma

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Recently, p63 (a p53 homologue) and its isoforms have become of diagnostic interest to clinicians. Among the p63 isoforms, \( \Delta Np63 \) and \( TA \)p63 have been the main focus due to their diagnostic and prognostic potential.

p63 comprises a group of at least 6 different proteins produced from a single gene with 2 promoters, P1 and P2, and characterized by alternative splicing of the primary RNA transcript. All p63 proteins share a common core domain, a DNA-binding domain, in the central region (Fig. 1). The 2 alternative promoters, which have different transcription start sites, give rise to 2 classes of p63 transcripts: transactivation (TA) isoforms, which are proteins with an N-terminal TA domain that are encoded from promoter P1; and \( \Delta N \) isoforms, which lack the TA domain and are encoded from promoter P2. In addition, the p63 isoforms, \( \alpha, \beta, \) and \( \gamma \), are produced by alternative splicing at the 3' end of the RNA, producing proteins with different C-terminal lengths.\(^1,3\)

In normal epidermal development, \( TA \)p63 isoforms appear to be the molecular switches that initiate an epithelial stratification program and inhibit terminal differentiation. This suggests that \( TA \)p63 isoforms must be counterbalanced by \( \Delta N \)p63 isoforms to allow cells to respond to the signals required for maturation of embryonic epidermis. Byrne et al.\(^5\) reported that \( \Delta N \)p63 isoforms, which are first expressed after 12 h of incubation in mouse embryos (E9.5) after the developing epidermis has committed to stratification, but prior to terminal differentiation, are thought to be involved in terminal differentiation.\(^5\)

\( \Delta Np63 \) appears to counteract the growth suppressive effects of \( TA \)p63 either through competitive binding on the p53-responsive element found in p53- and p63-target genes, such as insulin-like growth factor binding protein 3 (IGFBP-3),\(^6\) or through direct interaction with the TA forms leading to inactive oligo-
The p63 isoforms

Np63 isoform). The p63 isoforms

Np63 isoforms have oncogenic properties to carry out the positive regulatory function of the β-catenin signaling pathway in human squamous epithelial tumors. A number of reports have addressed the role of p63 isoforms in tumorigenesis in several organs; however, debate still exists for some issues. Developmentally-expressed TAp63 isoforms have been shown to be reactivated in the majority of well-differentiated head and neck squamous cell carcinomas. Furthermore, it has been suggested that deregulated TAp63 expression accelerates skin carcinogenesis and tumor progression. It has been suggested that Np63 is the major isotype expressed in epithelia and tumors of the esophagus, and elevated expression of p63 is an early event in the development of esophageal squamous cell carcinoma.

Altered expression of p63 isoforms has been reported in various tumors, such as non-Hodgkin lymphoma, cervical cancer, and thyroid cancer.

One report on NSCLCs has suggested that p63 genomic amplification and protein staining intensity are associated with better survival, and because there is a significant increase in the copy numbers in pre-invasive lesions, the p63 genomic amplification plays a role early in lung tumorigenesis and deserves further evaluation as a biomarker for lung cancer progression. Besides p63 amplification, overexpression of ∆Np63 has been documented as a critical step in the early development of NSCLC, and p63 amplification in particular might prove to be an excellent biomarker for SqCC progression.

The purpose of the present study was to elucidate the clinicopathologic significance related to the expression of p63 isoforms, especially ∆Np63, in NSCLC and to evaluate the relationships with the immunohistochemical patterns.

**MATERIALS AND METHODS**

**Patients and clinicopathologic data**

All patients with NSCLC who underwent lobectomy or pneumonectomy at Pusan National University Hospital in Busan, Korea during the period from June 1989 to October 2006 were considered for this study. After exclusion of cases in which there was insufficient pathologic material remaining for further study, 92 cases were selected for this study (81 males and 11 females). The mean age of the patients was 65.2 years (range, 40.0-85.0 years; median, 67.0 years). Tumors were staged according to the TNM classification of the International Union Against Cancer (UICC) staging system after review of the clinical, radiologic, and pathologic data were obtained; additional clinical information was extracted from the medical records.

