Role of ING4 in human melanoma cell migration, invasion and patient survival

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Inhibitor of growth (ING) 4 has been reported as a tumor suppressor and shown to diminish colony-forming efficiency, induce p53-dependent apoptosis and arrest cell cycle at G2-M phase. In this study, we investigated the role of ING4 in human melanoma pathogenesis. Using the tissue microarray technology, we found that ING4 expression is significantly decreased in malignant melanoma compared with dysplastic nevi (P < 0.0001, χ² test) and reduced ING4 staining is associated with melanoma thickness, ulceration (P = 0.034 and 0.002, respectively, χ² test) as well as poor overall and disease-specific 5-year survival of primary melanoma patients (P = 0.0002 and 0.001, respectively, χ² test). Cox regression analysis revealed that reduced ING4 staining is an independent factor for the poor prognosis of patients with primary melanomas. Furthermore, we found that overexpression of ING4 suppressed cell migration by 63% and inhibited the activity of Ras homolog family gene member A (RhoA) small GTPase protein and Rho-associated kinase (ROCK)-mediated formation of stress fiber in melanoma cells. Moreover, our data showed that overexpression of ING4 inhibited melanoma cell invasion by 43% compared with the control (P = 0.006, t-test) and ING4-overexpressing melanoma cells showed significantly reduced activity of matrix metalloproteinase (MMP)-2 and MMP-9. Taken together, this study highlights the importance of ING4 in melanoma pathogenesis and ING4 may serve as a promising prognostic marker and a potential therapeutic target for human melanoma.

Introduction

Melanoma is the most lethal form of skin cancer, which originates from the pigment-producing melanocytes. The incidence of melanoma has drastically increased over the past several decades. In the USA, the number of new melanoma cases in 2007 was estimated to be 59 940 and it was predicted to have 8110 deaths due to melanoma (1). Malignant melanoma is very resistant to the conventional radio-, chemothero- or immunotherapy (2–5). Although melanoma accounts for only 4% of all dermatological cancers, it is responsible for >80% of deaths from skin cancers and only 14% of patients with metastatic melanoma survive for >5 years (6).

The underlying mechanisms that regulate the progression of melanoma are still poorly understood. Many factors are involved in melanoma pathogenesis, including high levels of exposure to sunlight, histories of blistering sunburn before age 20, presence of multiple nevi or birthmarks and family history of melanoma (7–9). Previous studies indicated that inhibitor of growth (ING) family tumor suppressors might play an important role in melanoma development. Mutations of the ING1 gene abrogate nucleotide excision repair in melanoma cells. ING2, a DNA damage-inducible gene, is required for the initial DNA damage sensing and chromatin remodeling during nucleotide excision repair in melanoma cells (10). ING4 is located in chromosome 12p13 and encodes a 249-amino acid protein containing a C-terminal plant homeodomain finger motif and two nuclear localization signals (11). Overexpression of ING4 diminished colony-forming efficiency, decreased cell population in S phase and induced p53-dependent apoptosis (11). ING4 can physically interact with p300, a member of histone acetyltransferase complexes, and consequently enhance p53 acetylation at Lys382 (11). ING4 was also shown to physically interact with the p65 subunit of nuclear factor-kappa B (NF-kB) and regulate brain tumor angiogenesis through transcriptional repression of NF-kB-responsive genes, including interleukin 6, interleukin 8, cytochrome c oxidase subunit II (COX2) and colony-stimulating factor 3 (12). Compared with normal human brain tissue, expression of ING4 is significantly reduced in gliomas, and the extent of reduction correlates with the progression from lower to higher grades of tumors (12). In addition, ING4 acts as an adaptor protein to mediate the activity of hypoxia-inducible factor (13). In this study, we explored the role of ING4 in melanoma pathogenesis. First, we evaluated ING4 expression in different stages of human melanocytic lesions using tissue microarray (TMA) technology and immunohistochemistry. We found that ING4 expression was significantly decreased when dysplastic nevi transformed to malignant melanomas and ING4 expression was an independent prognostic factor for primary melanoma patients. We then demonstrated that ING4 overexpression suppressed melanoma cell migration, RhoA activity, stress fiber formation as well as inhibited cell invasion and matrix metalloproteinase (MMP) activities.

