Expression of ΔNp73 and TAp73α Independently Associated with Radiosensitivities and Prognoses in Cervical Squamous Cell Carcinoma

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Abstract

Purpose: The *p73* gene produces different protein isoforms using alternative promoters and splicing, which have different biological characteristics. This study was to investigate the expression patterns of two distinct p73 isoforms (ΔNp73 and TAp73α) in cervical squamous cell carcinomas (SCC) and the relationship between their expressions and prognostic significance in cervical SCC patients.

Experimental Design: We investigated the protein expressions of ΔNp73 and TAp73α in 117 cervical SCC and 113 normal cervical tissues using immunohistochemistry. The expression levels were analyzed with clinical variables and patients' survival.

Results: ΔNp73and TAp73α were significantly overexpressed in cervical SCC compared with those in normal cervical epithelium ($P < 0.001$). However, their expressions were inversely correlated ($P < 0.001$, $R = -0.368$) and associated with differential tumor radiosensitivity. Overexpression of ΔNp73 was significantly found in SCC resistant to irradiation ($P < 0.001$), whereas increase of TAp73α expression was observed in the majority of SCC sensitive to irradiation ($P < 0.001$). Multivariate and survival analyses indicated that the expressions of ΔNp73 and TAp73α were independently associated with prognosis: ΔNp73 was associated with recurrence of the disease [$P = 0.001$; odds ratio (OR), 4.857] and an adverse outcome ($P = 0.012$; OR, 4.676), whereasTAp73α predicted a better survival of cervical SCC patients ($P = 0.018$; OR, 0.065).

Conclusions: The *p73* gene might be an important determinant of cellular response to irradiation. The expressions of the two main isoforms (ΔNp73 and TAp73α) might be potential markers for predicting the prognosis and sensitivity to radiotherapy in patients with cervical SCC.

Cervical cancer is a common genital tract cancer in women. Its treatment includes mainly radical hysterectomy and/or radiotherapy. Radiotherapy is the mainstay of treatment, especially in advanced cervical cancer. The patients' survival rate can be determined by their responsiveness to radiotherapeutic treatment. Response of cancers to ionizing radiation varies widely, and this may be explained by differences in cancer cell death-inducing effectors. Multiple genetic and epigenetic changes in the cancer cell may contribute to radioresistance. Our previous study has shown an association between p73 expression and radiosensitivity of cervical cancers and suggested that p73 might play an important role in controlling cellular radiosensitivity (1).

p73 has been identified as a structural and functional homologue of the tumor suppressor protein p53 (2, 3). Despite their similarities, these two proteins are likely to display distinct functions, particularly in tumor formation and progression (4–6). Data from studies of human tumors and p73-deficient mice did not support the classic Knudsen-type tumor suppressor role for the *p73* gene. Inactivating mutations of p73 in human tumors are extremely rare, and p73-deficient mice lacks a spontaneous tumor phenotype (5). One possible explanation for the different roles of p53 and p73 in tumorigenesis possibly lies within their different genomic organization. Although p53 encodes one protein, p73 gives rise to multiple protein isoforms due to alternative promoter use and alternative mRNA splicing (6–8). The full-length wild-type TA isoform of p73 (TAp73) containing an NH_2-terminal transactivation domain (TA) can activate downstream target genes and induce apoptosis. In contrast, the NH_2-terminal truncated form (ΔNp73) lacking the transactivation domain acts as "dominant inhibitors" of the wild-type TAp73 and p53 and has antiapoptotic function. This suggested p73 holds dual roles where the TAp73 isoform harbors proapoptotic characteristic, whereas ΔNp73 holds antiapoptotic property.

