# Cytoplasmic expression of p33$^{ING1b}$ is correlated with tumorigenesis and progression of human esophageal squamous cell carcinoma

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**Abstract.** p33$^{ING1b}$, a newly discovered candidate tumor suppressor gene and a nuclear protein, belongs to the inhibitor of growth gene family. Previous studies have shown that p33$^{ING1b}$ is involved in the restriction of cell growth and proliferation, apoptosis, tumor anchorage-independent growth, cellular senescence, maintenance of genomic stability and modulation of cell cycle checkpoints. Loss of nuclear p33$^{ING1b}$ has been observed in melanoma, seminoma, papillary thyroid carcinoma, oral squamous cell carcinoma, breast ductal cancer and acute lymphoblastic leukemia. Inactivation and/or decreased expression of p33$^{ING1b}$ have been reported in various types of cancer, including head and neck squamous cell, breast, lung, stomach, blood and brain malignancies. Since little is known about the clinicopathological significance of p33$^{ING1b}$ in esophageal squamous cell carcinoma (ESCC), this study aimed to investigate the association of p33$^{ING1b}$ expression with clinicopathological variables and particularly interesting new cysteine-histidine rich protein (PINCH) in patients with ESCC. p33$^{ING1b}$ expression was examined by immunohistochemistry in 20 normal esophageal mucosa and in 64 ESCC specimens. The results revealed that the positive expression of p33$^{ING1b}$ protein in normal squamous cells was localized in the nucleus alone and the positive rate was 95%, while in ESCCs, the positive expression was mainly in the cytoplasm, together with nuclear expression, and the positive rate was 36% (P<0.0001). Furthermore, the cases with lymph node metastasis showed a higher frequency of positive cytoplasmic expression than those without metastasis (P=0.001). The cytoplasmic expression of p33$^{ING1b}$ was positively related to PINCH expression (P<0.0001) in ESCC, and the cases positive for both proteins had a high lymph node metastasis rate (P=0.001). In conclusion, p33$^{ING1b}$ cellular compartmental shift from the nucleus to the cytoplasm may cause loss of normal cellular function and play a central role in the tumorigenesis and metastasis of ESCC.

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## Introduction

Esophageal cancer ranks among the 10 most common types of cancer in the world. The vast majority of the tumors are squamous cell carcinomas. To date, surgical resection remains the first treatment. However, nearly 95% of surgically resected patients with advanced esophageal cancer succumb to recurrent or metastatic disease within 5 years (1). Accordingly, it is necessary to investigate the mechanism of tumorigenesis and metastasis of esophageal squamous cell carcinoma (ESCC).

Previous studies have revealed that oncogenes and tumor suppressor genes are implicated in tumorigenesis. Inactivation, by loss or mutation, of tumor suppressor genes is important in the genesis of many tumors. Tumor suppressor proteins negatively regulate cell growth through a variety of mechanisms controlling the cell cycle. The inhibitor of growth (ING) gene family is newly recognised to be a part of this evolutionarily old family of putative tumor suppressor genes. The currently identified members of this family are the ING1, ING2 (ING1-L), ING3, ING4 and ING5 genes. ING1, the first member of this family, was discovered through a subtractive hybridization assay between normal mammary epithelium and breast cancer cell lines and was shown to play an essential role in neoplastic transformation (2-6). ING1 has been mapped to

a locus on chromsome 13q33-34 and encodes four isoforms, p47$^{ING1a}$, p33$^{ING1b}$, p24$^{ING1c}$ and p27$^{ING1d}$, which vary in mass between 24 and 47 kDa (2,7). The p33$^{ING1b}$ protein is the best characterized and most widely expressed in normal tissue (8). Previous studies have shown that p33$^{ING1b}$ is involved in the restriction of cell growth and proliferation, apoptosis, tumor anchorage-independent growth, cellular senescence, maintenance of genomic stability and modulation of cell cycle checkpoints (9). A number of studies have been carried out on altered p33$^{ING1b}$ in relation to tumors. Loss of nuclear p33$^{ING1b}$ has been observed in melanoma, seminoma, papillary thyroid carcinoma, oral squamous cell carcinoma, breast ductal cancer and acute lymphoblastic leukemia (10,11). To date, inactivation and/or decreased expression of p33$^{ING1b}$ have been reported in various types of cancer, including head and neck squamous cell, breast, lung, stomach, blood and brain malignancies (7,12-16). To the best of our knowledge, although there are a few studies of p33$^{ING1b}$ in ESCC, little is known about its clinicopathological significance in ESCC.