Materials and methods

Patients for TMA study

Formalin-fixed and paraffin-embedded biopsies were obtained from the 1990–1998 archives of the Department of Pathology at Vancouver General Hospital. The use of human skin tissues in this study was approved by the medical ethical committee of the University of British Columbia and was performed in accordance with the Declaration of Helsinki guidelines. A total of 237 biopsies, including 66 dysplastic nevi, 118 primary melanomas and 53 metastatic melanomas, were used for TMA construction. Due to loss of biopsy cores, 50 dysplastic nevi, 101 primary melanomas and 49 metastatic melanomas could be evaluated for ING4 staining. Clinicopathological data were available for all melanoma cases.

TMA construction, immunohistochemistry and evaluation of immunostaining

TMA construction and immunohistochemistry of TMA were performed as described previously (14). We selected and marked the most representative tumor area on the hematoxylin and eosin-stained slide. A polyclonal rabbit anti-ING4 antibody (1:50 dilution; ProteinTech Group, Chicago, IL) was used for the immunohistochemistry staining. Negative controls were examined following the same procedure of test samples, except that ING4 antibody was omitted from the primary antibody incubation. The staining intensity and percentage of ING4-positive cells were evaluated blinded by three independent observers (including a dermatopathologist) simultaneously, and a consensus score was reached for each core. ING4 staining intensity was scored 0–3 (0, negative; 1, weak; 2, moderate and 3 strong). The percentage of ING4-positive stained cells was also scored into four categories: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). In the cases with a discrepancy between duplicated cores, the higher score from the two tissue cores was taken as the final score. The level of ING4 staining was evaluated by immunoreactive score (IRS) (15), which is calculated by multiplying the scores of staining intensity and the percentage of positive cells. Based on IRS, ING4 staining pattern was defined as: negative (IRS: 0), weak (IRS: 1–4), moderate (IRS: 5–8) and strong (IRS: 9–12).

Statistical analyses of TMA

The SPSS version 11.5 software (SPSS, Chicago, IL) was used for the statistical analysis and all tests of statistical significance were two sided. We used χ² test to compare ING4 staining in different melanocytic lesions, as well as the correlation between ING4 staining and the clinicopathological parameters of the primary melanoma patients. The Kaplan–Meier survival curve and log-rank test were used to evaluate the correlations between ING4 expression and patient survival. Finally, a Cox regression model was used for multivariate analysis. A P value of <0.05 was considered statistically significant.

Cell culture and transfection

MMR and SK-MEL-5 human melanoma cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.
(Invitrogen, Burlington, Ontario, Canada) in 5% CO₂ atmosphere at 37°C. Cells were grown to ~50% confluency before transfection of plasmid. Expression vector pCMV or pFlag-CMV-ING4 plasmids were transfected by Effectene reagent (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. Twelve hours after transfection, the medium containing transfection reagents was removed. The cells were rinsed by phosphate-buffered saline (PBS) and then incubated in fresh medium. The sulfurforhodamine B cell proliferation assay was performed as described previously (16).

**Western blot**

Cells were harvested and washed with PBS thrice. Whole-cell proteins were extracted as described previously (17). After protein concentration was determined by protein assay (Bio-Rad, Mississauga, Ontario, Canada), western blot analysis was performed as described previously (18). The following antibodies were used for western blot: mouse anti-Flag (1:1000; Applied Biological Materials, Vancouver, British Columbia, Canada), p53 (DO-1, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and actin (1:1000; Sigma, St Louis, MO). Infrared IR dye-labeled secondary antibody was applied to the blot for 1 h at room temperature and then signals were detected with Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE).

**Wound-healing assay**

Twenty-four hours after transfection with ING4 or empty vector, confluent MMRU cells and SK-MEL-5 were washed with PBS and a standard 200 μl pipette tip was applied to draw across the well to produce a 0.5-mm width wound at the center of each well. The wounded monolayer was washed twice to remove non-adherent cells and then incubated in fresh medium for another 24 h. Photographs were taken at the same position of the wounds at 0 and 24 h time points. The starting wound edges were defined in each photo by white lines based on the scratch at 0 h time point and the numbers of migrating cells across these white lines were counted to quantify the rate of cell migration.

