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Cytoplasmic expression of p33ING1b is correlated with tumorigenesis and progression of human esophageal squamous cell carcinoma

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Abstract. p33ING1b, a newly discovered candidate tumor suppressor gene and a nuclear protein, belongs to the inhibitor of growth gene family. Previous studies have shown that p33ING1b 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 p33ING1b 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 p33ING1b 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 p33ING1b in esophageal squamous cell carcinoma (ESCC), this study aimed to investigate the association of p33ING1b expression with clinicopathological variables and particularly interesting new cysteine-histidine rich protein (PINCH) in patients with ESCC. p33ING1b expression was examined by immunohistochemistry in 20 normal esophageal mucosa and in 64 ESCC specimens. The results revealed that the positive expression of p33ING1b 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 p33ING1b 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, p33ING1b 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.

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
which were matched with the primary tumors) taken from the study included 20 distant normal mucosa specimens (all of which were obtained from the Central laboratory, The First Hospital of Hebei Medical University. 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 µm) from paraffin-embedded tissue blocks were deparaffinised, hydrated and rinsed in distilled H₂O. 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% H₂O₂ 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. After 30 min of incubation with the primary antibody, the sections were washed in PBS and incubated with an avidin-biotin-peroxidase complex (Fuzhou Maxim Biology Technology Co., Ltd., Fuzhou, Fujian, China) at 1:100 dilution at 4°C overnight. A biotinylated secondary antibody (Fuzhou Maxim Biology Technology Co., Ltd., Fuzhou, Fujian, China) was then applied for 30 min followed by incubation with an avidin-biotin-peroxidase complex (Fuzhou Maxim Biology Technology 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 5 min. Sections were counterstained with hematoxylin, dehydrated and mounted. Sections of ESCCs known to stain positively for p33ING1 were used 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.

**p33ING1 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.
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 p33ING1b expression in normal esophageal mucosa and ESCC, and the correlation between p33ING1b 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

p33ING1b expression in normal mucosa and primary tumor. We examined p33ING1b protein expression in normal esophageal mucosa and ESCC. In the 20 specimens of normal mucosa, we found that the expression of p33ING1b 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 p33ING1b, 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 p33ING1b 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 p33ING1b expression in the nucleus (95% vs. 31%; \( \chi^2 = 24.898, \ P = 0.000 \)) and in the

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

Furthermore, we also observed the expression of p33ING1b at the invasive margin and the inner part of the tumor in all 64 ESCCs; there was no obvious difference between the two sites.

Table I. Correlation of p33ING1b protein expression with clinicopathological and biological variables in patients with ESCC.

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>$\chi^2$</th>
<th>P-value</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>50</td>
<td>32 (64)</td>
<td>18 (36)</td>
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<td>0.984</td>
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<tr>
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<td>14</td>
<td>9 (64)</td>
<td>5 (36)</td>
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<td></td>
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<tr>
<td>Age (years)</td>
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<td></td>
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<tr>
<td>≤50</td>
<td>19</td>
<td>13 (68)</td>
<td>6 (32)</td>
<td>0.223</td>
<td>0.637</td>
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<tr>
<td>&gt;50</td>
<td>45</td>
<td>28 (62)</td>
<td>17 (38)</td>
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<td>Tumor size (cm)</td>
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<td>≤3</td>
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<td>17 (65)</td>
<td>9 (35)</td>
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<td>0.855</td>
</tr>
<tr>
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<td>38</td>
<td>24 (63)</td>
<td>14 (37)</td>
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<tr>
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<td>2 (29)</td>
<td>0.208</td>
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<tr>
<td>Middle</td>
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<tr>
<td>Lower</td>
<td>21</td>
<td>13 (62)</td>
<td>8 (38)</td>
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<td>Lymph node status</td>
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<td>34 (77)</td>
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<td>Grade</td>
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<tr>
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<td>27 (69)</td>
<td>12 (31)</td>
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<td></td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>2 (40)</td>
<td>3 (60)</td>
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<td></td>
</tr>
<tr>
<td>PINCH</td>
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<td></td>
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</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>26 (93)</td>
<td>2 (7)</td>
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<tr>
<td>Positive</td>
<td>36</td>
<td>15 (42)</td>
<td>21 (58)</td>
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</table>

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

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. INGI is the first member of the ING family, has been mapped to a locus on chromosome 13q33-34 and encodes four isoforms, p47ING1, p33ING1b, p24ING1b, and p27ING1d. Currently, p33ING1b 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 p33ING1b and identified that there was a loss of nuclear p33ING1b expression in invasive malignant melanoma compared with normal cutaneous melanocytes or the melanocytes of benign melanocytic naevi, and enhancement of cytoplasmic p33ING1b expression in invasive malignant melanoma (29). In another study, Hoque et al examined the mRNA expression of p33ING1b by reverse transcription-PCR of 28 oral squamous cell cancers and found 2 (7%) tumors with loss of p33ING1 expression (30). Thereafter, our research group explored 49 oral squamous cell carcinoma specimens for p33ING1b expression by immunohistochemistry and found that 37 (76%) of the primary tumors were negative for p33ING1b expression although the majority (90%) of normal mucosa specimens showed p33ING1b-positive expression in the nucleus (11). Recently, Luo et al also identified that p33ING1b 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, p33ING1b expression was only present in the nuclei of the epithelial cells and 19 (95%) cases were positive for p33ING1b (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 p33ING1b expression; this was significant difference (95 vs. 36%; \( \chi^2 = 21.263, P = 0.000 \)).

Results from previous studies have shown that p33ING1b, 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 in tumorigenesis. Thus, the loss of p33ING1b function may have similar consequences to loss of TP53 function and may contribute to tumorigenesis by augmenting genomic instability and refractivity to pro-apoptotic stimuli (9,32). The observation by other groups of loss of p33ING1b expression in oral squamous cell carcinoma (37). In the present study, we also observed that high cytoplasmic expression of p33ING1b was significantly correlated with lymph node metastasis, but no significant correlation was found between cytoplasmic expression of p33ING1b and 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 p33ING1b 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 p33ING1b and PINCH cooperate in the metastasis of ESCC. Taken together with the results of our previous study of p33ING1b expression in oral squamous cell carcinoma (11), we propose that, during tumor development and metastasis, p33ING1b 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, p33ING1b 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\textsuperscript{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.

Acknowledgements

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