Oncogenes and Tumor Suppressors

Actin-Binding Protein, Espin: A Novel Metastatic Regulator for Melanoma

Takeshi Yanagishita1,3, Ichiro Yajima1,5, Mayuko Kumasaka1,5, Yoshiyuki Kawamoto2, Toyonori Tsuzuki4, Yoshinari Matsumoto3, Daisuke Watanabe6, and Masashi Kato1

Abstract

Espin is a multifunctional actin-bundling protein with multiple isoforms, and has special connections to hair cell stereocilia and microvillar specializations of sensory cells in the inner ear. However, there have been no reports showing the expression and function of Espin in cancers, including melanoma. Here, it is demonstrated that Espin expression is significantly increased in melanomas that spontaneously developed in RET-transgenic mice (RET-mice). Importantly, the invasion capacity of Espin-depleted Mel-ret melanoma cells derived from a tumor of the RET-mouse was dramatically less than that of control melanoma cells with reductions of lamellipodia, focal adhesion kinase (FAK), and GTP-Rac1 activities. Correspondingly, the ratio of metastatic foci in Espin-depleted Mel-ret melanoma cells was significantly less than that of control melanoma cells in an in vivo melanoma metastasis model. Moreover, Espin could be a novel biomarker of melanoma in humans, because our immunohistochemical analysis data reveal that percentages of Espin-positive cells in human primary and metastatic melanomas were significantly higher than that of cells in melanocytic nevi. Together, these results indicate that Espin is not only a metastatic regulator for melanoma but also a potential biomarker of disease progression.

Implications: Actin-binding protein Espin is expressed in melanoma, affects metastasis, and is a potential target for melanoma therapy. Mol Cancer Res; 12(3); 440–6. ©2013 AACR.

Introduction

The incidence of melanoma has increased by 3.1% every year and its incidence rate has doubled over a 10-year period (1). Melanoma is the most serious skin cancer and is highly invasive and resistant to conventional therapy (2). Identification of a molecule associated with melanoma growth, progression, and metastasis will provide new insights into the design of a biomarker and a novel therapeutic strategy for melanoma. Preventing or eliminating metastasis is one of the most important challenges for therapeutic intervention. Several regulatory molecules for cancer invasion and metastasis have recently been reported as candidates for clinical applications (3). However, an effective therapy for melanoma has not yet been established (2).

The ESPIN gene has various isoforms and encodes an actin-filament–binding protein (Supplementary Fig. S1; refs. 4, 5). All isoforms of ESPIN encode the same region of a 116–amino acid actin-bundling module (ABM), which is necessary for potent actin-bundling and microvillar parallel actin bundle (PAB) elongating activities (4, 5). Mutation in Wiskott–Aldrich syndrome protein homology 2 (WH2) and ABM domains of ESPIN gene causes hereditary deafness and vestibular dysfunction accompanied by stereociliary shortening in jerker mice and humans (5, 6). Espins are also capable of affecting the actin cytoskeleton, resulting in a special connection to microvillar specializations of sensory cells such as taste receptor cells, solitary chemoreceptor cells, and Merkel cells (7). On the other hand, dynamic remodeling of the actin cytoskeleton is required for cancer invasion. To activate cancer invasion, remodeling of the actin cytoskeleton results in the formation of lamellipodia, sheets of F-actin, regulated by Rho family GTPases, including Rac1, whose activity is regulated by focal adhesion kinase (FAK; ref. 8). These previous findings indicate the possibility that Espin is functionally correlated with the invasion of cancer. To our knowledge, however, there is no information about the expression levels of Espin in cancer cells or its role in cancers including melanoma.

We previously established RET-transgenic mice of line 304/B6 (RET-mice) carrying oncogenic RET (RFP/RET) under the control of the metallothionein-I promoter (9). Hyperpigmented skin, benign melanocytic tumors, and melanomas with metastases develop stepwise in RET-mice...
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(9). Because histopathologic characteristics of melanoma in RET-mice are similar to those of melanoma in humans, RET-mice have been used as a standard model of cutaneous melanoma in recent studies (9, 10). In this study, we examined the effect of Espin on the pathogenesis of melanoma and found for the first time a correlation between Espin and cancer.