The surgically resected specimens were immediately fixed in 10% buffered formalin (pH 7.0). All sections containing both tumor and surrounding lung tissues were embedded in paraffin. The pathologic diagnosis was based on the third edition of the World Health Organization classification. There were 47 cases of SqCC and 45 cases of AC. Additionally, a portion of the fresh specimens from cancer foci and the surrounding lung tissues were frozen at -80°C for future studies.

**Microarray construction**

The representative tumor areas to be sampled for tissue microarray (TMA) were carefully selected and marked on the H&E

Fig. 1. The p63 gene contains 15 exons and encodes at least six protein isoforms that are classified into two groups based on whether they encode the transcription activation domain (TA isoform) or no (∆N isoform). The p63 isoforms α, β, and γ, are produced by alternative splicing at the 3′ end of the RNA, producing proteins with different C-terminal lengths. TA, transactivation domain; DBD, DNA binding domain; OD, oligomerization domain; SAM, sterile alpha motif domain (Modified from King et al.).
slides. TMAs were assembled using a tissue-arraying instrument consisting of thin-walled stainless biopsy needles and stylets used to empty and transfer the needle contents. The assembly was held in an X-Y position guide that was manually adjusted by micrometers. Briefly, the instrument was used to create holes in a recipient paraffin block and to acquire tissue cores from the donor block using a thin-walled needle. The cylindrical sample was retrieved from the selected region in the donor block and extruded directly into the recipient block with defined array coordinates. A solid stylet, closely fitted in the needle, was used to transfer the tissue cores into the recipient block. Taking tumor heterogeneity into account, we used a large-diameter stylet (2.0 mm). The study specimens were routinely oversampled with two replicate core tumor region samples (different areas) from each donor block. Forty-eight cores from 24 tumors were included in each tissue array block. Multiple 4-μm sections were cut with a microtome.

Immunohistochemistry

Sections from TMA blocks were transferred to poly-L-lysine-coated glass slides and air-dried overnight at 37°C. They were then dewaxed in xylene (three changes), rehydrated in a graded series of decreasing ethanol concentrations, and rinsed in Tris-buffered saline (TBS; pH 7.4). Endogenous peroxidase activity was inactivated with 5% hydrogen peroxide in methanol for 15 min at 37°C.

Antigen retrieval, except for anti-proliferating cell nuclear antigen (PCNA), was performed using a 5-min microwave treatment in TBS. The following primary antibodies were used: anti-p63, anti-AΔp63, anti-TAp63, anti-p53, E-cadherin, and anti-PCNA. Each antibody was incubated with the tissue sections at room temperature. Immunohistochemical procedures were performed using the Envision detection kit (DAKO, Glostrup, Denmark). Detailed information concerning the antibodies used is summarized in Table 1.

Reaction products were visualized by exposing sections to 3-amino-9-ethylcarbazole or diaminobenzidine. Nuclei were lightly counterstained for approximately 20 s with Mayer’s hematoxylin. Sections were then mounted in diluted malinol after the application of Universal Mount (DAKO, Carpinteria, CA, USA). Appropriate positive control specimens were used in each staining batch.

Immunohistochemistry scoring

By light microscopy, all the tissue sections were scored semi-quantitatively considering the proportion of tumor cells showing immunoreactivity. The scoring procedure was implemented by three pathologists. Two scoring methods were separately applied to a given group (p63, AΔp63, TAp63, and p53) and to PCNA. In the former, positive tumor cells showed dark brown nuclear immunoreactivity. The cases showing >10% immunoreactive cells in 2 cores of the same case were considered positive, while the rest were considered negative. The PCNA index was measured as the percentage of nuclear immunoreactive cells among 1,000 tumor cells.

