Human cutaneous malignant melanoma is a type of skin cancer resulting from uncontrolled proliferation of melanocytes in the epidermis. The incidence of melanoma is increasing most rapidly in the Caucasian population among all malignancies except for lung cancer in women (1, 2). Melanoma is a life-threatening disease due to its ability to metastasize rapidly to other organs and high resistance to conventional radiotherapy and chemotherapy (3, 4). Patients with metastatic melanoma have a poor prognosis with a median survival of <5% of the patients surviving >5 years (5–7). Therefore, understanding of the molecular events during the tumor initiation and progression will be helpful for prevention and treatment of melanoma.

The ING3 gene is mapped to 7q31.3, consists of 12 exons, and encodes a 46.8 kDa protein, which shares the conserved carboxyl-terminal plant homeodomain and a nuclear localization sequence with other ING family members (8). ING3 is ubiquitously expressed in normal human tissues and can modulate p33-mediated transcription, cell cycle control, and apoptosis in RKO cells (9). As a critical chromatin acetylation regulator, ING3 is predominantly present in the NuA4 histone acetyltransferase multisubunit complex and required for the histone acetyltransferase activity of Tip60 (10, 11). It is also reported to link the function of p53 to histone acetylation (11). The yeast homologue of ING3, yng2, is also an important component of yeast NuA4 complex and abrogation of yng2 function results in a deficient genome-wide nucleosomal histone H4 acetylation and an increased sensitization to DNA damage in S phase (12). In melanoma cells, we have previously shown that ING3 promotes UV-induced apoptosis via a Fas/caspase-8–dependent pathway, independently of functional p53 (13).

ING3 is significantly reduced in human head and neck squamous cell carcinomas and higher mortality has been observed in cases with decreased ING3 expression (14). In malignant melanoma, although a frequent copy number gain has been reported at the chromosome 7q31-q34 region that contains the ING3 gene with the comparative genomic hybridization analysis (15), ING3 expression level and the cytogenetic characteristics of the ING3 gene in melanoma has never been revealed. To investigate the role of ING3 in the...
development of melanoma, we used tissue microarray (TMA) technology and immunohistochemistry to evaluate ING3 expression in different stages of human melanocytic lesions and analyzed the correlation between ING3 expression and clinicopathologic variables and patient survival.

Materials and Methods

Construction of tissue microarray. Formalin-fixed, paraffin-embedded tissues from 58 dysplastic nevi, 114 primary melanomas, and 50 metastatic melanomas were used for this study. All specimens were obtained from the 1990 to 1998 archives of the Department of Pathology, Vancouver General Hospital. The use of human skin tissues was approved by the medical ethical committee of the University of British Columbia and was done in accordance with the Declaration of Helsinki guidelines. The most representative tumor area was carefully selected and marked on the H&E-stained slides. The TMAs were constructed as previously described with duplicate 0.6-mm-thick tissue cores taken from each biopsy specimen (16, 17). Three composite high-density TMA blocks containing 107, 126, and 111 cases from a total of 344 patients were designed. Multiple 4-μm sections were cut with a Leica microtome and transferred to adhesive-coated slides.

Immunohistochemistry. TMA slides were dewaxed at 55°C for 30 min and washed with xylene. Tissues were rehydrated by a series of washes in 100%, 95%, and 80% ethanol, and distilled water. Antigen retrieval was done by heating the samples at 95°C for 30 min in 10 mmol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. After blocking with universal blocking serum for 30 min, slides were incubated with a polyclonal rabbit anti-ING3 antibody (Protein Tech Group) at 4°C overnight. The sections were then incubated with biotin-labeled secondary antibody and streptavidin-peroxidase for 30 min each, followed by developing with 3,3′-diaminobenzidine substrate and counterstained with hematoxylin. Slides were finally dehydrated and sealed with coverslips. As negative controls, the ING3 antibody was omitted during primary antibody incubation.

