Elevated expression of ΔNp63 in advanced esophageal squamous cell carcinoma

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Despite recent advances in the treatment of esophageal squamous cell carcinoma (ESCC), the fight against ESCC is greatly confounded by the lack of an effective early detection strategy. Esophageal squamous cell carcinoma is considered to be an aggressive carcinoma based on its malignant transformation, carcinogenesis, invasion, and lymph node metastasis.1,2–4

A member of the p53 transcription factor family, p63 (TP63), shares significant homology with p53 and plays a role in maintaining the viability and proliferative capacity of basal epithelial cells.5–7 Alternative splicing of the TP63 gene produces transcripts encoding two isoforms, one with (TAp63) and one without (ΔNp63) the transactivation domain.4,6–8 The 3q27–q29 chromosomal region containing the TP63 gene is frequently amplified early in the development of squamous carcinoma7,8, and may play important roles in a variety of squamous cell carcinomas (SCCs) including lung, head and neck, bladder, and cervical cancers.9–11

Indeed, the elevated expression of the ΔN isoform of p63 serves as a diagnostic marker used to distinguish SCC from adenocarcinoma in lung cancer, as well as CK5/6 or CK14.12–15 ΔNp63 serves as a transactivator through a second transactivation domain and also acts as a dominant-negative transcriptional repressor that inhibits p53- or TAp63-mediated transcription, activities that are consistent with its potential role as an oncogene.15,16,17 Although these findings suggest that ΔNp63 is an SCC oncogene, the biological roles and the pathologic relevance of p63 in tumorigenesis have not been fully elucidated.18,19

Additionally, the loss of p53 function is considered to play an essential role in carcinogenesis and the progression of esophageal cancer malignancies.20,21 The TAp63 isoforms are inhibited by interactions with cancer-associated p53 mutants.11,22

In this report, we investigate the association between the expression of ΔNp63 and ESCC biology. We assess the expression of ΔNp63 in ESCC tumors using immunohistochemistry and clinicopathological studies.

Materials and Methods

Cell lines and plasmids. The human ESCC cell lines, TE-1 and TE-8, were obtained from RIKEN BRC (Tsukuba, Japan) and previously described.23 TE-1 and TE-8 cells were cultured in RPMI-1640 with 10% FBS at 37°C in a 5% CO2 incubator. Human cDNA encoding FLAG-tagged ΔNp63 was subcloned into the LPCX retroviral expression vector (Takara Bio, Shiga, Japan). The sequence of the above construct was verified using DNA sequencing.
Patients. This study included 160 consecutive patients with ESCC who underwent surgical resection at Gunma University Hospital (Maebashi, Japan) between September 2000 and January 2010. All patients underwent R0 resection without preoperative adjuvant therapy. No patient was excluded from this investigation. The age of the patients ranged from 56 to 84 years (median age, 64 years). The study was approved by the institutional review board of Gunma University. The authors’ approach to the evaluation and resection of these tumors has been described previously. Formalin-fixed and paraffin-embedded primary tumor samples from the 160 patients were examined. All surgical specimens were reviewed and classified according to the WHO system by an experienced pathologist who was unaware of the clinical or imaging findings. Pathological TNM stages were established using the International System for Staging adopted by the AJCC and the UICC. All patients had tumors with SCC histology, of which 64 were stage T1 and 96 were stage T2.

