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Elevated expression of ΔNp63 in advanced esophageal squamous cell carcinoma

Yuji Kumakura,[1] Susumu Rokudai,[2] Misaki Iijima,[1] Bolag Altan,[3] Tomonori Yoshida,[1] Halin Bao,[1] Takehiko Yokobori,[4] Makoto Sakai,[1] Makoto Sohda,[1] Tatsuya Miyazaki,[1] Masahiko Nishiyama[2] and Hiroyuki Kuwano[1]

Departments of [1]General Surgical Science; [2]Molecular Pharmacology and Oncology, Gunma University, Maebashi; [3]Department of Oncology Clinical Development, Graduate School of Medicine, Gunma University, Maebashi; [4]Research Program for Omics-based Medical Science, Division of Integrated Oncology Research, Gunma University Initiative for Advanced Research, Maebashi, Japan

Key words

Carcinogenesis, esophageal cancer, squamous cell carcinoma, TP63, ΔNp63/p40

Correspondence

Yuji Kumakura, Department of General Surgical Science, Gunma University, 3-39-22 Showa, Maebashi, Gunma 371-8511, Japan.
Tel: +81-27-220-8224; Fax: +81-27-220-8230;
E-mail: bokuha_kuma_kura@yahoo.co.jp

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This study aims to explore the expression level of ΔNp63 in esophageal squamous cell carcinoma (ESCC). To investigate the association between ΔNp63 (p40) expression and ESCC biology, we compared the levels of ΔNp63 expression in normal and tumor tissues, with a specific focus on the diagnostic value of ΔNp63 in ESCC. We analyzed 160 consecutive patients with ESCC who underwent surgical resection without neoadjuvant chemotherapy at Gunma University Hospital (Maebashi, Japan) between September 2000 and January 2010. The clinicopathological characteristics and survival of patients were subclassified based on the expression of ΔNp63 as determined by immunohistochemistry, indicating that ΔNp63 was highly expressed in 75.6% (121/160) of ESCC patients. Clinicopathological analysis of ΔNp63 expression showed that ΔNp63-positive tumors significantly correlated with two important clinical parameters: T factor ($P = 0.0316$) and venous invasion ($P = 0.0195$). The 5-year overall survival rates of advanced ESCC patients with positive and negative expression of ΔNp63 were 35.6% and 71.7%, respectively. Multivariate analysis revealed that the expression of ΔNp63 was identified as an independent prognostic factor ($P = 0.0049$) in advanced ESCC. In line with this, ΔNp63α-transduced ESCC cell lines increased tumor growth in a soft agar colony formation assay. We report here for the first time that ΔNp63 expression increases the oncogenic potential of ESCC and is an independent marker for predicting poor outcome in advanced ESCC. Our findings suggest that ΔNp63 could serve as a new diagnostic marker for ESCC and might be a relevant therapeutic target for the treatment of patients with this disease.

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]

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.[3] Alternative splicing of the *TP63* gene produces transcripts encoding two isoforms, one with (TAp63) and one without (ΔNp63) the transactivation domain.[4–6] The 3q27–q29 chromosomal region containing the *TP63* gene is frequently amplified early in the development of squamous carcinoma[7,8] 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.[8,12–15] *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.[5,16,17] 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% CO_2 incubator. Human cDNA encoding FLAG-tagged ΔNp63α was subcloned into the LPCX retroviral expression vector (Takara Bio, Shiga, Japan). The sequence of the above construct was verified using DNA sequencing.

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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.[23] 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.[24] 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.[25,26] 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. [26,27] In short, total RNA was