Particularly interesting new cysteine-histidine rich protein (PINCH) is a newly discovered adapter protein, which consists primarly of five LIM (double zinc finger) domains, and the gene is located on chromosome 2q12.2. PINCH protein is able to interact directly with integrin-linked kinase (ILK) and Nck-2 protein, and is associated with integrin signaling and the growth factor signaling pathway (17-19). It has been observed that PINCH expression is upregulated in numerous types of malignancy, including oral and esophageal squamous cell carcinoma, colorectal, pancreatic, skin, breast, lung, prostate cancer and endometrioid endometrial carcinoma, as well as gliomas (20-27). PINCH localizes to the peritumoral stromal cells, particularly at the invasive edges of the tumor (20). Furthermore, PINCH is an independent prognostic factor in patients with colorectal cancer (21). Our previous study on the same series of cases used in the present study demonstrated that PINCH expression was upregulated in ESCC compared with normal esophageal squamous cells and the strong expression of PINCH was correlated with lymph node metastasis (26). Recent studies have shown that the genesis and metastasis of tumors are the result of the interaction between tumor cells and tumor-associated stromal cells (28). Therefore, it is of significance to explore whether there is a correlation between p33$^{ING1b}$ expression in tumor cells and PINCH expression in the stromal cells in human ESCC.

The aim of the present study was to investigate p33$^{ING1b}$ expression in ESCC compared with normal esophageal mucosa, and further to analyze the correlation between p33$^{ING1b}$ expression in ESCC and clinicopathological variables, including gender, age, tumor size, location, lymph node status and the grade of differentiation, as well as PINCH expression status.

## Patients and methods

*Patients*. Formalin-fixed paraffin-embedded tissue samples were obtained from 64 ESCC patients who underwent surgical resection at the First Hospital of Hebei Medical University (Shijiazhuang, Hebei, China), between 2000 and 2004. The study included 20 distant normal mucosa specimens (all of which were matched with the primary tumors) taken from the margin of distant resection. The primary tumors were located in the upper, middle and lower sections of the esophagus in 7, 36 and 21 cases, respectively, and 20 cases involved lymph node metastasis. None of the patients had received preoperative radiotherapy or chemotherapy. The patients' gender, age, tumor size, location, lymph node status and the grade of differentiation were obtained from surgical and/or pathological records at the hospital. The mean age of the patients was 59.5 years old (range 41-78 years). According to the WHO classification, the tumor differentiation was graded as grade I (high differentiation: 20 cases), grade II (moderate differentiation: 39 cases) and grade III (low differentiation: 5 cases). All pathological slides, including normal specimens and tumors, were confirmed by two pathologists (Z.L. Zhu and Z.M. Wang). The study was approved by the ethical committee of the First Hospital of Hebei Medical University, Shijiazhuang, Hebei, China. Written informed consent was obtained from the patients.

Data of PINCH immunohistological staining in ESCC were obtained from our previous study carried out at the Central laboratory, The First Hospital of Hebei Medical University. According to the intensity of PINCH staining in the tumor-associated stromal cells, PINCH expression was graded as negative group (none or <20% positive cells) and positive group (≥20% positive cells) (26).