Evaluation of immunostaining. The evaluation of ING3 nuclear and cytoplasmic expression was made blinded by two independent observers (including one dermatopathologist) simultaneously, and a consensus score was reached for each core. The nuclear or cytoplasmic ING3 staining was scored into four grades according to the following staining intensities: 0, 1+, 2+, and 3+. Percentages of nuclear or cytoplasmic ING3-positive cells were also scored into four categories: 0 (0%), 1 (1-33%), 2 (34-66%), and 3 (67-100%). In the cases with a discrepancy between duplicated cores, the higher score from the duplicate tissue cores was taken as the final score. The sum of the intensity and percentage scores was used as the final ING3 nuclear or cytoplasmic staining score (17), defined as follows: 0 to 2, negative or weak; 3 to 4, moderate; and 5 to 6, strong.

Statistical analysis. Statistical analysis was done with the SPSS 11.5 software. The χ² test was used to compare the quantitative differences of nuclear or cytoplasmic ING3 expression in different stages of melanoma progression. The Spearman test was used to analyze the correlation between nuclear and cytoplasmic ING3 expression and the correlation between ING3 expression and the clinicopathologic variables, including age, gender, tumor thickness, ulceration, and tumor location. The Kaplan-Meier method and log-rank test were used to evaluate the correlations between nuclear ING3 staining and patient survival. Cox regression model was used for multivariate analysis. P < 0.05 is considered significant.

Table 1. Clinicopathologic features of 114 primary melanomas

<table>
<thead>
<tr>
<th>No. patients (%)</th>
<th>Age, y</th>
<th>Gender</th>
<th>Tumor thickness, mm</th>
<th>Ulceration</th>
<th>Tumor subtype</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤56</td>
<td>Male</td>
<td>&lt;2.0</td>
<td>Absent</td>
<td>SSM</td>
<td>Sun exposed</td>
</tr>
<tr>
<td>No. patients (%)</td>
<td>56 (49)</td>
<td>64 (56)</td>
<td>76 (67)</td>
<td>98 (86)</td>
<td>52 (46)</td>
<td>19 (17)</td>
</tr>
<tr>
<td></td>
<td>&gt;56</td>
<td>Female</td>
<td>≥2.0</td>
<td>Present</td>
<td>LMM</td>
<td>Sun protected</td>
</tr>
<tr>
<td>No. patients (%)</td>
<td>58 (51)</td>
<td>50 (44)</td>
<td>38 (33)</td>
<td>16 (14)</td>
<td>17 (15)</td>
<td>95 (83)</td>
</tr>
</tbody>
</table>

Abbreviations: SSM, superficial spreading melanoma; LMM, lentigo malignant melanoma. *Includes desmoplastic melanoma, acro lentigous melanoma, and nodular melanoma. †Sun-protected sites: trunk, arm, leg, and feet. Sun-exposed sites: head and neck.

Fig. 1. Representative images of ING3 immunohistochemical staining in human melanocytic lesions. A, strong nuclear ING3 staining in dysplastic nevi. B, moderate nuclear ING3 staining in primary melanoma. C, weak nuclear ING3 staining in metastatic melanoma.

Imaging, Diagnosis, Prognosis
Results

Clinicopathologic features of TMAas. Due to loss of biopsy cores or insufficient tumor cells present in the cores, 58 cases of dysplastic nevi, 114 cases of primary melanomas, and 50 cases of melanoma metastases could be evaluated for ING3 staining. As shown in Table 1, for the 114 primary melanoma cases, there were 64 men and 50 women, with age ranging from 21 to 93 years (median, 56 years). For melanoma staging, we used Breslow thickness as our criteria for evaluating ING3 expression: 76 tumors were <2.0 mm thick (low-risk melanoma), and 38 were thicker than 2.0 mm (high-risk melanoma). Nineteen melanomas were located in sun-exposed sites (head and neck), and 95 were located in sun-protected sites (other locations). Ulceration was observed in 16 cases.