TAp73, similar to p53, can be activated in response to DNA damage, and the activation of TAp73 leads to the induction of ΔNp73 (9). Interestingly, ΔNp73, acting as a dominant-negative inhibitor, can interfere with p53 from binding to the

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p53-responsive elements and also inhibit the transcription of the TAp73 isoforms. Recent studies have revealed that ΔNp73 could be rapidly degraded in response to strong DNA damage, hence releasing its dominant-negative effect exerted on p53 and TAp73 and allowing cell cycle arrest and apoptosis to proceed (10, 11). Therefore, ΔNp73 is suggested to be part of a dominant-negative feedback loop that regulates the function of both p53 and TAp73. This regulatory mechanism can be overcome in case of strong DNA damage.

The expression pattern of ΔNp73 in tumors is likely to be an important determinant of cellular response to the treatments. ΔNp73 is frequently overexpressed in multiple primary tumor types and cancer cell lines, but is barely detected in normal human tissues (12, 13). A relative increased ΔNp73 expression has been associated with tumor progression and poor prognosis in several human cancers, including neuroblastoma, lung, and ovarian carcinomas (13–15). In addition, alterations in the relative levels of ΔNp73 and TAp73 have been shown to correlate with prognosis in some cancers, suggesting that the disruption of the balance between ΔNp73 and TAp73 isoforms may be of importance in tumorigenesis and in resistance to chemotherapy (16, 17). However, the relative expressions of ΔNp73 and TAp73 in cervical cancers have not yet been reported.

In this study, we analyzed the expression of ΔNp73 in comparison with that of TAp73α in cervical squamous cell carcinoma (SCC) and normal cervices by immunohistochemical staining. The relationship between the expressions of the two p73 isoforms and their prognostic significance was also evaluated in cervical cancer.

Materials and Methods

Cervical cancer and control specimens. One hundred seventeen cervical cancers and 113 normal cervices were recruited at the Department of Obstetrics and Gynaecology, Queen Mary Hospital, The University of Hong Kong from 1998 to 2002. They were completely independent and not overlapped with the specimens in the previous study regarding the p73 expression (1). They were recoded without disclosure of the identity of the patients. The use of the clinical specimens in the present study was approved by the local institutional ethics committee (Institutional Review Board no. UW 06-005 T/1030). Among 117 cancer patients, 33 patients were treated by surgery alone, and 84 patients were treated with radiotherapy, while some of them had additional treatment of chemotherapy and/or surgery. The mean age of patients was 56.2 years (range, 28-93 years). The stage of cancer was diagnosed according to the criteria of the International Federation of Gynaecology and Obstetrics classification: 78 (66.7%) cases at early stages (I -IIa) and 39 (33.3%) cases at advanced stages (IIb and above). Histologic cell type of the cancer was SCCs. Normal cervical tissues from 113 patients who had undergone hysterectomy for benign gynecologic diseases were included as normal controls. The mean age was 47.4 years (range, 31-65 years).

The relevant clinical information of all cases was retrieved from the medical records. Eighty-four cervical cancer patients treated with radiotherapy were divided into radiosensitive (33 cases) and radioresistant (51 cases) groups based on the histologic findings of residual tumor cells in the cervical biopsy specimens taken after the completion of radiotherapy (1).

Immunohistochemistry. The immunohistochemical assessment was done on tumor specimens obtained before the initiation of the treatments. Formalin-fixed and paraffin-embedded cervical tissues were retrieved from the Department of Pathology, Queen Mary Hospital, The University of Hong Kong. They were sectioned at 5 μm thick and mounted on aminopropyltriethoxysilane (Sigma, St. Louis, MO)–coated slides. All specimens were stained with H&E for histopathologic evaluation. The consecutive section was used in immunohistochemical staining, which was done by a streptavidin-biotin-peroxidase complex method as described previously (18). Antigen retrieval was done by microwave pretreatment in 0.01 mol/L citrate buffer 15 minutes for ΔNp73 or 9 minutes for TAp73α. Mouse monoclonal antibodies of ΔNp73 (raised against a synthetic peptide LYVGDPARHLAT corresponding to amino acid residues 2-13 of human ΔNp73; Calbiochem, San Diego, CA) and TAp73α (raised specific for the