*Immunohistological staining and evaluation*. Tissue sections (5 $\mu$m) from paraffin-embedded tissue blocks were deparaffinised, hydrated and rinsed in distilled $H_2O$. In order to expose masked epitopes, the sections were boiled in citrate buffer (pH 9.0) in a high pressure cooker for 20 min, and then kept at room temperature for 30 min prior to washing with phosphate-buffered saline (PBS, pH 7.4). The activity of endogenous peroxidase was blocked with 3% $H_2O_2$ in methanol for 10 min and then the sections were washed three times in PBS. After blocking with 1.5% horse serum in PBS for 10 min, the sections were incubated with a goat polyclonal p33ING1 antibody raised against a peptide mapping at the C-terminal of p33ING1 of human origin (C-19, sc-7566; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:100 dilution at 4˚C overnight. A biotinylated secondary antibody (Fuzhou Maixim Biology Technology Co., Ltd., Fuzhou, Fujian, China) was then applied for 30 min followed by incubation with an avidin-biotin-peroxidase complex (Fuzhou Maxim Biotechnology Co., Ltd.) for 30 min. The sections were rinsed in PBS between the incubation steps. The peroxidase reaction was developed using diaminobenzidine (Beijing Zhongshan Biotechnology Co., Ltd, Beijing, China) for 8 min. Following counterstaining with hematoxylin, the sections were dehydrated and mounted. Sections of ESCCs known to stain positively for p33$^{ING1b}$ were included as negative (using PBS instead of the primary antibody) and positive controls in all runs. There was no staining in the negative controls, while the positive controls showed clear staining.

p33$^{ING1b}$ immunohistological staining was evaluated by two independent pathologists (Z.L. Zhu and Z.M. Wang) in a blind fashion without knowledge of any clinicopathological information. In normal squamous cells, only nuclear staining was observed, while in tumors, cytoplasmic staining alone or staining in the nucleus and cytoplasm were observed. According to the rate of positive staining, we graded p33$^{ING1b}$

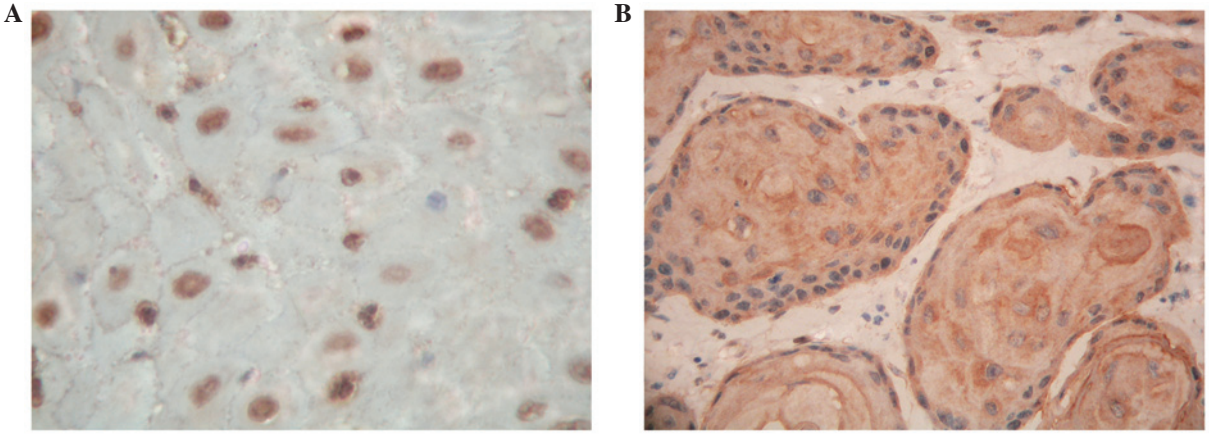


Figure 1. Positive p33ING1b immunohistochemical staining (A) in the nuclei of cells in normal esophageal mucosa and (B) mainly in the cytoplasm of esophageal squamous cell carcinoma (ESCC).