**RhoA pull-down assay**

MMRU melanoma cells were transfected with vector pCMV or pFlag-CMV-ING4 at 50% confluency for 24 h. Then, cells were serum starved for 30 min. Cells were then lysed in 300 μl RIPA lysis buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 10 mM MgCl₂, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate, 10 μg/ml of aprotinin and leupeptin and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were cleared by centrifugation at 12 000 g for 15 min at 4°C and the supernatants were used directly for the pull-down assay. The supernatants were incubated with 20 μg glutathione S-transferase fusion protein immobilized to glutathione Sepharose 4B beads at 4°C for 1 h. Bound proteins were then separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with monoclonal mouse anti-RhoA antibody (1:250; Santa Cruz Biotechnology). Total RhoA expression was evaluated by western blot analysis using the anti-RhoA antibody. The bands were quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

**Cell invasion assay**

Boydén chamber assay was used for the cell invasion analysis as described previously (19). Briefly, 18 μl of 5 mg/ml matrigel (BD Biosciences, Mississauga, Ontario, Canada) in serum-free medium was applied to the upper compartment of 24-well Transwell culture chambers (with 8.0 μm pore size polycarbonate membrane). MMRU cells (5 × 10⁵) suspended in 250 μl of serum-free medium were loaded on the upper compartment, and the lower compartment was filled with 750 μl of complete medium. After 24 h incubation, cells were fixed with 10% trichloroacetic acid at 4°C for 1 h. Non-invaded cells were removed from the upper surface of the filter carefully with a cotton swab. Invaded cells on the lower side of the filter were stained with 0.5% crystal violet for 2 h and the retained dye on the filters was extracted by 30% acetic acid followed by reading the absorbance at 590 nm.

**Zymography**

Zymography assay was carried out following the protocol described previously (19). MMRU cells were transfected with empty pCMV vector or ING4 plasmid. Serum-free medium was applied to the cells overnight and the proteins in the conditioned medium were concentrated with YM-3 Centricon membranes (Millipore, Billerica, MA) at 7000g for 4 h at 4°C. Proteins (5 μg) were loaded on a 10% polyacrylamide gel containing 0.1% gelatin (Sigma). After electrophoresis, the gel was incubated in Triton X-100 exchange buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM CaCl₂ and 2.5% Triton X-100] for 30 min followed by 10 min wash with the incubation buffer (same buffer without Triton X-100) thrice. The gel was then incubated in incubation buffer overnight at 37°C, stained with 0.5% Coomassie blue R250 (Sigma) for 1 h and destained with 30% methanol and 10% acetic acid for 1 h. Gelatinolytic activity was shown as clear areas in the gel. Recombinant MMP-2 and MMP-9 (R&D Systems, Minneapolis, MN) were used as positive controls.

**Results**

**Clinicopathological features of primary melanoma biopsies**

The clinicopathological features of the 101 primary melanoma biopsies were summarized in supplementary Table I (available at Carcinogenesis Online). There were 62 men and 39 women with age ranging from 21 to 93 years (median, 57 years). For the tumor thickness, 35 cases were ≤1.0 mm, 31 cases were 1.01–2.0 mm, 17 cases were 2.01–4.0 mm and 18 cases were >4.0 mm. Tumor ulceration was present in 19 cases at diagnosis. For the histological subtype, there were 45 cases of superficial spreading melanoma, 18 lentigo maligna melanomas, 16 nodular melanomas and other 22 cases unspecified. Nineteen melanomas were located in sun-exposed sites, such as head and neck, and the other 82 were located in sun-protected sites.

**ING4 expression is decreased in malignant melanoma**

ING4 is a nuclear protein as it contains the nuclear localization sequence (11,20). We found that ING4 staining was mainly localized in the nucleus in melanocytic lesions (Figure 1). Various levels of ING4 staining were observed in dysplastic nevi and malignant melanoma biopsies (Figure 1). Strong and moderate ING4 staining decreased from 98% in dysplastic nevi to 67% in primary melanomas and 0.5% Triton X-100 in PBS) was then applied to the cells for 10 min at 4°C.

**Immunofluorescence**

MMRU cells were transfected with vector pCMV or pFlag-CMV-ING4 and subcultured onto coverslips in six-well plate. After 6 h, the cells were serum starved overnight and stimulated with complete medium containing 10% fetal bovine serum for a designated period. Fixation solution (2% paraformaldehyde and 0.5% Triton X-100 in PBS) was then applied to the cells for 10 min at 4°C.

![Fig. 1. ING4 expression is significantly reduced in malignant melanomas.](https://academic.oup.com/carcin/article-abstract/29/7/1373/2476507)
53% in metastatic melanomas. Significant differences in ING4 staining were observed between dysplastic nevi and primary melanoma \( (P = 0.000027, \chi^2 \text{ test}) \) and between dysplastic nevi and metastatic melanoma \( (P = 0.000003, \chi^2 \text{ test}) \), but not between primary melanoma and metastatic melanoma \( (P = 0.409, \chi^2 \text{ test}) \) (Figure 2A). Unlike ING1b and ING3 which showed nuclear-to-cytoplasm shift in melanoma cells (21–23), we did not observe nuclear-to-cytoplasm shift for ING2 from our previous work (24) or ING4 in melanoma cells in this study (data not shown).