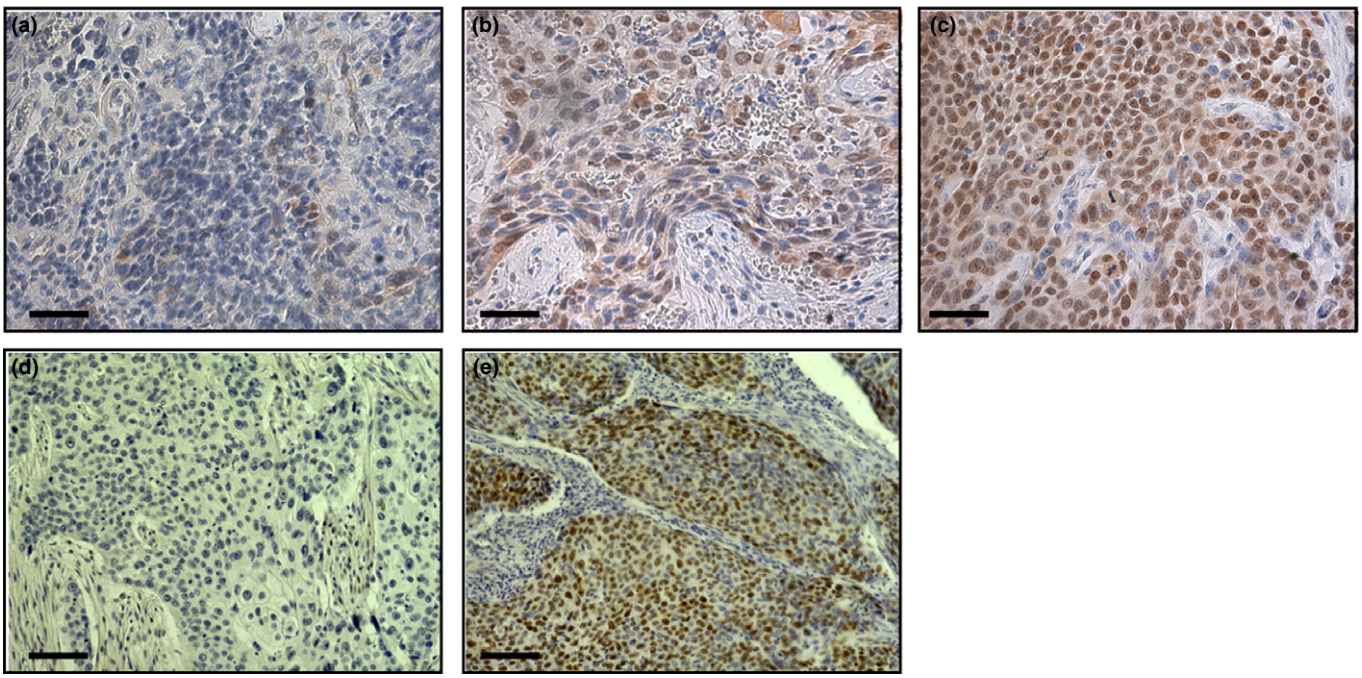

Fig. 1. Representative immunohistochemical staining of tissue samples from a patient with esophageal squamous cell carcinoma. (a–c) Immunostaining of ΔNp63 shows a nuclear immunostaining pattern with positive ΔNp63 expression: score 0, 0% positive cells (a); 1, 1–25% (b); and 2, >25% (c). (d,e) Immunostaining of p53 with positive p53 expression: score 0 (d) and score 1 (e).

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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′-GGAAAACAATGCCCAGACTC-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.[18] 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 specimens (Fig. S1a); elevated 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).[28,29]

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 ($P = 0.032$) and venous invasion ($P = 0.019$). Tumors that were positive for ΔNp63 expression were more common in

Table 1. Demographics of 160 patients with esophageal squamous cell carcinoma (ESCC) according to ΔNp63 expression

Variable	Total ESCC ($n = 160$)			Superficial ESCC ($n = 64$)			Advanced ESCC ($n = 96$)		
	Positive ($n = 121$), n (%)	Negative ($n = 39$), n (%)	P-value	Positive ($n = 54$), n (%)	Negative ($n = 10$), n (%)	P-value	Positive ($n = 67$), n (%)	Negative ($n = 29$), n (%)	P-value
Age									
≤65 years	63 (39.4)	21 (13.1)	0.846	28 (43.8)	3 (4.7)	0.304	35 (36.5)	18 (18.8)	0.503
>65 years	58 (36.3)	18 (11.3)		26 (40.6)	7 (10.9)		32 (33.3)	11 (11.5)	
Sex									
Male	108 (67.5)	35 (21.9)	0.931	50 (78.1)	9 (14.1)	0.585	58 (60.4)	26 (27.1)	>0.99
Female	13 (8.1)	4 (2.5)		4 (6.3)	1 (1.6)		9 (9.4)	3 (3.1)	
T factor									
T1	54 (33.8)	10 (6.3)	0.032†						
T2–4	67 (41.9)	29 (18.1)							
N factor									
N0	50 (31.3)	10 (6.3)	0.073	34 (53.1)	4 (6.3)	0.292	16 (16.7)	6 (6.3)	0.731
N1–3	71 (44.4)	29 (18.1)		20 (31.3)	6 (9.4)		51 (53.1)	23 (24.0)	
M factor									
M0	100 (62.5)	30 (18.8)	0.434	49 (76.6)	8 (12.5)	0.354	50 (52.1)	22 (22.9)	>0.99
M1	21 (13.1)	9 (5.6)		5 (7.8)	2 (3.1)		17 (17.7)	7 (7.3)	
Lymphatic permeation									
Positive	102 (63.8)	34 (21.3)	0.657	36 (56.3)	7 (10.9)	>0.99	66 (68.8)	27 (28.1)	0.186
Negative	19 (11.9)	5 (3.1)		18 (28.1)	3 (4.7)		1 (1.0)	2 (2.1)	
Venous invasion									
Positive	88 (55.0)	35 (21.9)	0.019†	26 (40.6)	6 (9.4)	0.732	62 (64.6)	29 (30.2)	0.318
Negative	33 (20.6)	4 (2.5)		28 (43.8)	4 (6.3)		5 (5.2)	0 (0.0)	
Differentiation									
Well or moderately	94 (58.8)	25 (15.6)	0.097	45 (70.3)	6 (9.4)	0.192	49 (51.0)	19 (19.8)	0.455
Poorly	27 (16.9)	14 (8.8)		9 (14.1)	4 (6.3)		18 (18.8)	10 (10.4)	
TP53									
Positive	80 (50.0)	27 (16.9)	0.718	35 (54.7)	6 (9.4)	>0.99	45 (46.9)	21 (21.9)	0.608
Negative	41 (25.6)	12 (7.5)		19 (29.7)	4 (6.3)		22 (22.9)	8 (8.3)	