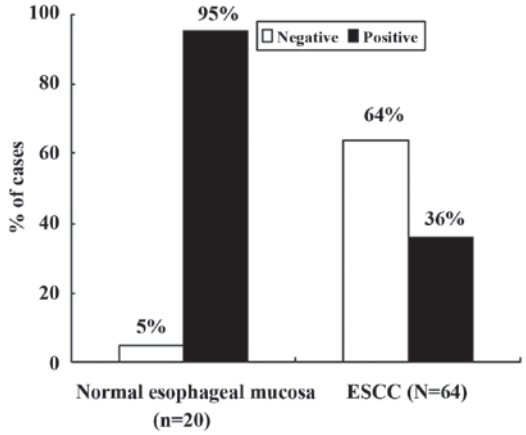


Figure 2. Frequency of p33ING1b immunohistochemical staining in normal esophageal mucosa and esophageal squamous cell carcinoma (ESCC).

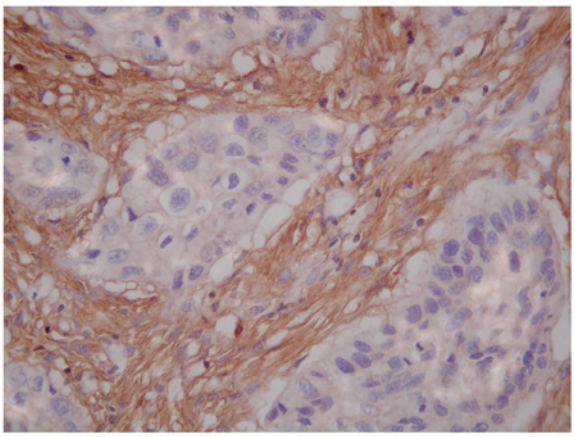

Figure 3. Expression of particularly interesting new cysteine-histidine rich protein (PINCH) was positive in the stromal cells of esophageal squamous cell carcinoma (ESCC).

expression as negative (no positive cells or <5% positive cells), weak (5-25% positive cells), moderate (26-50% positive cells) and strong positive (>50% positive cells). In statistical analysis, taking into account similar clinicopathological features and facilitating statistical analysis, we considered negative as the negative staining group, and weak, moderate and strong positive as the positive staining group. In order to avoid artificial effects, cells on the margins of sections and in areas with poorly presented morphology were not counted.

*Statistical analysis*. The statistical analyses were performed using SPSS version 13.0 software. The Chi-square test was used to examine the correlation between the frequencies of $p33^{ING1b}$ expression in normal esophageal mucosa and ESCC, and the correlation between $p33^{ING1b}$ expression in cancer and clinicopathological variables or PINCH expression. All P-values cited were two-sided and P<5% was considered to indicate a statistically significant difference.

## Results

*$p33^{ING1b}$ expression in normal mucosa and primary tumor*. We examined $p33^{ING1b}$ protein expression in normal esophageal mucosa and ESCC. In the 20 specimens of normal mucosa, we found that the expression of $p33^{ING1b}$ was only present in the nuclei of epithelial cells and there was no cytoplasmic staining (Fig. 1A). Of the specimens, 1 case was negative (5%) and 19 cases were positive (95%), including 3 (15%) weak, 5 (25%) moderate and 11 (55%) strong staining. However, in the primary cancers, none of the tumors exhibited nuclear staining alone. There were 41 cases negative and 23 positive for $p33^{ING1b}$, including 5 (8%) weak, 6 (9%) moderate and 12 (19%) strong staining cases. Among the 23 positive cases, 20 cases showed nuclear and cytoplasmic staining, mainly in the cytoplasm (Fig. 1B) and 3 cases had cytoplasmic staining alone.