**ING4 expression is correlated with melanoma tumor thickness and ulceration**

We also conducted the correlation analysis between ING4 expression and clinicopathological parameters, including age, gender, tumor thickness, ulceration, histological subtype and tumor site in 101 primary melanoma biopsies. Reduced ING4 expression is significantly correlated with tumor thickness and ulceration \( (P = 0.034 \text{ and } 0.002, \chi^2 \text{ test}) \) (Figure 2B and C), but not correlated with patient’s age, gender, histological subtype or tumor site \( (P > 0.1 \text{ for all, } \chi^2 \text{ test}) \) (supplementary Table I, available at Carcinogenesis Online).

**Correlation between ING4 expression and patient survival**

To study whether ING4 expression is correlated with melanoma patient survival, we constructed Kaplan–Meier survival curves in patients with primary melanoma and metastatic melanoma, respectively. Our overall and disease-specific survival analysis data from primary melanoma group are shown in Figure 3. Patients with moderate to strong ING4 staining showed significantly better overall and disease-specific 5-year survival rate (85 and 91%) than patients with negative to weak ING4 staining did (52 and 67%) \( (P = 0.0002 \text{ and } 0.001, \chi^2 \text{ test}) \) (Figure 3). In metastatic melanoma group, both overall and disease-specific 5-year survival rate were similar in patients with moderate to strong ING4 staining (15 and 30%) and those with negative to weak ING4 staining (13% for both) \( (P > 0.6 \text{ for both, log-rank test}) \) (data not shown). Next, we performed Cox regression multivariate analysis to study the effect of ING4 expression in patient survival together with patient’s age, gender, tumor thickness, ulceration, histological subtype and tumor site in 101 primary melanomas. Our results indicated that ING4 expression is an independent prognostic factor for both overall \( (\text{Relative risk } = 2.50, 95\% \text{ confidence interval } = 1.09 – 5.74, P = 0.031) \) and disease-specific 5-year survival \( (\text{Relative risk } = 2.97, 95\% \text{ confidence interval } = 1.02 – 8.17, P = 0.038) \) (Figure 3).
8.63, \( P = 0.045 \) (supplementary Table II, available at Carcinogenesis Online).

**ING4 inhibits melanoma cell migration**

As cell motility is an important factor for tumor progression and ING4 expression is significantly reduced in malignant melanoma, we investigated the role of ING4 in melanoma cell migration. First, we transfected melanoma MMRU cells with pFlag-CMV-ING4 and found that ING4 was overexpressed in this cell line compared with vector control (Figure 4A). In the wound-healing assay, the control cells transfected with vector alone healed the wound completely 24 h after scratch, whereas the ING4-overexpressing cells were unable to heal the wound (Figure 4B). Quantitative analysis of the number of the cells that migrated into the wound area indicated that ING4 inhibited cell migration by 63% in MMRU cell line (\( P = 0.0006 \), \( t \)-test) (Figure 4C). This inhibition was not due to ING4 inhibition on cell proliferation, as the proliferation of ING4-transfected cells is only \( \sim 10\% \) slower than the vector control (Figure 4D), although overexpression of ING4 started to show significant inhibition on cell proliferation 36 h after transfection (\( P = 0.224 \) and 0.015 for 24 and 36 h after transfection, respectively, \( t \)-test). To confirm that ING4 inhibitory effect on cell migration is not cell line specific, we performed the cell migration assay in another wild-type p53 melanoma cell line SK-MEL-5. Our data showed that overexpression of ING4 inhibited SK-MEL-5 cell migration by 66% compared with the vector control (\( P = 0.001 \), \( t \)-test) (supplementary Figure S1, available at Carcinogenesis Online).