†Fisher's exact test.

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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.

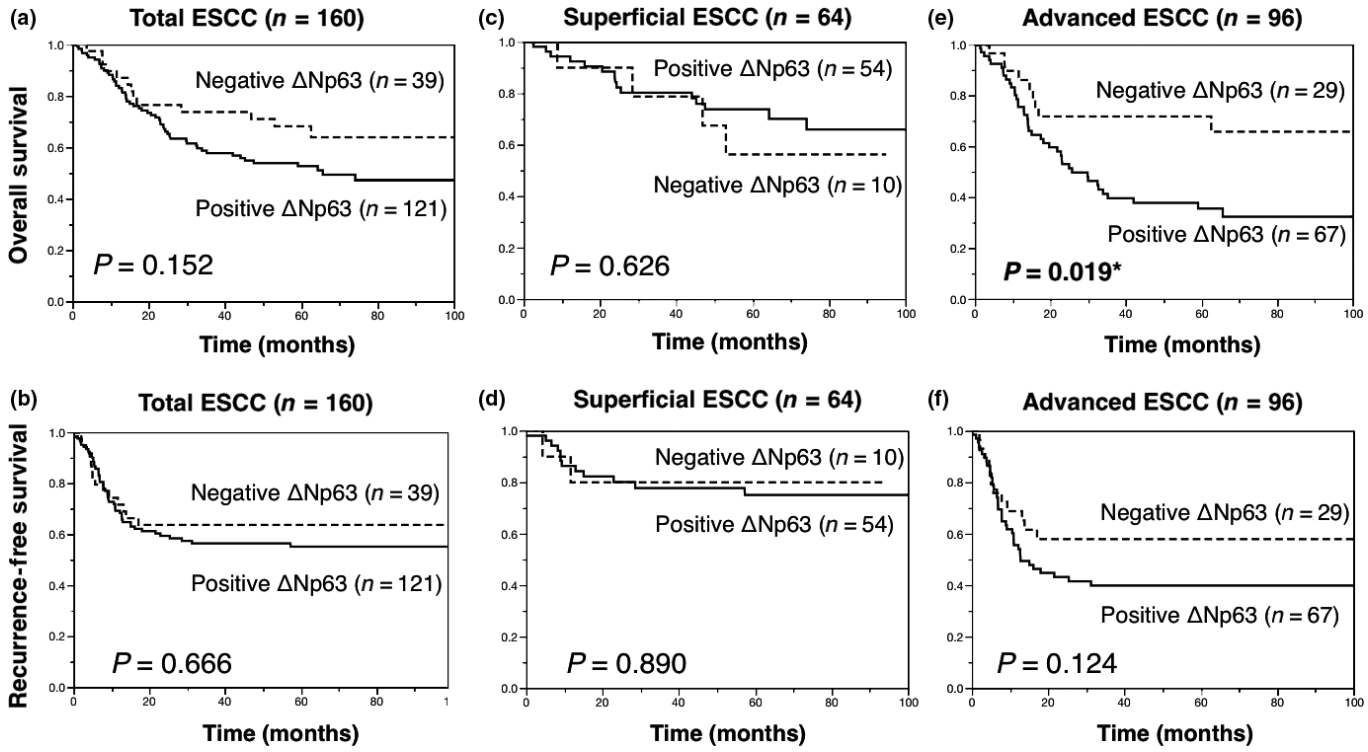

Fig. 2. Kaplan–Meier analysis of all cases (n = 160), superficial esophageal squamous cell carcinoma (ESCC) (n = 64), and advanced ESCC (n = 96) in terms of overall survival (a,c,e) and recurrence-free survival (b,d,f) with respect to ΔNp63 expression. A statistically significant difference in overall survival was observed between advanced ESCC patients with and without tumor expression of ΔNp63 (P = 0.019) (e).

patients with stage T2–4 disease (69.8%; 67/96) than in those negative for ΔNp63 expression (30.2%; 29/96). Negative ΔNp63 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.