As shown in Fig. 2, which presents the frequency of $p33^{ING1b}$ expression in normal mucosa and ESCC, the rate of positive expression in the normal mucosa specimens was 95% (19/20), which was significantly higher than that in the ESCC specimens (36%, 23/64; $\chi^2$=21.263, P<0.0001). We further compared nuclear and cytoplasmic staining separately between the normal mucosa and ESCC specimens; the results showed that the frequency of positive $p33^{ING1b}$ expression in the nucleus (95% vs. 31%; $\chi^2$=24.898, P=0.000) and in the

Table I. Correlation of p33$^{ING1b}$ protein expression with clinicopathological and biological variables in patients with ESCC.

| Variables | N | p33$^{ING1b}$ expression | | $\chi^2$ | P-value |
|---|---|---|---|---|---|
| | | Negative (%) | Positive (%) | | |
| Gender | | | | | |
| Male | 50 | 32 (64) | 18 (36) | 0.000 | 0.984 |
| Female | 14 | 9 (64) | 5 (36) | | |
| Age (years) | | | | | |
| ≤50 | 19 | 13 (68) | 6 (32) | 0.223 | 0.637 |
| >50 | 45 | 28 (62) | 17 (38) | | |
| Tumor size (cm) | | | | | |
| ≤3 | 26 | 17 (65) | 9 (35) | 0.033 | 0.855 |
| >3 | 38 | 24 (63) | 14 (37) | | |
| Location | | | | | |
| Upper | 7 | 5 (71) | 2 (29) | 0.208 | 0.901 |
| Middle | 36 | 23 (64) | 13 (36) | | |
| Lower | 21 | 13 (62) | 8 (38) | | |
| Lymph node status | | | | | |
| Non-metastasis | 44 | 34 (77) | 10 (23) | 10.673 | 0.001 |
| Metastasis | 20 | 7 (35) | 13 (65) | | |
| Grade | | | | | |
| I | 20 | 12 (60) | 8 (40) | 1.853 | 0.396 |
| II | 39 | 27 (69) | 12 (31) | | |
| III | 5 | 2 (40) | 3 (60) | | |
| PINCH | | | | | |
| Negative | 28 | 26 (93) | 2 (7) | 17.927 | <0.0001 |
| Positive | 36 | 15 (42) | 21 (58) | | |

ESCC, esophageal squamous cell carcinoma; PINCH, particularly interesting new cysteine-histidine rich protein.

cytoplasm (0 vs. 36%; $\chi^2$=9.898, P=0.002) was significantly different.

Furthermore, we also observed the expression of p33$^{ING1b}$ at the invasive margin and the inner part of the tumor in all 64 ESCCs; there was no obvious difference between the two sites.

*p33$^{ING1b}$ protein expression in relation to clinicopathological variables and PINCH expression in ESCCs*. Cytoplasmic staining of p33$^{ING1b}$ occurred only in cancers and also dominated over nuclear staining, although nuclear staining appeared in the majority of the cases with cytoplasmic expression. For further statistical analysis, regardless of nuclear staining, we investigated only p33$^{ING1b}$ cytoplasmic staining (23 cases of positive staining) in relation to clinicopathological variables (Table I).

As shown in Table I, the cases with lymph node metastasis had a higher frequency of p33$^{ING1b}$ positive expression than those without metastasis in the lymph nodes (65% vs. 23%; $\chi^2$=10.673, P=0.001). p33$^{ING1b}$ expression was not significantly correlated with gender (P=0.984), age (P=0.637), tumor size (P=0.855), tumor location (P=0.901) or grade of differentiation (P=0.396).

The results also revealed that p33$^{ING1b}$ expression was positively related to the PINCH expression (Fig. 3) in all 64 ESCCs (Table I). Of the 36 cases with PINCH-positive expression, 21 (58%) cases were p33$^{ING1b}$ positive and 15 (42%) cases were p33$^{ING1b}$ negative. However, in the 28 cases with PINCH-negative expression, there were 2 (7%) cases of p33$^{ING1b}$ positive and 26 (93%) cases of p33$^{ING1b}$ negative ($\chi^2$=17.927, P=0.000). Moreover, we found that the cases positive for both proteins had the highest frequency of lymph node metastasis (13/20, 65%), the cases negative for both proteins had the lowest frequency of metastasis (2/20, 10%) and cases positive for either protein had a moderate frequency (5/20, 25%; $\chi^2$=14.550, P=0.001).