ESCC patients received radiotherapy as postoperative treatment. No patient was excluded from this investigation. The age of the patients ranged from 56 to 84 years (median age, 64 years). The study was approved by the institutional review board of Gunma University. The authors’ approach to the evaluation and resection of these tumors has been described previously. Formalin-fixed and paraffin-embedded primary tumor samples from the 160 patients were examined. All surgical specimens were reviewed and classified according to the WHO system by an experienced pathologist who was unaware of the clinical or imaging findings. Pathological TNM stages were established using the International System for Staging adopted by the AJCC and the UICC. All patients had tumors with SCC histology, of which 64 were stage T1 and 96 were stage T2–4. The day of surgery was the starting day for the measurement of postoperative survival. The follow-up duration ranged from 22 to 3983 days (median, 1470 days). In terms of postoperative treatment, 49.4% (79/160) of all ESCC patients received postoperative chemotherapy (mainly 5-fluorouracil-based chemotherapy), 43.8% (53/121) of positive ΔNp63 expressors and 66.7% (26/39) of negative ΔNp63 expressors (Table S1). None of 160 ESCC patients received radiotherapy as postoperative treatment. The study was carried out according to the Reporting Recommendations for Tumour Marker Prognostic Studies guidelines. We used the term “prognostic marker” according to the guidelines.

Immunohistochemistry. Immunohistochemical analysis was carried out on formalin-fixed and paraffin-embedded ESCC sections. The sections were deparaffinized, blocked in Protein Block Serum-Free Reagent (Dako, Carpinteria, CA, USA) for 30 min, and incubated overnight with diluted primary antibodies at 4°C in a humidified chamber. Staining reactions were developed using Histofine Simple Stain MAX-PO Multi Kit (Nichirei, Tokyo, Japan) for immunohistochemistry. Meyer’s hematoxylin (IHC World, Woodstock, MD, USA) was used as a nuclear counterstain.

Expression of ΔNp63 and p53 was determined by immunohistochemical staining with an anti-ΔNp63 rabbit polyclonal antibody (1:100 dilution) and an anti-p53 mouse mAb (DO7, 1:50 dilution; Dako) as previously described. ΔNp63 expression was considered positive if nuclear staining was present, and was scored semiquantitatively based on the percentage of positive cells as follows: 0, 0%; 1, 1%–25%; and 2, >25%. Tumors scored as 0 were defined as negative expression, and those scored as 1 or 2 were defined as positive expression. P53 microscopic examination for the nuclear reaction product was undertaken and scored. P53 expression in >10% of tumor cells was defined as positive expression. The tissue sections were assessed using light microscopy in a blinded fashion by at least two of the authors.

Statistical analysis. Fisher’s exact test was used to examine the association of two categorical variables. The correlation between different variables was analyzed using the non-parametric Spearman’s rank test. Follow-up for these 160 patients was undertaken using patient medical records. The Kaplan–Meier method was used to estimate survival as a function of time, and survival differences were analyzed using the log-rank test. The day of surgery was defined as the starting day for the measurement of postoperative survival. Overall survival (OS) was determined as the time from tumor resection to the time of death from any cause. Disease-free survival was defined as the time between tumor resection and the first disease progression or death. Multivariate analyses were carried out using a stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analyses were undertaken using JMP (SAS Institute, Cary, NC, USA).

Quantitative real-time RT-PCR. The method for real-time RT-PCR was described previously. In short, total RNA was

Fig. 1. Representative immunohistochemical staining of tissue samples from a patient with esophageal squamous cell carcinoma. (a–e) Immunostaining of ΔNp63 shows a nuclear immunostaining pattern with positive ΔNp63 expression: score 0, 0% positive cells (a); 1, 1%–25% (b); and 2, >25% (c). (d,e) Immunostaining of p53 with positive p53 expression: score 0 (d) and score 1 (e).
prepared from surgically resected samples using an RNeasy Mini kit (Qiagen, Hilden, Germany). Relative RNA quantities were measured by a Universal Probe Library Set (Roche, Penzberg, Germany) with KAPA Master Mix (KAPA Biosystems, Wilmington, MA, USA) on a StepOne real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Primers for ΔNp63 were: forward, 5'-GGAAAACAATGCCCA-GACTC-3' and reverse 5'-CTGCTGGTCCATGCTGTTC-3'. The Universal Probe Library Human ACTB Gene Assay (Roche) was used for an endogenous normalization control.