Statistical analysis

The Pearson chi-square test was used to evaluate the statistical significance of AΔp63 expression related to clinicopathologic parameters, and differences in the expression of p53, p63, and AΔp63 according to histologic types of NSCLC. In addition, Student’s t-test was also performed to determine differences in the PCNA indexes according to histologic types. Regression analysis was used to evaluate the effect of AΔp63 on p63, and scatter diagrams were constructed for the Pearson correlation between PCNA and AΔp63, p63, and p53. Follow-up information was also obtained for survival analysis. Patient survival was calculated as the time between surgery and death. Patients who were still alive at the time of data collection were censored in the statistical analysis. Survival curves were plotted according to the Kaplan-Meier method. Differences between the sur-

Table 1. Summary of antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63</td>
<td>Mmab</td>
<td>4A4</td>
<td>DAKO, Denmark</td>
<td>1:100</td>
<td>RT, 60 min</td>
</tr>
<tr>
<td>AΔp63</td>
<td>Rpab</td>
<td>Poly6190</td>
<td>BioLegend, USA</td>
<td>1:200</td>
<td>RT, 60 min</td>
</tr>
<tr>
<td>TAp63</td>
<td>Gpab</td>
<td>SC-8608</td>
<td>Santa Cruz, USA</td>
<td>1:50</td>
<td>RT, 120 min</td>
</tr>
<tr>
<td>p53</td>
<td>Rmab</td>
<td>SP5</td>
<td>Lab Vision, USA</td>
<td>1:100</td>
<td>RT, 60 min</td>
</tr>
<tr>
<td>PCNA</td>
<td>Mmab</td>
<td>PC10</td>
<td>SIGMA, USA</td>
<td>1:2,000</td>
<td>Overnight</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mmab</td>
<td>4A2C7</td>
<td>ZYMED, USA</td>
<td>1:200</td>
<td>RT, 60 min</td>
</tr>
</tbody>
</table>

Mmab, monoclonal mouse antibody; Rpab, polyclonal rabbit antibody; Gpab, polyclonal goat antibody; Rmab, monoclonal rabbit antibody.
vival curves were analyzed using the log-rank test. All statistical analyses were performed on a personal computer with the SPSS statistical package (release 15.0; SPSS, Inc., Chicago, IL, USA). P-values <0.05 were regarded as statistically significant. All statistical tests were two-sided.

RESULTS

Expressions of ΔNp63 and TAp63 in NSCLCs

Immunoreactivity to anti-ΔNp63 antibody was found in 42.8% (41/92) of NSCLC cases. The expression of ΔNp63 was observed only in the nuclei located in the basal and suprabasal layers without localization in the cytoplasm or cell membrane. Carcinoma cells showed diffuse, strong nuclear positivity in many cases of SqCC (39/47 [82.9%]); however, only 2 cases of AC (4.4% of 45 cases) showed moderate nuclear staining. The expression of ΔNp63 was significantly higher in SqCCs than in ACs (p<0.01). Immunoreactivity to anti-TAp63 antibody, however, failed in this experiment except for a diffuse, non-specific pattern. Instead, weak bands for TAp63 on Western blot analysis from tumor samples were demonstrated (data not shown). Fig. 2 illustrates the immunohistochemical results for anti-ΔNp63 antibody.

Relationship between ΔNp63 expression and clinicopathologic parameters

Histologic type, gender, pathologic T stage (pT), p53 expression, and p63 expression showed correlation with ΔNp63 expression (Table 2). With respect to tumor grade, the expression of ΔNp63 was highest in moderately differentiated tumors (p<0.01). The expression of ΔNp63 was also higher in male patients (40 of 81 cases [49.4%]) than in female patients (1 out 11 cases [9.0%]; p<0.05). The pT1 cases showed lower ΔNp63 expression compared to cases with pT2 or more (p<0.05). Among pathologic parameters, PCNA, p53, p63, and ΔNp63 had higher nuclear expression in SqCC than in AC (p<0.05, respectively; Table 3). Furthermore, the expression of p63, ΔNp63, and p53 were correlated with the PCNA proliferation index (p<0.01, respectively).