## Discussion

Studies have shown that the evolution and development of ESCC results from multiple stepwise alterations of cellular and molecular pathways in the squamous cells (1). Genetic changes may cause some individuals to be more sensitive to these environmental factors, although lifestyle factors account for the majority of ESCCs. The activation of oncogenes and inactivation of tumor suppressor genes (TSGs) are implicated in tumorigenesis. Tumor suppressor genes are often referred to as 'gatekeepers' as they are able to prevent tumor genesis and development by direct control of the cell cycle. The ING gene family is a newly discovered TSG class. The currently identified members of this family are the ING1, ING2, ING3,

ING4 and ING5 genes. ING1 is the first member of the ING family, has been mapped to a locus on chromsome 13q33-34 and encodes four isoforms, $p47^{ING1a}$, $p33^{ING1b}$, $p24^{ING1c}$ and $p27^{ING1d}$. Currently, $p33^{ING1b}$ is the most widely studied in malignancies and is a focus of medical studies (2-7). Nouman *et al* studied 76 melanocytic lesions by immunohistochemistry for the expression of $p33^{ING1b}$ and identified that there was a loss of nuclear $p33^{ING1b}$ expression in invasive malignant melanoma compared with normal cutaneous melanocytes or the melanocytes of benign melanocytic naevi, and enhancement of cytoplasmic $p33^{ING1b}$ expression in invasive malignant melanoma (29). In another study, Hoque *et al* examined the mRNA expression of $p33^{ING1b}$ by reverse transcription-PCR of 28 oral squamous cell cancers and found 2 (7%) tumors with loss of $p33^{ING1}$ expression (30). Thereafter, our research group explored 49 oral squamous cell carcinoma specimens for $p33^{ING1b}$ expression by immunohistochemistry and found that 37 (76%) of the primary tumors were negative for $p33^{ING1b}$ expression although the majority (90%) of normal mucosa specimens showed $p33^{ING1b}$-positive expression in the nucleus (11). Recently, Luo *et al* also identified that $p33^{ING1b}$ expression was lost in the nucleus in 115 of 217 cases of human non-small cell lung cancer (31). In the present study, we used immunohistological staining and observed that, in 20 cases of normal mucosa, $p33^{ING1b}$ expression was only present in the nuclei of the epithelial cells and 19 (95%) cases were positive for $p33^{ING1b}$ (including 11 cases of strong staining). By contrast, in 64 primary tumor samples, none of the cancers showed nuclear staining alone and 41 (64%) cases had negative $p33^{ING1b}$ expression; this was significant difference (95 vs. 36%; $\chi^2$=21.263, P=0.000).

Results from previous studies have shown that $p33^{ING1b}$, as a candidate type II TSG, is involved in a variety of processes, including DNA repair, cell cycle control, senescence, apoptosis and chromatin remodeling, which are critical points for genomic integrity and stability (9). $p33^{ING1b}$ gene and TP53 products are interrelated and the optimum functioning of both is required for efficient cell growth suppression. Moreover, the tumor suppression of TP53 and the transactivation activity of WAF1 are partially dependent upon the fidelity and activity of $p33^{ING1b}$. Thus, the loss of $p33^{ING1b}$ function may have similar consequences to loss of TP53 function and may contribute to tumorgenesis by augmenting genomic instability and refractivity to pro-apoptotic stimuli (9,32). The observation by other groups of loss of $p33^{ING1b}$ expression in tumors and our results in the present study, indicate that the loss of $p33^{ING1b}$ nuclear expression in tumors may be a key point in tumorigenesis.