**Anchorage-independent growth (soft agar colony formation assay).** TE-1 and TE-8 cells were retrovirally transduced with ΔNp63. For soft agar assays, the cells were grown in triplicate for 10 days, after which anchorage-independent growth was quantified with a CytoSelect-96 kit (Cell Biolabs, San Diego, CA, USA).

**Results**

**Immunohistochemical analyses.** Clinicopathological data and surgically resected tumor specimens were collected from the 160 patients included in this study. The majority of patients were men (89.4%). All patients received radical surgery, having evidence of superficial ESCC (pathological stage T1, 40.0%) or advanced ESCC (pathological stage T2–4, 60.0%). Squamous cell carcinomas frequently display evidence of simultaneous upregulation of both TAp63 and ΔNp63. ΔNp63 is a putative diagnostic marker for pulmonary SCC. Representative examples of ΔNp63 protein expression in tumor tissue samples are shown in Figure 1. ΔNp63 was observed in the cell nuclei of the basal and parabasal layers of non-cancerous and cancerous esophageal cell layers (Fig. S1b). ΔNp63 expression was observed in 75.6% (121/160) of ESCC tumor tissues, predominantly localized in the nucleus. The aberrant expression of ΔNp63 was observed early in carcinogenesis; cases that maintained such expression in advanced cancer might have a poor prognosis. There is a factor related to the stability of ΔNp63 that may result in a poor prognosis in cases where ΔNp63 expression is maintained. A similar predominant expression of ΔNp63 is found in atypical nuclei of precancerous cases like intra-epithelial neoplasia, compared to normal esophagus, in which p63 staining is restricted to the proliferating basal and parabasal esophageal cell layers (Fig. S1b).

**Clinicopathological factors.** The clinicopathological factors and tumor expression of ΔNp63 of the 160 patients included in this study are shown in Table 1. A clinicopathological analysis of ΔNp63 expression showed that ΔNp63-positive tumors significantly correlated with two important clinical parameters: T factor and venous invasion. Tumors that were positive for ΔNp63 expression were more common in

| Variable                  | Total ESCC (n = 160) | Superficial ESCC (n = 64) | Advanced ESCC (n = 96) |
|---------------------------|----------------------|---------------------------|------------------------|
|                           | Positive (n = 121),  | Positive (n = 54),       | Positive (n = 67),     |
|                           | Negative (n = 39),   | Negative (n = 10),       | Negative (n = 29),     |
|                           | n (%)                | n (%)                     | P-value                |
| Age                       |                      |                           |                        |
| ≤65 years                 | 63 (39.4)            | 28 (43.8)                 | 35 (36.5)              |
| >65 years                 | 58 (36.3)            | 26 (40.6)                 | 32 (33.3)              |
| Sex                       |                      |                           |                        |
| Male                      | 108 (67.5)           | 50 (78.1)                 | 58 (60.4)              |
| Female                    | 13 (8.1)             | 4 (6.3)                   | 9 (9.4)                |
| T factor                  |                      |                           |                        |
| T1                        | 54 (33.8)            | 4 (6.3)                   | 9 (9.4)                |
| T2-4                      | 67 (41.9)            | 29 (18.1)                 | 3 (3.1)                |
| N factor                  |                      |                           |                        |
| N0                        | 50 (31.3)            | 34 (53.1)                 | 16 (16.7)              |
| N1–3                      | 71 (44.4)            | 20 (31.3)                 | 51 (53.1)              |
| M factor                  |                      |                           |                        |
| M0                        | 100 (62.5)           | 49 (76.6)                 | 50 (52.1)              |
| M1                        | 21 (13.1)            | 5 (7.8)                   | 17 (17.7)              |
| Lymphatic permeation      |                      |                           |                        |
| Positive                  | 102 (63.8)           | 36 (56.3)                 | 66 (68.8)              |
| Negative                  | 19 (11.9)            | 18 (28.1)                 | 1 (1.0)                |
| Venous invasion           |                      |                           |                        |
| Positive                  | 88 (55.0)            | 26 (40.6)                 | 62 (64.6)              |
| Negative                  | 33 (20.6)            | 28 (43.8)                 | 5 (5.2)                |
| Differentiation           |                      |                           |                        |
| Well or moderately        | 94 (58.8)            | 45 (70.3)                 | 49 (51.0)              |
| Poorly                    | 27 (16.9)            | 9 (14.1)                  | 18 (18.8)              |
| TP53                      |                      |                           |                        |
| Positive                  | 80 (50.0)            | 35 (54.7)                 | 45 (46.9)              |
| Negative                  | 41 (25.6)            | 19 (29.7)                 | 22 (22.9)              |