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Remarkably, patient survival was significantly associated with ΔNp63 expression and N factor, as assessed by univariate analysis. Multivariate analysis confirmed that ΔNp63 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 ΔNp63 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 ΔNp63 expression. Unlike in advanced ESCC cases, ΔNp63 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 ΔNp63 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 ΔNp63 (Fig. 2). The 5-year OS rates of advanced ESCC with high and low expression of ΔNp63 were 35.6% and 71.7%, respectively ($P = 0.019$). The prognosis of the ΔNp63-positive group was worse than that of the ΔNp63-negative group, although the difference was not statistically significant ($P = 0.152$).

In order to confirm the functional relevance of ΔNp63 expression during ESCC tumorigenicity, we monitored the colony formation of TE-1 and TE-8 ESCC cells transduced with Δ*Np63* retrovirus. As shown in Figure 3(a,b), Δ*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 Δ*Np63*α increases tumor formation.

Overall, our results indicate that Δ*Np63* has oncogenic activity in ESCC both in clinicopathological relevance and *in vitro*, and further suggest that Δ*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 ΔNp63-positive tumors than did the group with early stage cancer. In addition, we found that ΔNp63 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 ΔN 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 ΔNp63 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 ΔNp63 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 ΔNp63 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 ΔNp63-expression cases in advanced ESCC are correlated with poor prognosis, and that ΔNp63 is involved in the early development of SCC.[7,8] The exact mechanism needs to be clarified that ΔNp63-expression remains in advanced ESCC with poor prognosis. In superficial ESCC, ΔNp63 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 ΔNp63 could play a role in carcinogenesis and tumor progression in ESCC.[26,34]

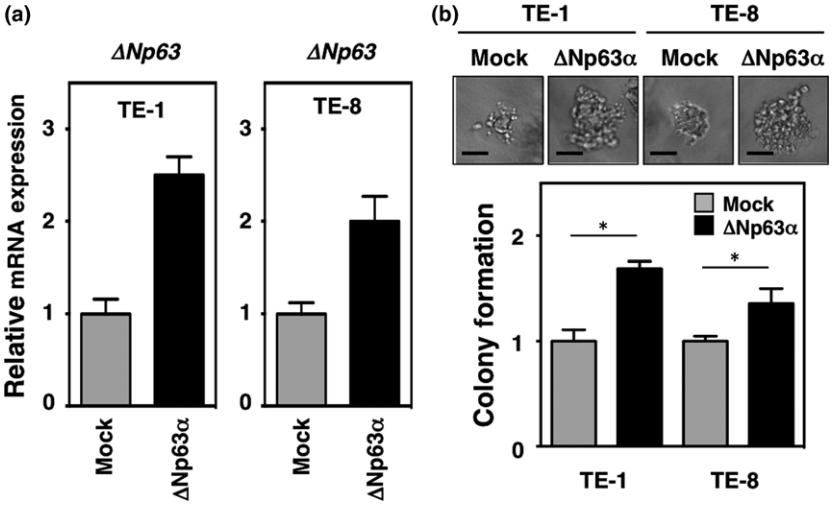

Fig. 3. Δ*Np63* increases esophageal squamous cell carcinoma tumorigenesis *in vitro*. (a) Esophageal squamous cell carcinoma cell lines, TE-1 and TE-8, were retrovirally infected with LPCX empty vector (Mock) and Δ*Np63*α. Total RNAs were quantified by real-time RT-PCR analysis and the induction level of Δ*Np63* was determined by the relative Ct method. (b) Growth of TE-1 and TE-8 cells transduced with ΔNp63 were monitored by anchorage-independent soft agar colony formation assay. Standard deviations are plotted. *$P < 0.05$.

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Our results showed that the proportion of positive ΔNp63 expression was high in patients without venous invasion compare 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.[36] The correct balance between the tumor-suppressive role of the TA isotype and the oncogenic role of the ΔN 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.[37] Hence, the oncogenic activity of ΔNp63 might be a consequence of the physical association between ΔNp63 and mutant p53.[25,38] 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.[39] 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.[26]

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 ΔNp63 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).

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