Reduced ING3 nuclear staining correlates with melanoma progression. Various levels of ING3 staining were observed in dysplastic nevi and melanoma biopsies (Fig. 1). Strong nuclear ING3 staining was recorded in 60%, 33%, and 10% of the biopsies in dysplastic nevi, primary melanomas, and melanoma metastases, respectively (Fig. 2A). Significant differences for nuclear ING3 staining (negative-to-moderate versus strong staining) were observed between dysplastic nevi and primary melanomas ($P < 0.05$, $\chi^2$ test), between primary melanomas and melanoma metastases ($P < 0.05$, $\chi^2$ test) and between dysplastic nevi and melanoma metastases ($P < 0.001$, $\chi^2$ test). In addition, the reduced nuclear ING3 expression was positively associated with the increased cytoplasmic ING3 expression in either primary melanomas or metastatic melanomas (Fig. 2C and D; $P = 0.001$ and 0.042, respectively, Spearman test).

Reduced ING3 nuclear staining correlates with tumor location. To assess whether reduced ING3 staining correlates with clinicopathologic variables of the patients, we examined the ING3 nuclear staining in 114 primary melanomas. Because UV radiation is the main environmental factor for melanoma formation, we first analyzed the ING3 staining in sun-exposed or sun-protected sites. Reduced nuclear ING3 staining (negative-to-moderate versus strong staining) significantly correlated with the location of primary melanomas (Fig. 3A; $P = 0.021$, Spearman test). Thirty-eight percent (36 of 95) of tumors had strong ING3 nuclear staining in sun-protected sites (trunk, arm, leg, feet, etc.), whereas strong ING3 nuclear expression was observed in only 10% (2 of 19) of tumors at sun-exposed sites (head and neck). However, the increased cytoplasmic ING3 staining did not significantly correlate with the location of primary melanomas (Fig. 3B; $P > 0.05$, Spearman test), although the strong ING3 cytoplasmic staining increased from 38% in tumors at sun-protected sites to 55% in tumors at sun-exposed sites. On the other hand, neither nuclear nor cytoplasmic ING3 staining significantly correlated with other
clinicopathologic variables, including tumor thickness, subtype, ulceration, or patient's age or gender (data not shown).

**Reduced nuclear ING3 staining correlates with disease-specific 5-year patient survival.** To evaluate whether the reduced nuclear ING3 staining in human primary melanomas correlates with a worse prognosis, Kaplan-Meier survival curves were constructed using overall or disease-specific 5-year survival to evaluate the biopsies with strong nuclear ING3 staining to those with negative-to-moderate nuclear ING3 staining. The overall 5-year survival rate dropped from 87% in patients with the strong nuclear ING3 staining to 74% in patients showing negative-to-moderate nuclear ING3 staining, but they were not significantly correlated ($P = 0.119$, log-rank test; data not shown). On the other hand, the disease-specific 5-year survival rate dropped from 97% in patients showing strong nuclear ING3 staining to 82% in those with negative-to-moderate nuclear ING3 staining, and this correlation between the reduced ING3 nuclear staining and a poorer 5-y disease-specific survival is statistically significant ($P = 0.0226$, log-rank test; Fig. 4A). We noticed that the majority of the death cases occurred in the high-risk group (tumor thickness $\geq 2.0$ mm), whereas only 1 of 76 patients in the low-risk group (tumor thickness $< 2.0$ mm) died. Therefore, we analyzed the correlation between the reduced nuclear ING3 staining with the disease-specific survival in high-risk primary melanomas. We found that the survival rate was 93% in patients with strong nuclear ING3 staining compared with 44% in patients with negative-to-moderate nuclear ING3 staining, and this correlation is statistically significant ($P = 0.004$, log-rank test; Fig. 4B).

We then examined whether the nuclear ING3 expression is an independent prognostic marker for melanoma. We did a multivariate Cox regression analysis, including ING3 nuclear staining, age, gender, tumor thickness, ulceration, and location of the tumors, for 114 primary melanomas. Besides the tumor thickness to be an independent prognostic marker for disease-specific survival, the nuclear ING3 level showed significance in predicting the patient outcome independently of other clinicopathologic variables for disease-specific survival (relative risk, 9.31; 95% confidence interval, 1.13-76.5; $P = 0.038$; Table 2). In high-risk primary melanoma, the nuclear ING3 expression showed a borderline significance in predicting the patient outcome as an independent factor (relative risk, 8.34; 95% confidence interval, 0.959-72.6; $P = 0.055$; Table 2), whereas tumor thickness did not show significance in predicting patient outcome independently of other clinicopathologic variables for disease-specific survival ($P = 0.151$; Table 2).