†Fisher’s exact test.
patients with stage T2–4 disease (69.8%; 67/96) than in those negative for DNp63 expression (30.2%; 29/96). Negative DNp63 expression was more prevalent in samples from more extensive venous invasion: 28.4% (35/123) in carcinomas with venous invasion but only 10.8% (4/37) in those without venous invasion.

Table 2. Univariate and multivariate survival analyses in patients with advanced esophageal squamous cell carcinoma

| Variable       | Overall survival | Recurrence-free survival |
|----------------|------------------|--------------------------|
|                | Univariate       | Multivariate             | Univariate       | Multivariate             |
|                | 5-year rate, %   | P-value                  | HR (95% CI)      | P-value                  | 5-year rate, %   | P-value                  | HR (95% CI)      | P-value                  |
| Age            |                  |                          |                 |                          |                  |                          |                 |                          |
| ≤65 years      | 47.3             | 0.910                    |                 |                          | 36.6             | 0.112                    |                 |                          |
| >65 years      | 45.7             |                          |                 |                          | 52.3             |                          |                 |                          |
| T factor       |                  |                          |                 |                          |                  |                          |                 |                          |
| T2             | 62.8             | 0.810                    |                 |                          | 58.8             | 0.110                    |                 |                          |
| T3–4           | 43.0             |                          |                 |                          | 40.3             |                          |                 |                          |
| N factor       |                  |                          |                 |                          |                  |                          |                 |                          |
| N0             | 67.3             | 0.042†                   | 1.90            | 0.090                    | 70.2             | 0.002†                   | 2.60            | 0.020†                   |
| N1–2           | 41.3             |                          | (1.02–5.19)     |                          | 36.2             |                          | (1.15–6.96)     |                          |
| M factor       |                  |                          |                 |                          |                  |                          |                 |                          |
| M0             | 51.0             | 0.100                    |                 |                          | 50.9             | 0.003†                   | 1.95            | 0.033†                   |
| M1             | 33.3             |                          |                 |                          | 21.7             |                          | (1.06–3.51)     |                          |
| Venous invasion|                  |                          |                 |                          |                  |                          |                 |                          |
| Positive       | 46.3             | 0.910                    |                 |                          | 43.4             | 0.920                    |                 |                          |
| Negative       | 50.0             |                          |                 |                          | 50.0             |                          |                 |                          |
| DNp63          |                  |                          |                 |                          |                  |                          |                 |                          |
| High expression| 35.6             | 0.005†                   | 2.56            | 0.006†                   | 37.9             | 0.113                    |                 |                          |
| Low expression | 71.7             |                          | (1.29–5.65)     |                          | 57.9             |                          |                 |                          |

†Fisher’s exact test.