**ING4 inhibits RhoA activity and reduces ROCK-mediated stress fiber formation**

RhoA–ROCK pathway induces stress fiber reorganization and thus plays an important role in cell migration. To address whether the negative effect of ING4 on melanoma cell migration was associated with this pathway, we first used the RhoA pull-down assay to study the correlation between ING4 overexpression and RhoA activity. We found that active RhoA expression was greatly decreased in ING4-overexpressing cells compared with the vector-transfected cells (Figure 5A). Moreover, immunofluorescent staining revealed that serum stimulation induced much more stress fiber formation in vectortransfected MMRU cells than ING4-overexpressing cells. In addition, we found that ROCK inhibitor Y27632 abolished the effect of ING4 in stress fiber formation (Figure 5B). The relative fluorescence intensity of F-actin in cells transfected with ING4 was reduced by 50% compared with cells transfected with vector, whereas there is no significant difference between cells transfected with ING4 and vector after treatment with ROCK inhibitor Y27632 (Figure 5C).

**ING4 regulates cell invasion and MMP activity**

To study the role of ING4 in melanoma cell invasion, we performed the Boyden chamber assay and zymography. We found that cell invasion was reduced by 43% in ING4-overexpressing MMRU cells compared with the vector control (\( P = 0.006 \), \( t \)-test) (Figure 6A). Since MMPs play a crucial role in cell invasion, we then carried out the zymography assay to compare the activity of MMP-2 and MMP-9 in ING4-overexpressing and vector control cells. As shown in Figure 6B, MMP-2 and MMP-9 gelatinolytic activities were decreased by 25 and 61% in ING4-overexpressing cells compared with the vector control, respectively (\( P = 0.013 \) and 0.007, respectively, \( t \)-test) (Figure 6B and C).

**Discussion**

ING family members play important roles in cellular stress response to ultraviolet irradiation and melanoma pathogenesis. We have shown previously that ING1b and ING2 enhance the repair of ultraviolet-damaged DNA and promote apoptosis by activating the p53-dependent mitochondrial pathway (18,25–27). ING3 can also promote ultraviolet-induced apoptosis by activating the p53-independent Fas/caspase-8 pathway (17). In addition, we showed that nuclear expression of ING2 and ING3 is significantly reduced in human melanomas (23,24). In this study, we investigated the role of ING4 in human melanoma pathogenesis in vivo and in vitro. Our data demonstrated for the first time that ING4 expression is significantly decreased concomitant with melanoma progression and reduced ING4 expression is closely correlated with a poorer 5-year survival of primary melanoma...
patients. We also revealed that ING4 inhibits melanoma cell migration, cell invasion and the activity of MMP-2 and MMP-9.

Reduced ING4 expression in malignant melanoma described in this study is consistent with our previous findings that ING2 and ING3 expressions are significantly reduced in human malignant melanomas (23,24), suggesting that ING tumor suppressors play important roles in melanoma tumorigenesis. Our data are also in agreement with the findings by Garkavtsev et al. (12) who showed that ING4 expression level is dramatically reduced in gliomas both at messenger RNA and protein levels. The reduction of ING4 expression in melanoma can possibly be explained by the findings that deletion or mutation at chromosome 12p12–13, which includes ING4 gene, occurs frequently in a number of human tumors (28). Hassler et al. (29) studied 18 glioblastoma multiforme patients and found that loss of the ING4 gene, together with VEGF and bFGF, was associated with neangiogenesis. Gunduz et al. (28) reported that loss of heterozygosity at 12p12–13 region occurred in 66% head and neck squamous cell carcinoma patients and that 76% tumor tissues had decreased ING4 messenger RNA expression compared with matched normal samples. In another study, Kim et al. (30) found deletion of the ING4 locus in 10–20% of human breast cancer cell lines and primary breast tumors by comparative genomic hybridization analysis. These studies imply that deletion of ING4 gene may be a common event in tumorigenesis. However, whether ING4 is mutated or deleted in human melanoma remains to be determined.

Our data showed significant differences for ING4 staining pattern between dysplastic nevi and primary melanoma, but not between

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**Fig. 5.** ING4 reduces RhoA activity and stress fiber formation via ROCK. (A) ING4 reduces RhoA activity determined by RhoA pull-down assay (left panel). Total RhoA was used as loading control (right panel). (B) ING4 reduces stress fiber formation mediated by ROCK. Cells were transfected with vector or ING4, respectively, followed by serum starvation overnight and serum stimulation for 30 min. For ROCK inhibitor treatment, cells were pretreated with serum-free medium containing 10 μM Y27632 for 2 h after transfection with vector or ING4, serum starved overnight and then incubated with complete medium containing 10% fetal bovine serum and 10 μM Y27632 for 30 min. Magnification, ×400. (C) Quantitation of (B).