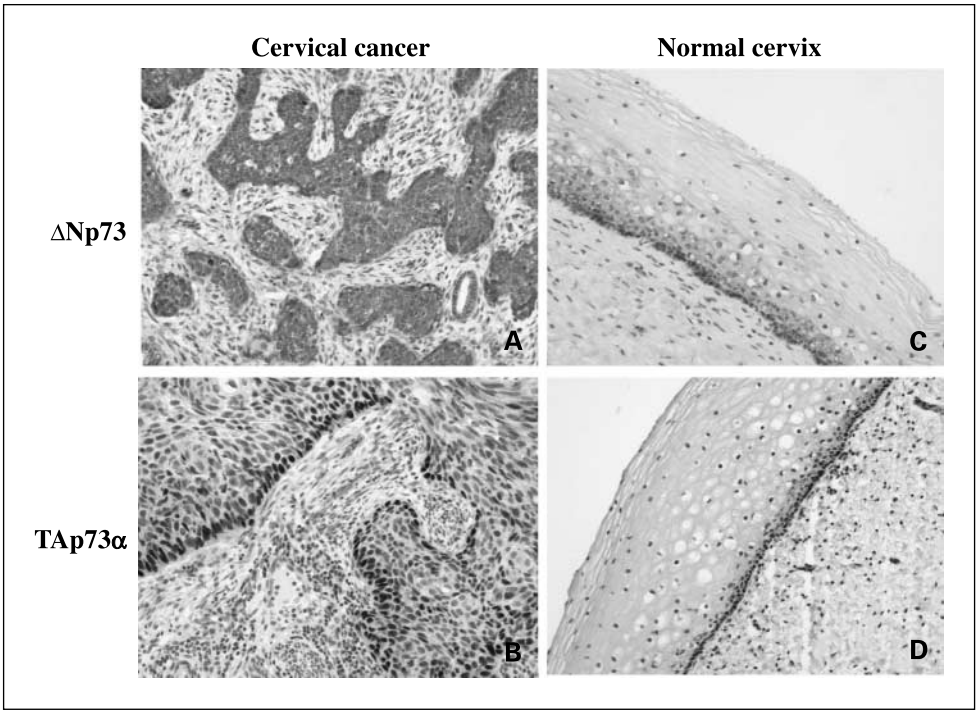

Fig. 1. Immunohistochemical analyses of ΔNp73 and TAp73α expression in cervical SCC (*A* and *B*) and normal cervix (*C* and *D*).

Table 1.

A. Immunohistochemical analyses of ΔNp73 and TAp73α expressions in cervical SCCs and normal cervices

Immunohistochemistry	ΔNp73		TAp73α	
	Cancer (%)	Normal (%)	Cancer (%)	Normal (%)
Positive				
≤50%	69 (59.0)	106 (93.8)	81 (69.2)	105 (92.9)
>50%	48 (41.0)	7 (6.2)	36 (30.8)	8 (7.1)
Total	117	113	117	113
χ^2 test	$P < 0.001$		$P < 0.001$	

B. Correlation analysis between the expressions of two isoforms in cervical SCCs

Immunohistochemistry	ΔNp73	
	≤50%	>50%
TAp73α		
≤50%	38	43
>50%	31	5
Total	69	48
χ^2 test	$P < 0.001$	

full-length alpha; Zymed, San Francisco, CA) were diluted in 1:100. Cases of cervical cancer known to be immunoreactive for these two antibodies were used as positive controls. Negative control, where primary antibody was omitted, was also included in each experiment.

Evaluation. Sections were examined at high power (×400), and 10 fields were chosen randomly. Cells were judged as positive for ΔNp73 or TAp73α expressions when the cytoplasm or nuclei were stained. The immunoreactivity of both isoforms was estimated with respect to the sum of the values of both percentage and intensity of the positive cells as described previously (18). Each case with positive staining in >50% of cancer cells or normal epithelial cells was considered as strongly positive for statistical analysis in this study.