**Discussion**

Acquired resistance to apoptosis is a major hallmark of cancers (18), which allows cancer cells to escape the apoptotic death induced by anticancer agents and enables the establishment of metastasis. The human cutaneous malignant...
neoplasia. Supportively, the reduced nuclear ING3 expression staining in cytoplasm. This suggests that the ING3 nuclear-to-cytoplasm translocation remains to be determined. The reason for ING3 nuclear-to-cytoplasmic translocation in 114 cases of primary melanoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>All primary melanomas</th>
<th>High-risk melanomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.471 (0.151-1.47) 0.194</td>
<td>0.477 (0.145-1.56) 0.222</td>
</tr>
<tr>
<td>Age</td>
<td>0.648 (0.159-2.64) 0.545</td>
<td>0.430 (0.097-1.92) 0.268</td>
</tr>
<tr>
<td>Thickness</td>
<td>54.8 (6.43-470) 0.000</td>
<td>0.391 (0.108-1.41) 0.151</td>
</tr>
<tr>
<td>Site</td>
<td>0.910 (0.304-2.72) 0.865</td>
<td>1.30 (0.397-4.28) 0.662</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0.708 (0.238-2.30) 0.566</td>
<td>0.760 (0.216-2.74) 0.685</td>
</tr>
<tr>
<td>ING3</td>
<td>9.31 (1.13-76.5) 0.038</td>
<td>8.34 (0.959-72.6) 0.055</td>
</tr>
</tbody>
</table>

NOTE: Coding of variables: ING3 nuclear staining was coded as 0 (negative to moderate staining) and 1 (strong staining); gender was coded as 0 (male) and 1 (female); age was coded as 0 (56 y) and 1 (<56 y); thickness was coded as 0 (>2.0 mm) and 1 (<2.0 mm) in all primary melanomas or 0 (>4.0 mm) and 1 (<4.0 mm) in high-risk melanomas (thickness >2.0 mm); ulceration was coded as 0 (absent) and 1 (present); site was coded as 0 (head and neck) and 1 (other sites).

Table 2. Multivariate Cox regression analysis of ING3 subcellular distribution in 114 cases of primary melanoma

ING3 in Human Melanoma

melanoma is a particularly aggressive type of cancer in this regard. Its metastatic form is very resistant to conventional radiotherapy and chemotherapy (19–21). The novel tumor-suppressor ING3 is inducible by UV irradiation or treatment with genotoxic drugs, and overexpression of ING3 dramatically enhances UV-induced apoptosis (13). In this study, using the tissue microarray and immunohistochemistry technology, we for the first time showed that ING3 nuclear expression is remarkably reduced in human melanomas compared with dysplastic nevi (Fig. 2A). This finding is consistent with the report by Gunduz et al. (14) showing that ING3 is reduced in head and neck squamous cell carcinoma. However, different mechanisms may be involved for the reduction of ING3 expression in head and neck squamous cell carcinoma and melanoma. Fifty percent of the head and neck squamous cell carcinoma cases showed reduced or absent ING3 mRNA and 48% of cases had allelic deletion in 7q31 region (14), whereas in melanoma the reduced nuclear ING3 expression is positively correlated with the increased cytoplasmic ING3 level, indicating an obvious translocation of ING3 from nucleus to cytoplasm compartment (Fig. 2C). Similarly, the mRNA level of ING1b, an ING3 homologue, is decreased in 61% of human exocrine pancreatic carcinoma (22), 42% of non-small cell lung cancer (23), and 44% of primary breast cancers (24), whereas the translocation of ING1b from nucleus to cytoplasm was also observed in the invasive breast cancer (25), childhood acute lymphoblastic leukemia (26), and melanoma (27), which may be caused by the sequestration of ING1b by 14-3-3 proteins in cytoplasm (28). The reason for ING3 nuclear-to-cytoplasm translocation remains to be determined.