**Fig. 6.** ING4 overexpression inhibits melanoma cell invasion and the activity of MMP-2 and MMP-9. (A) MMRU cells were transfected with vector or ING4. Then, cells were suspended in serum-free medium and seeded on matrigel, incubated at 37°C for 24 h, stained by crystal violet and quantified. Columns, means from triplicate wells; bars, standard deviation. (B) ING4 inhibits MMP-2 and MMP-9 activity in MMRU cells by zymography. (C) Quantitation of (B). Columns, means from three independent experiments; bars, standard deviation.
primary melanoma and metastatic melanoma, suggesting that ING4 inactivation might be a critical requirement for the transformation from nevus to malignant tumor. Recent studies indicated that cellular senescence is a critical mechanism for opposing neoplastic transformation by the activation of oncogenic pathways (6,31–33). NF-κB is shown to be involved in the senescence of normal human epidermal keratinocytes by upregulating the expression of the manganese superoxide dismutase (34). Since ING4 was reported to downregulate NF-κB-downstream genes (12), it is reasonable to assume that ING4 regulates cellular senescence by suppressing NF-κB activity, thus inhibiting the malignant cell transformation.

In this study, we found that overexpression of ING4 inhibits melanoma cell migration in both melanoma cell lines, MMRU and SK-MEL-5 (Figure 4B, supplementary Figure S1, available at Carcinogenesis Online). Cell migration is the central step in tumor metastasis and regulated by various factors. NF-κB, an important transcription factor, was shown to be involved in this regulation through activation of RhoA GTPase. It is reported previously that ING4 binds to RelA subunit of NF-κB and acts as a negative regulator of NF-κB activity (12). Our data showed that ING4 inhibited RhoA activity (Figure 5A), which is believed to be able to promote reorganization of stress fiber during cell migration. Therefore, it is possible that ING4 inhibits melanoma cell migration by downregulating RhoA activity through NF-κB pathway. Furthermore, we found that ROCK inhibitor abrogated the inhibition of ING4 on stress fiber formation, suggesting that ING4 mediates melanoma cell migration via the RhoA–ROCK-signaling pathway (Figure 5B and C).

Our data showed that ING4 overexpression suppresses melanoma invasion that is consistent with the report by Shen et al. (35) who showed that ING4 interacts with liprin z1 to inhibit cell migration and cell invasion in RKO, HEK-293 and U-87 MG cell lines. We also found that overexpression of ING4 inhibited the gelatinolytic activities of both MMP-2 and MMP-9. Previous study showed that the number of MMP-2-positive cells increased with increasing atypia in different stage of melanocytic lesions by immunohistochemical staining (36), suggesting that MMP-2 expression is involved in melanogenesis. MMP-9 was also found to be expressed in the vertical growth phase of primary melanomas, indicating that MMP-9 plays a key role in melanoma cell invasion (37) and thus in melanoma progression (19,38). The inhibitory effect of ING4 on MMP-2 and MMP-9 activities at least partially contribute to the suppression of melanoma cell invasion, and this is consistent with our TMA data showing the negative correlation between ING4 expression and melanoma tumor thickness. The inhibitory effect of ING4 in cell invasion can also explain our observation that reduced ING4 expression significantly correlates with a poorer 5-year survival of patients with primary melanoma.

Other tumor-suppressive functions of ING4 may also contribute to the inverse correlation between ING4 expression and patient survival. ING4 induces cell cycle arrest, enhances chemosensitivity (39), suppresses tumor angiogenesis (12), the loss of cell contact inhibition (30), and the activation of hypoxia-inducible factor (13,40). It is not surprising that inactivation of ING4 leads to the abrogation of these tumor-suppressive functions and results in tumor progression and poorer survival outcome. As treatment history was not available, we were not able to determine whether chemoresistance of melanoma after ING4 inactivation was partially responsible for the poor patient survival. We found that ING4 expression is not correlated with the survival of patients with metastatic melanoma. This is because most patients with metastatic melanoma die within a few months (41). Currently, there are no prognostic factors that can substantially predict survival rate by a few months in patients with metastatic melanoma (42).

In summary, we demonstrated that ING4 plays an important role in human melanoma pathogenesis. Reduced ING4 expression may facilitate tumor progression by enhancing cell migration and cell invasion. Our results imply that ING4 may serve as a promising prognostic marker as well as a potential therapeutic target for malignant melanoma.

**Supplementary material**

Supplementary Figure S1, Tables I and II can be found at http://carcin.oxfordjournals.org/

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