Statistical analysis. All statistical analyses were done using Statistical Package for the Social Sciences 11.5 (SPSS, Inc., Chicago, IL). Receiver operating characteristic curve analysis was used to discriminate the genes expression cutoff point. The genes expression cutoff points were found to be at 50% expression with respect to tumor radiosensitivity and patients' prognostic outcome of which these two clinical factors were our initial major concerns of this study. Statistical significance was evaluated using Pearson's χ^2 test or Fisher's exact test where applicable. Survival probabilities were calculated according to the Kaplan-Meier method, and differences between the groups were analyzed by log-rank test. The Cox regression model was used for multivariate analysis to assess the independence of different prognostic factors. The statistical difference was considered significant when $P < 0.05$.

Results

Differential expressions of ΔNp73 and TAp73α in cervical SCC and normal cervix. The protein expressions of both ΔNp73 and TAp73α were successfully evaluated in 117 cervical cancers

Table 2. Association analyses between the expression levels of ΔNp73 and TAp73α and the clinical characteristics of cervical SCC patients

Characteristics	Total	ΔNp73 expression			TAp73α expression		
		≤50%	>50%	*P*	≤50%	>50%	*P*
All cases	117	69 (59.0%)	48 (41.0%)		81 (69.2%)	36 (30.8%)	
Age (y)							
<55	60	41 (59.4%)	19 (39.6%)		40 (49.4%)	20 (55.6%)	
≥55	57	28 (40.6%)	29 (60.4%)	0.027	41 (50.6%)	16 (44.4%)	0.339
Stage							
Early	78	50 (72.5%)	28 (58.3%)		55 (67.9%)	23 (63.9%)	
Late	39	19 (27.5%)	20 (41.7%)	0.082	26 (32.1%)	13 (36.1%)	0.413
Radiosensitivity							
Sensitive	33	27 (81.8%)	6 (18.2%)		8 (24.2%)	25 (75.8%)	
Resistant	51	15 (29.4%)	36 (70.6%)	<0.001	48 (94.1%)	3 (5.9%)	<0.001
Recurrence							
−	93	62 (66.7%)	31 (33.3%)		61 (65.6%)	32 (34.4%)	
+	24	7 (29.2%)	17 (70.8%)	0.001	20 (83.3%)	4 (16.7%)	0.136

and 113 normal cervical tissues, respectively. The immunoreactivity of ΔNp73 was diffused in the tumor and found in both cytoplasm and nuclei of the cells (Fig. 1A), whereas the positive TAp73α staining was found to be heterogeneous and particular in the invasive borders of the tumor clusters (Fig. 1B). The staining was restricted to the nuclei of the cells. Overexpression of the two p73 isoforms was found in the majority of the cancer cases. Forty-eight (41.0%) and 36 (30.8%) cancers showed strong ΔNp73 and TAp73α expressions, respectively (Table 1A). Interestingly, cancers that expressed a higher level of ΔNp73 tended to have a lower level of TAp73α or vice versa. The expression of the two isoforms were inversely correlated (χ^2 test, $P < 0.001$; $R = -0.368$; Table 1B).

In contrast, weak expression of both p73 isoforms was observed in most of the normal cervical tissues. The expression of ΔNp73 was found in suprabasal and intermediate layers (Fig. 1C), which is the transit amplifying population of cervical epithelium. Unlike ΔNp73, the expression of the TAp73α was primarily detected in the basal cells of normal cervical epithelium (Fig. 1D). This suggested that TAp73α expressed in nonproliferating cells at the base of the cervical squamous epithelium. Only 8 (7.1%) and 7 (6.2%) normal cervical samples displayed strong expression of ΔNp73 or TAp73α (>50% of normal epithelia cells showed positive staining), respectively. No correlation between the expressions of the two isoforms was observed in the normal controls (Fisher's exact test, $P = 1.0$). The two p73 isoforms were differentially expressed in the SCC when compared with those in the normal controls (χ^2 test, $P < 0.001$ for both ΔNp73 and TAp73α).