Although the nuclear ING3 expression was significantly reduced in melanomas compared with dysplastic nevi, only 60% (35 of 58) of the dysplastic nevi showed strong nuclear ING3 expression and 24% (14 of 58) of them had strong ING3 staining in cytoplasm. This suggests that the ING3 nuclear-to-cytoplasm translocation may be an early event in melanocytic neoplasia. Supportively, the reduced nuclear ING3 expression was positively correlated with the tumor location at sun-exposed sites (Fig. 4A), indicating a crucial role of UV radiation in regulating nuclear ING3 level. However, although increased strong cytoplasmic ING3 staining was observed in tumors at sun-exposed sites, the correlation between cytoplasmic ING3 expression and sun exposure was not significant (Fig. 3B). One possible explanation for this discrepancy is that the nuclear-to-cytoplasm translocation of ING3 may be a partial reason for reduced nuclear ING3 expression after UV irradiation because 32% (6 of 19) of tumors at sun-exposed sites with reduced nuclear expression did not show strong cytoplasmic ING3 staining. In addition, we notice that 13% (15 of 114) of primary melanomas and 16% (8 of 50) of metastatic melanomas with the reduced nuclear ING3 expression did not show a significant increase of the cytoplasmic ING3 level. Therefore, mechanisms other than the nuclear-to-cytoplasm shift could be involved in the loss of nuclear ING3 expression in these tumors.

The defects in the apoptosis pathway, including Apaf-1, PUMA, and XAF1, mostly attribute to the initiation step in melanoma pathogenesis (29), whereas activated survival pathway by increased integrin-linked kinase and phospho-Akt levels or reduced PTEN expression often correlates with tumor progression (16, 17). In this study, the reduced nuclear ING3 expression is correlated with the progression from primary melanoma to metastatic melanoma, indicating that ING3 plays a role not only in the initiation of melanoma, but also in melanoma progression. In agreement with the correlation between reduced ING3 expression and a poorer patient survival in head and neck squamous cell carcinoma (14), the reduced nuclear ING3 expression is significantly correlated with a poorer 5-year disease-specific survival of patients with primary melanoma (Fig. 4A) and is likely an independent factor predicting patient outcome (Table 2).

We have recently found that nuclear ING2 expression was significantly reduced in human melanomas (30). In an earlier report, Nouman et al. (27) also showed that nuclear ING1b expression was significantly decreased and shifted to cytoplasm in melanocytic lesions. These results together with our current data suggest that the ING family proteins play a crucial role in the development of melanoma. This is also supported by our in vitro studies showing that both ING1b and ING2 significantly enhance the repair of UV-damaged DNA and promote apoptosis in melanoma cells in a p53-dependent pathway (31–34), whereas ING3 promote UV-induced apoptosis by activating the p53-independent Fas/caspase-8 pathway (13). Because the p53 gene is rarely mutated in melanoma (35–37), the aberrant expression of ING family tumor suppressors may be an important step during melanoma development. Further analysis on the correlation between ING protein expression and their mutational status will provide new insight on the mechanisms of aberrant expression of ING proteins in melanoma.

In conclusion, the data presented in this report showed that nuclear ING3 expression is significantly reduced with human melanoma progression. The reduced nuclear ING3 level was correlated with the increased cytoplasmic ING3 level. Strikingly, the reduced nuclear ING3 expression correlates with a worse 5-year disease-specific survival of primary melanoma patients and is an independent prognostic factor for primary melanomas. These data, together with the regulation of ING3 in cell growth...
and apoptosis, indicate that ING3 may play an important role in both melanoma initiation and progression and serve as a promising prognostic marker and therapeutic target for malignant melanoma.

References


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Prognostic Significance of Nuclear ING3 Expression in Human Cutaneous Melanoma

Yemin Wang, Derek L. Dai, Magdalena Martinka, et al.


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