Topographic distribution of p63 and ΔNp63

The double staining method was applied to evaluate the topographic patterns of p63, ΔNp63, and E-cadherin expression. The results suggested that p63 and ΔNp63 had a similar distribution and that E-cadherin was expressed evenly at the surface of the epithelium (Fig. 3). Regression analysis of ΔNp63 on p63, as shown in Fig. 4, suggested that ΔNp63 was quantita-

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Fig. 2. Immunohistochemical staining for p63, ΔNp63, p53, and PCNA shows strong nuclear staining in the nests of carcinoma. E-cadherin shows strong membranous staining. SqCC, squamous cell carcinoma; AC, adenocarcinoma; PCNA, proliferating cell nuclear antigen.
tively a predominant isoform among the p63 family in NSCLC (R-square=0.801, p<0.01).

Survival analysis

The length of survival in months was defined as the time interval between surgical resection and either death or last follow-up. Observation was censored at the last follow-up when patients were alive or had died from something other than cancer (n.b., none of the patients in the current study developed another type of cancer). Overall survival curves for several significant parameters are shown in Fig. 5. Our study showed that △Np63 was not related to survival (Fig. 5A, p>0.05), but that TNM stage (Fig. 5B, p<0.05), pN (Fig. 5C, p<0.05), and pT (Fig. 5D, p<0.05) were independent prognostic factors in NSCLC.

Table 2. Relationship between △Np63 expression and clinicopathological parameters in non small cell lung carcinomas

<table>
<thead>
<tr>
<th>Parameters</th>
<th>△Np63*</th>
<th>Histologic type</th>
<th>No. cases</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic type</td>
<td></td>
<td>SqCC (n=47)</td>
<td>AC (n=45)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Male</td>
<td>41 (51)</td>
<td>10 (91)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Female</td>
<td>40 (49)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td>T1</td>
<td>T2-4</td>
<td></td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td>19 (73)</td>
<td>32 (48)</td>
<td></td>
</tr>
<tr>
<td>pN</td>
<td></td>
<td>N0</td>
<td>N1-3</td>
<td></td>
</tr>
<tr>
<td>pN</td>
<td></td>
<td>33 (56)</td>
<td>18 (35)</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td>Negative</td>
<td>49 (55)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td>40 (45)</td>
<td>1 (33)</td>
<td></td>
</tr>
<tr>
<td>p63</td>
<td></td>
<td>Negative</td>
<td>40 (98)</td>
<td>11 (22)</td>
</tr>
<tr>
<td>p63</td>
<td></td>
<td>1 (2)</td>
<td>40 (78)</td>
<td></td>
</tr>
</tbody>
</table>

SqCC, squamous cell carcinoma; AC, adenocarcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

DISCUSSION

The p53 family consists of p53, p63, and p73.\textsuperscript{19} p63 is a p53-

Table 3. Relationship between other parameters and histologic types of non small cell lung carcinoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Histologic type</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63*</td>
<td>42 (89)</td>
<td>7 (16)</td>
</tr>
<tr>
<td>△Np63*</td>
<td>40 (85)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>p53*</td>
<td>27 (57)</td>
<td>13 (29)</td>
</tr>
<tr>
<td>PCNA</td>
<td>46.00±20.77</td>
<td>35.06±22.23</td>
</tr>
</tbody>
</table>

*The values are expressed as the number of positive cases (%); \textsuperscript{1}The values are expressed as mean±SD.

SqCC, squamous cell carcinoma; AC, adenocarcinoma; PCNA, proliferating cell nuclear antigen.