Clinical correlations of ΔNp73 and TAp73α expressions. To determine the clinical implications of the two p73 isoform expressions in cervical cancers, clinical correlation and patient's survival analyses were done. Overexpression of ΔNp73 had significant correlation with the patient's age at first diagnosis with cancer (χ^2 test, $P = 0.027$; Table 2). Patients diagnosed to have cancer at an older age (≥55 years) tended to have higher ΔNp73 expression than those at age < 55 years (odds ratio, 2.235; 95% confidence interval, 1.054-4.741). No significant difference was found between the expressions of the two isoforms and the disease stages (χ^2 test, $P = 0.082$ and $P = 0.413$ for ΔNp73 and TAp73α, respectively). With respect to tumor radiosensitivity, both p73 isoform expressions were analyzed in 84 patients who had undergone radiotherapy (Table 2). The SCC cases resistant to radiotherapy were found to have overexpression of ΔNp73 (70.6%, 36 of 51); yet, such an expression pattern was observed in only 18.2% (6 of 33) of the SCC sensitive to radiotherapy (χ^2 test, $P < 0.001$; odds ratio, 10.8; 95% confidence interval, 3.704-31.492). Conversely, only 5.9% (3 of 51) of radioresistant SCC had increased TAp73α expression, whereas 75.8% (25 of 33) of radiosensitive SCC showed TAp73α (χ^2 test, $P < 0.001$; odds ratio, 0.02; 95% confidence interval, 0.05-0.082).

The expression patterns of both p73 isoforms were significantly associated with the patient's survival, but they represented different prognostic significance (Fig. 2A and B). Overexpression of ΔNp73 was correlated with an adverse outcome of the patients (log-rank test, $P < 0.001$), whereas the TAp73α overexpression predicted the better survival (log-rank test, $P = 0.003$). To further study the prognostic significance of these two competing expression factors, the SCC cases were categorized into three groups: cases with overexpression of