Notably, in the present study, we also observed that 23 (36%) tumor samples had cytoplasmic expression of $p33^{ING1b}$, including 20 cases with nuclear and cytoplasmic staining and 3 cases with cytoplasmic staining alone. From these results, a doubt may be raised as to whether the $p33^{ING1b}$ cytoplasmic expression was specific or background staining. In order to clarify this issue, we re-observed the staining results of all sections and confirmed the specificity of the cytoplasmic staining of $p33^{ING1b}$ for the following reasons: firstly, the negative controls did not show any cytoplasmic staining; and secondly, there was no cytoplasmic staining in the normal epithelial cells. Furthermore, this evidence has been confirmed in certain tumors, including melanoma (10), brain tumor (15), breast cancer (7), oral squamous cell carcinoma (11) and acute lymphoblastic leukemia (14), where $p33^{ING1b}$ was also found to localize mainly in the cytoplasm. In addition, we also identified that the cases with lymph node metastasis had a higher frequency of positive $p33^{ING1b}$ expression in the cytoplasm than those without metastasis (65% vs. 23%; $\chi^2$=10.673, P=0.001). This result suggests a role for $p33^{ING1b}$ cytoplasmic expression in promoting metastasis of the ESCCs. Therefore, from the results of the present study and other studies, the $p33^{ING1b}$ cellular compartment shift from the nucleus to the cytoplasm may cause loss of normal cellular function and play a central role in tumorigenesis and progression.

However, the mechanism behind this shift of $p33^{ING1b}$ protein from the nucleus to the cytoplasm is not fully understood. Riabowol's research group has reported that $p33^{ING1b}$ particularly binds to members of the 14-3-3 family through phosphorylation at serine residue 199 (33). Studies revealed that 14-3-3 family members primarily reside in the cytoplasm and are associated with phosphorylated ligands involved in numerous cellular processes, including regulation of the cell cycle and DNA damage checkpoints. Binding to 14-3-3 causes tethering of significant amounts of $p33^{ING1b}$ in the cytoplasm (33,34). Moreover, other studies have demonstrated that cytoplasmic $p33^{ING1b}$ may be imported into the nucleus through interactions between its intrinsin nuclear location signal and karyopherins $\alpha$2 and $\beta$1. In the nucleus, lamin A binds and targets ING1 and regulates its levels and biological function (35,36). Therefore, 14-3-3, karyopherins $\alpha$2 and $\beta$1, and lamin A are involved in the cytoplasmic accumulation of $p33^{ING1b}$ in tumors. However, the function of cytoplasmic $p33^{ING1b}$ is unclear and requires further study.

There have been a few studies on the correlation between $p33^{ING1b}$ expression and clinicopathological variables. Li *et al* found that high expression of cytoplasmic $p33^{ING1b}$ was significantly correlated with poor differentiation, T staging, lymph node metastasis and TNM staging in head and neck squamous cell carcinoma (37). In the present study, we also observed that high cytoplasmic expression of $p33^{ING1b}$ was significantly correlated with lymph node metastasis, but no significant correlation was found between cytoplasmic expression of $p33^{ING1b}$ and other clinicopathological variables, including gender, age, tumor size, tumor location and the grade of differentiation.

In the present study, we also found that the cytoplasmic expression of $p33^{ING1b}$ had a positive correlation with PINCH expression in the primary tumors. More importantly, we further observed that cases positive for both proteins had the highest frequency of lymph node metastasis (65%), cases negative for both proteins had the lowest frequency of metastasis (10%) and cases positive for either protein had a moderate frequency (25%). The results suggest that $p33^{ING1b}$ and PINCH cooperate in the metastasis of ESCC. Taken together with the results of our previous study of $p33^{ING1b}$ expression in oral squamous cell carcinoma (11), we propose that, during tumor development and metastasis, $p33^{ING1b}$ in the tumor cells interacts with PINCH by a signaling pathway in the associated-tumor stroma, particularly at the site of cell adhesion. PINCH may be a marker for stroma manifesting the ability to facilitate metastasis in human ESCC. If so, $p33^{ING1b}$ and PINCH may be

considered as novel biomarkers for the target of therapy. Thus, it is necessary to further study this issue in a large number of samples to verify this result.

The results suggest that p33$^{ING1b}$ cellular compartment shift from the nucleus to the cytoplasm causes a loss of normal cellular function and may play a central role in the tumorigenesis and metastasis in human ESCC, particularly in combination with PINCH expression.

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