Fig. 3. Double immunohistochemical staining for combinations of p63, △Np63, and E-cadherin shows similar topographical distributions for p63 and △Np63. (A) for △Np63 and E-cadherin; (B) for p63 and E-cadherin.
homologous nuclear protein that appears to play a crucial role in the regulation of stem cell commitment in squamous cells and other epithelia.\textsuperscript{1}

The TAp63 (or TAp73) isotypes are thought to function like p53 because they may transactivate p53 downstream targets, induce apoptosis, and mediate cell cycle control. However, the ΔNp63 (or ΔNp73) isoforms have been shown to display opposing functions as oncoproteins.\textsuperscript{30} Moreover, p63 up-regulation in human tumors is often concomitant with overexpression of the dominant negative isoform, ΔNp63. ΔNp63 is preferentially expressed in some tumors, suggesting that this p63 isoform may act as an oncogene.\textsuperscript{21,22} However, a previous report showing TAp63 expression in gastric cancer suggested that TAp63 isoforms may also be involved in tumor progression.\textsuperscript{23} This might be true in thyroid tumors, in which ΔNp63 is not expressed.\textsuperscript{24}

It has been shown that staining intensity for p63 is directly correlated with p63 amplification in NSCLCs; however, this correlation did not hold for SqCCs (r=0.04, p=0.72). It has been suggested that this inconsistency points to an unknown mechanism of p63 gene expression independent of genomic amplification.\textsuperscript{16}

One study showed that p53 alterations were more prevalent in SqCC than in AC, and that p53 was a significant marker in

\begin{align*}
\text{Proportion of p63} &= 10.84 + 1.01 \times \Delta Np63 \\
R^2 &= 0.806 (p=0.000)
\end{align*}

Fig. 4. Regression analysis to evaluate the effect of ΔNp63 on p63.

Fig. 5. Overall survival curves for ΔNp63 (A), pathologic stage (B), pN (C), and pT (D).
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AC patients with a poor prognosis, but not in patients with SqCC. Although it has been reported that lung cancer patients with positive ΔNp73 expression have a poorer prognosis than those with a negative ΔNp73 expression, univariate analysis has demonstrated that positive expression of ΔNp63 is not a significant independent factor for predicting poor prognosis. The current study failed to detect the expression of TA isoforms immunohistochemically. A weak positive band for the TA form by Western blot analysis was identified (data not shown). The strength of the band for NSCLC was even weaker than normal lung tissues and transitional cell carcinoma of urinary bladder as a positive control. This analysis suggested that the expression of TAp63 would be decreased during tumorigenesis in NSCLCs. Further, there was a significant correlation between the expression of the p63 and ΔNp63 isoforms in NSCLCs. SqCCs showed an intimate relationship with ΔNp63 isoforms, which were considered as major forms of the p63 expressed in this study. The present work indicates that the unexplained 20% of p63 expression reflects expression of other isoforms. Finally, in view of previous studies, we arrived at several possible inferences. ΔNp63 functions initially as the major oncoprotein in SqCCs, and as a result, its elevated expression represses the expression of TAp63. Simultaneously, ΔNp63 executes competitive binding to the p53 responsive element in promoter P2, so the expression of p53 was stimulated. ΔNp63 appeared to play an important role in the proliferation of tumor cells as judged from the correlation between the PCNA index and ΔNp63. Both ΔNp63 and p63 had no significant influence on patient survival on univariate analysis. Nevertheless, it should be remembered that there have been debates concerning the prognostic value of ΔNp63 in the setting of NSCLC.

The level of expression of ΔNp63 in moderately differentiated lung tumors was higher than poorly differentiated tumors, although the correlation was paradoxical. The significance was not determined in this study, but may be related to several factors including relatively fewer cases of poorly differentiated tumors and subjectivity in determining histologic grades of NSCLC. Thus, further detailed studies need to be conducted in a larger number of patients with high grade tumors.

In summary, we suggest that ΔNp63 isoforms have oncogenic properties and play a role in the progression of NSCLC, particularly SqCC. However, much more research remains to be done concerning the relationship between p63 isoforms and NSCLC.

REFERENCES