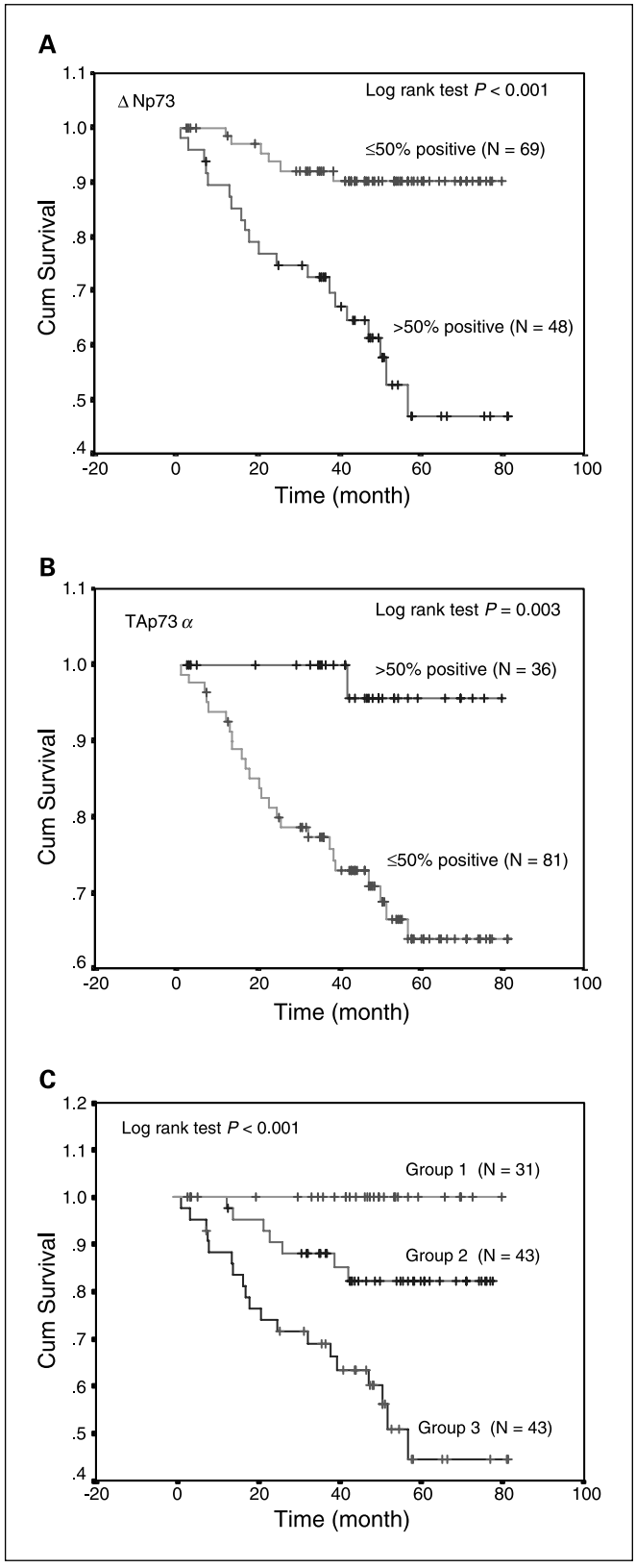

Fig. 2. Cumulative survival curves. Analyses of cervical SCC patient's survival with the expression of ΔNp73 (*A*) or TAp73α (*B*). The expression level was stratified by ≤50% or >50% of positive statining. *C*, survival analysis among patients stratified into three groups: group 1, TAp73α expression >50% and ΔNp73 expression ≤50%; group 2, case not fit into group 1 or 3; group 3, TAp73α expression ≤50% and ΔNp73 expression >50%.

TAp73α and diminished expression of ΔNp73, cases with reduced expression of TAp73α and overexpression of ΔNp73, cases not fitted into either group. Survival analysis indicated that patients with overexpression of TAp73α and diminished expression of ΔNp73 had much better survival than patients of the other two groups (log-rank test, $P < 0.001$; Fig. 2C).

Survival and association analyses were done between the patients without and with recurrence of disease, including local and distant recurrence. In our SCC cases, 20 of them had recurrent disease in distant metastasis at lymph nodes, pelvic, liver, lung, brain, and bone; four cases had local recurrence at parametrium, vagina, and vaginal vault; the rest of the cases were not found to have disease recurrence at the point when this study completed. The results showed that patients with recurrence of disease had adverse outcome (χ^2 test, $P < 0.001$; Fig. 3A). The expression of ΔNp73 was found significantly higher in patients with recurrence of disease (17 of 24, 70.8%) than in patients without recurrence (31 of 93, 33.3%; Fisher's exact test, $P = 0.001$; odds ratio, 4.857; 95% confidence interval, 1.823-12.943), whereas the expression of TAp73α did not correlate with the disease recurrence (Fisher's exact test, $P = 0.136$; Table 2).

Besides the expressions of ΔNp73 and TAp73α, the other two variables (disease stage and tumor radiosensitivity) were also significantly associated with patient's survival by univariate analysis (χ^2 test, $P = 0.001$ and $P = 0.036$, respectively; Fig. 3B and C). Furthermore, multivariate analysis showed that the disease stages and ΔNp73 and TAp73α expressions were independently correlated with the survival of the patients undergone radiotherapeutic treatment (Cox regression model, $P = 0.005$, $P = 0.012$, and $P = 0.018$ for disease stage and ΔNp73 and TAp73α expressions, respectively; Table 3) and indicated that these factors were important determinants in the survival of cervical cancer patients.