Fig. 2. Kaplan–Meier analysis of all cases (n = 160), superficial esophageal squamous cell carcinoma (ESCC) (n = 64), and advanced ESCC (n = 96) in terms of overall survival (a,c,e) and recurrence-free survival (b,d,f) with respect to DNp63 expression. A statistically significant difference in overall survival was observed between advanced ESCC patients with and without tumor expression of DNp63 (P = 0.019) (e).
Remarkably, patient survival was significantly associated with \( \Delta Np63 \) expression and N factor, as assessed by univariate analysis. Multivariate analysis confirmed that \( \Delta Np63 \) expression and disease stage were independent prognostic factors in lung SCC patients with poor progression-free survival and OS (Table 2). Multivariate analysis confirmed that the expression of \( \Delta Np63 \) was an independent prognostic factor for poor OS in advanced ESCC \((P = 0.006) \) but not superficial ESCC (Table 2), whereas N factor had no significant correlation (Table 2). Additionally, there were no statistically significant differences in age, sex, lymph node metastasis, or distant metastasis with respect to the level of \( \Delta Np63 \) expression. Unlike in advanced ESCC cases, \( \Delta Np63 \) was not prognostic factor in superficial ESCC cases (Table S2).

**Prognosis.** The 5-year OS rate for all patients was 56.7%, and the 5-year OS rate and median survival time for advanced ESCC patients was 48.4% and 35.2 months, respectively. Table 2 shows the results of univariate and multivariate analyses of OS in advanced ESCC patients. The univariate analysis revealed that \( \Delta Np63 \) expression and N factor had a significant relationship with OS.

Figure 2 shows the Kaplan–Meier survival curve for ESCC patients as defined by the expression of \( \Delta Np63 \) (Fig. 2). The 5-year OS rates of advanced ESCC with high and low expression of \( \Delta Np63 \) were 35.6% and 71.7%, respectively \((P = 0.019) \). The prognosis of the \( \Delta Np63 \)-positive group was worse than that of the \( \Delta Np63 \)-negative group, although the difference was not statistically significant \((P = 0.152) \).

In order to confirm the functional relevance of \( \Delta Np63 \) expression during ESCC tumorigenicity, we monitored the colony formation of TE-1 and TE-8 ESCC cells transduced with \( \Delta Np63 \) retrovirus. As shown in Figure 3(a,b), \( \Delta Np63 \) expression in either TE-1 or TE-8 cells led to increased anchorage-independent colony formation in soft agar, suggesting that the upregulation of \( \Delta Np63 \) increases tumor formation.

Overall, our results indicate that \( \Delta Np63 \) has oncogenic activity in ESCC both in clinicopathological relevance and in vitro, and further suggest that \( \Delta Np63 \) could be a critical driver of tumor propagation in ESCC.

**Discussion**

In this study, we observed that the group of patients with advanced ESCC had a significantly lower frequency of \( \Delta Np63 \)-positive tumors than did the group with early stage cancer. In addition, we found that \( \Delta Np63 \) expression in advanced ESCC tumors is associated with a poor prognosis.

The function of \( p63 \) is indispensable to normal epidermal stratification and the proliferative potential of epithelial stem cells, whereas the \( \Delta N \) isoform of \( p63 \) is thought to maintain the proliferative potential of basal regenerative cells, including stem cells in the skin, thymus, breast, prostate, and urothelial stratified epithelium.\(^{(3,10,30–32)} \) Our results suggest that \( \Delta Np63 \) expression is of diagnostic value in ESCC and is an independent prognostic factor in advanced ESCC, although these results were not statistically significant for the total cases of ESCC.

The expression of \( \Delta Np63 \) has been reported as a highly specific diagnostic marker of various human SCCs, including lung, head and neck, bladder, and cervical cancers.\(^{(8–11)} \) In fact, the amplification and overexpression of \( p63 \) has been frequently observed in lung cancers and in head and neck cancers.\(^{(11)} \)

The expression of \( \Delta Np63 \) is reported to be significantly lower in invasive ESCC and to have a negative correlation with poor prognosis \(^{(33)} \) whereas the expression of \( p63 \) decreases during the progression of lung, breast, and bladder cancer. Moreover, the loss of \( p63 \) expression is associated with poor prognosis in some cases.\(^{(31,34,35)} \) In this study, our results suggested that the remaining \( \Delta Np63 \)-expression cases in advanced ESCC are correlated with poor prognosis, and that \( \Delta Np63 \) is involved in the early development of SCC.\(^{(17,8)} \) The exact mechanism needs to be clarified that \( \Delta Np63 \)-expression remains in advanced ESCC with poor prognosis. In superficial ESCC, \( \Delta Np63 \) expression was not found to be a prognostic factor (Table S2). Although functional evaluation of \( p63 \) was not fully studied and the bias of postoperative treatment could not be completely excluded, we concluded that \( \Delta Np63 \) could play a role in carcinogenesis and tumor progression in ESCC.\(^{(26,34)} \)