Discussion

The *p73* gene has a complex genomic structure, which accounts for the expression of different isoforms. The transactivating full-length TAp73 shows proapoptotic effects, whereas the NH_2 terminally truncated ΔNp73 has an evident antiapoptotic function (19). Although the *p73* gene is rarely mutated or deleted in human cancers, recent studies have suggested that the disruption of the balance between TAp73 and ΔNp73 protein stability may be of importance in tumorigenesis and in resistance to chemotherapy than direct gene mutation (16, 17). An increased expression of p73 has been suggested in different types of human malignancies when compared with the corresponding normal tissues, but the status of the different p73 isoforms in tumors remains an open question due to the complicated genomic organization of this gene. We previously showed that the TAp73α expression was significantly increased in cervical cancers, and the expression predicted a better prognosis in patients with radiotherapy (1). In the present study, we further investigated the ΔNp73 expression in cervical cancers and its clinical correlation in comparison with the expression of TAp73α.

We found that both ΔNp73 and TAp73α isoforms were overexpressed in cervical SCC but less frequently detected in normal cervical epithelium. However, the expressions of these two isoforms were inversely correlated, indicating that their expressions might be up-regulated differently in SCC. Considering the different functions of these two p73 isoforms, up-regulation of ΔNp73 and TAp73α might promote the oncogenic or proapoptotic activity, respectively, in the

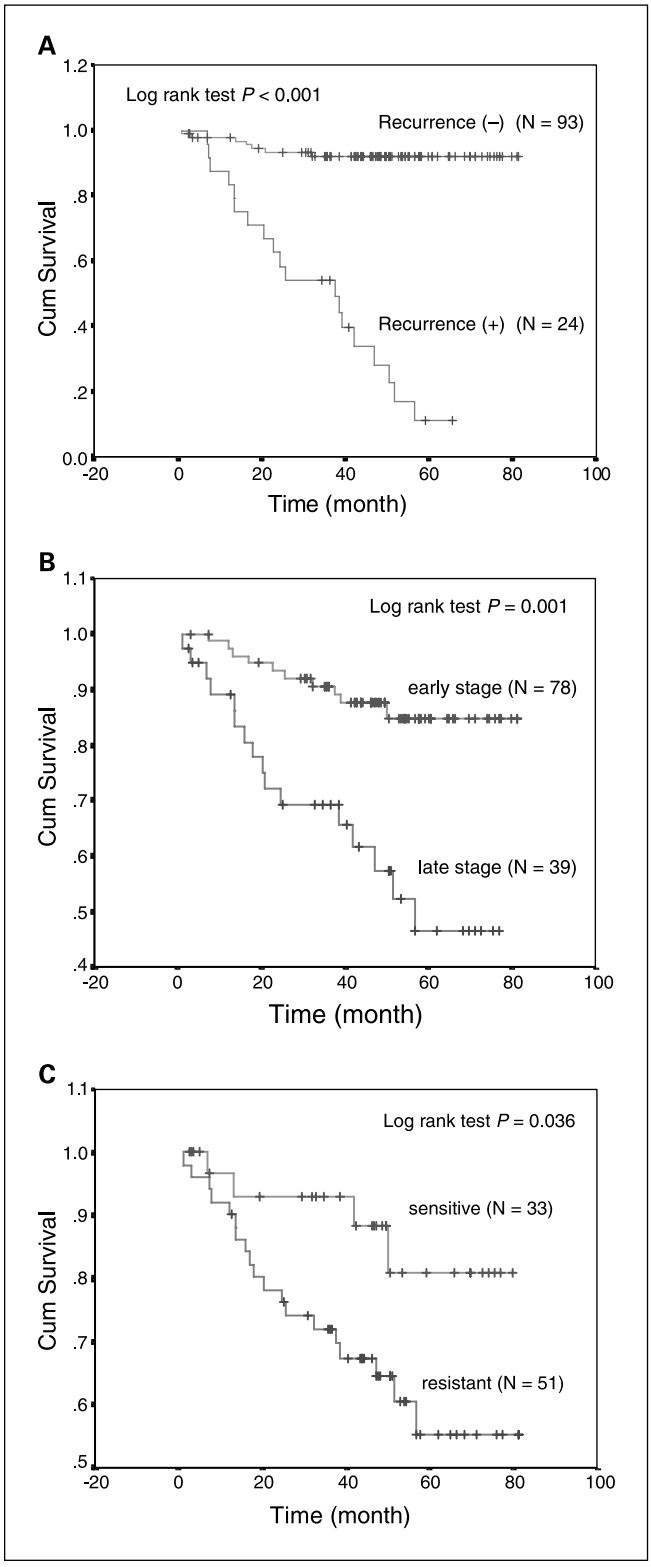

Fig. 3. Survival analyses in relation to the disease recurrence (*A*), stages (*B*), and radiosensitivity (*C*) of cervical SCC patients.

Table 3. Multivariate survival analyses of various prognostic factors

Variable	Characteristics		Odds ratio (95% confidence interval)	*P*
	Unfavorable	Favorable		
ΔNp73	Positive >50%	Positive ≤50%	4.676 (1.399-15.630)	0.012
TAp73α	Positive ≤50%	Positive >50%	0.065 (0.007-0.626)	0.018
Stage	Late	Early	3.540 (1.452-8.630)	0.005
Radiosensitivity	Resistant	Sensitive	0.345 (0.091-1.306)	0.117

pathogenesis of cervical SCC. Although the expressions of two isoforms were found correlated with the SCC radiosensitivity, overexpression of ΔNp73 was detected mainly in radioresistant cases (70.6%), whereas increased TAp73α expression was found in majority of the radiosensitive cases (75.8%). The latter was consistent with our previous published findings (1). Furthermore, overexpression of ΔNp73 was also a potential indicator in predicting the risk of the disease recurrence, as it was observed in majority of the patients with recurrence of disease (70.8%). The survival analysis results showed that both isoforms had different prognostic significance. The ΔNp73 expression was associated with an adverse outcome, whereas the TAp73α expression predicted a better survival of cervical cancer patients. The prognostic significance of both p73 isoforms remained even after adjustment for the clinical and pathologic variables. Our results indicated that the presence of two distinct protein isoforms (ΔNp73 and TAp73α) was crucial in cervical SCC, and the role of p73 in tumor progression and prognosis could be related to the interplay of the ΔNp73 and TAp73α isoforms.

Increased expression levels of p73 have been frequent observed in many other human tumors. In some studies, overexpression of p73 was even correlated with an advanced tumor stage or poor prognostic variables (20). However, most of previous studies measured the total levels of p73 expression. Up-regulation of ΔNp73 isoforms might thus contribute to the elevated "p73" levels found in those studies. Until recently, only a few studies specifically measured the TAp73 and/or ΔNp73 in a limited number of tumors (13–16, 21, 22). In the present study, we used two specific antibodies to identify the distinct isoforms of ΔNp73 and TAp73α expressions in cervical SCC. We showed that the functional status of ΔNp73 and TAp73α might be important determinants of cellular response to radiotherapy and prognosis. Although the expression of ΔNp73 conferred the radioresistance and the risk of disease recurrence, the TAp73α protein synergized with radiotherapeutic treatment. This implied that the natural, or pharmacologically regulated, relative balance of these two isoforms might influence the clinical outcome. This was also an important and clinically relevant finding, which suggested the use of the status of two isoforms in evaluating the treatment regimens and as prognostic markers to predict the outcome of the patients with cervical SCC.

Our results suggested that interfering the expression or function of ΔNp73 and TAp73α might render cancer cells more responsive to the radiotherapy and reduce their aggressiveness and metastatic capacity. The study of the differential p73 isoform status may help us to understand the molecular links among apoptosis, tumorigenesis, and the treatment resistance and hence provide the foundation to tailor the therapeutic approaches for cancer therapy. Further investigations should focus on the functions of these distinct p73 isoforms in cervical tumorigenesis and the mechanism in regulating the cellular radiation response.

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