**Fig. 3.** \( \Delta Np63 \) increases esophageal squamous cell carcinoma tumorigenesis *in vitro.* (a) Esophageal squamous cell carcinoma cell lines, TE-1 and TE-8, were retrovirally infected with LPCX empty vector (Mock) and \( \Delta Np63 \). Total RNAs were quantified by real-time RT-PCR analysis and the induction level of \( \Delta Np63 \) was determined by the relative Ct method. (b) Growth of TE-1 and TE-8 cells transduced with \( \Delta Np63 \) were monitored by anchorage-independent soft agar colony formation assay. Standard deviations are plotted. \(* P < 0.05.\)
Our results showed that the proportion of positive ΔNp63 expression was high in patients without venous invasion compared to patients with venous invasion. However, T factor and ΔNp63 are significantly correlated (Table S3, P < 0.0001), we evaluate that venous invasion was affected by T factor.

Although high expression of ΔNp63 correlates with poor prognosis in patients with a variety of tumors, this correlation is stronger in patients whose tumors also have a mutation in the p53 gene. The correct balance between the tumor-suppressive role of the TA isotype and the oncogenic role of the AN isotype, along with the tissue-specific environment, may be essential for the proliferation and differentiation of both epithelial stem cells and cancer stem cells. Whereas we could not establish a significant correlation between ΔNp63 and mutant p53 (Fig. 1, Table 1), the interaction between mutant p53 and p63 is reported to regulate the expression of p63 target genes to enhance invasion and metastasis. Hence, the oncogenic activity of ΔNp63 might be a consequence of the physical association between ΔNp63 and mutant p53.

Recent evidence indicates that inhibition of the p53/NF-Y complex by mutant gain of p53 function enhances expression of platelet-derived growth factor receptor-β and promotes metastasis in a subset of pancreatic cancers. Syntaxin binding protein 4 is reported to physically interact with ΔNp63 and to play a crucial role in driving lung SCC growth through platelet-derived growth factor receptor-α signaling in a ΔNp63-dependent manner, which is consistent with a putative diagnostic role for ΔNp63.

The results of this study differ with respect to prognostic factor findings from those of other studies. Although previous reports described a poor prognosis for ΔNp63-negative cases, similar to our study, lower expression was more frequently observed in cases with worse tumor invasion. Our clinical samples are newer than those of previous reports, and there might be a smaller number of cases with early stage ESCC that are treatable by endoscopic submucosal dissection. The limitation of this study is that it is retrospective in nature and was undertaken at a single center with a small number of cases. Despite the retrospective analysis, none of the cases involved neoadjuvant chemotherapy, and the clinical conditions did not differ.

We report here for the first time that ΔNp63 expression increases the oncogenic potential of ESCC and serves as an independent prognostic marker for predicting poor outcome in advanced ESCC. Our findings suggest that ΔNp63 is a new diagnostic marker for ESCC and might be a relevant therapeutic target for the treatment of patients with this disease.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Representative pictures of ANp63 immunohistochemistry in non-cancerous (a) and pre-cancerous (b) specimens from patients with esophageal squamous cell carcinoma.

Table S1. Postoperative chemotherapy among 160 patients with esophageal squamous cell carcinoma.

Table S2. Univariate and multivariate survival analysis in patients with superficial esophageal squamous cell carcinoma.

Table S3. Correlation of T factor and venous invasion in patients with esophageal squamous cell carcinoma (n = 160).