Materials and Methods

Cells
A murine Mel-ret melanoma cell line derived from a tumor in the RET-mouse (11) and human melanoma cell lines (G361, SK-Mel28, Colo679, HMVII, A375P, and A375M) (10) were cultured in RPMI 1640 supplemented with 10% FBS. G361, SK-Mel28, Colo679, and HMVII were provided by the Riken Bioresource Cell Bank and A375P and A375M were kindly provided by Dr. Dorothy C Bennett (St. George’s, University of London, UK). Primary culture of normal human epithelial melanocytes (NHEM) was performed according to the manufacturer’s protocol (Cell Applications).

Immunoblotting, immunohistochemistry, and immunofluorescence analysis

An anti-Espin-ru rabbit polyclonal antibody was developed using peptides (SSSTGSKSFN) in this study (Supplementary Fig. S1). Rabbit polyclonal antibodies against phosphorylated tyrosine 397 in FAK (Invitrogen), phosphorylated tyrosine 181 in paxillin (Epitomics), mouse monoclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology), FAK (BD Transductions Laboratories), vinculin (Sigma), and paxillin (BD) were used as primary antibodies. A pull-down assay was performed using RhoA and Rac1 Activation Assay Kits (Cell Biolabs) according to the manufacturer’s protocol. Immunohistochemical estimation of ESPIN (positive/negative) was performed using the software program WinROOF (Mitani Corporation) according to the method previously reported (12). Methods for immunoblotting, immunofluorescence analysis, and immunohistochemistry using human tissue array slides (ME103; US Biomax, Inc.) are described in Supplementary Materials and Methods.

Wound-healing assay and in vitro invasion assay

Wound-healing assays using stably expressed short hairpin RNA (shRNA) and control Mel-ret cells seeded on type I collagen (10 μg/mL)–coated 6-well plastic dishes at a density of 2 × 10⁵ cells per well were performed according to the method previously described (13). In vitro invasion assays using 2 × 10⁵ cells with stably expressed shRNA and 2 × 10⁵ control Mel-ret cells were performed according to the method previously described (14).

Melanoma xenograft studies

Mel-ret melanoma cells (1 × 10⁶ in PBS) stably transfected with Espin shRNA or control shRNA were injected into the lateral veins of 6-week-old female mice (n = 5 in each group), and the mice were sacrificed at 42 days after inoculation. Green fluorescence protein (GFP)-positive metastatic melanomas in lungs were analyzed by MZ16F light microscopy (Leica Microsystems).

Ethics statement

This study was performed in Chubu University, Japan. The study was approved by the Animal Care and Use Committee (approval no. 2410030), Recombination DNA Advisory Committee (approval no. 10-08), and Ethical Committee (approval no. 20008-10) at the Chubu University.

Results

Expression and localization of Espin in murine melanomas

We first showed that Espin transcript expression levels in melanomas were more than 200-fold higher than those in benign melanocytic tumors in RET-mice by quantitative real-time PCR (qRT-PCR) analysis using primers (primer set total Espin shown in Supplementary Fig. S1), which recognize all isoforms of the Espin transcript (Supplementary Fig. S2A). These results indicated that the Espin transcript is highly expressed in melanomas in RET-mice. We then examined the isoform of the Espin transcript that is highly expressed in melanoma using various primers (primer sets 1, 2A-A, 2B-B, 1–3, and 4) shown in Supplementary Fig. S1. Transcript expression levels of Espin-1, -2, and -3 and Espin-4 isoforms detected by primer sets 1–3 and 4 (Supplementary Fig. S1) in melanomas were 286- and 28-fold higher than those in benign tumors, respectively (Supplementary Fig. S2B). However, transcript expression levels of Espin-1, Espin-2A, and -2A’ and -Espin-1, 2B, and -2B’ isoforms detected by primer sets 1, 2A-2A’, and 2B-2B’ (Supplementary Fig. S1) in melanomas were comparable with those in benign tumors (Supplementary Fig. S2C). These results showed higher expression of Espin-3, lower expression of Espin-4, and no expression of Espin-1 and Espin-2 transcripts in melanomas. Because ESPIN-1 and -3, but not ESPIN-2 and -4, were detected in humans in previous studies (5, 7), we newly developed a rabbit polyclonal anti-Espin-ru antibody targeting the common sequence for Espin-3 protein in mice and humans (Supplementary Figs. S1 and S3).

Corresponding to the results of qRT-PCR analysis, immunoblot analysis using anti-Espin-ru-antibody revealed a more than 40-fold increased Espin protein expression level in melanomas compared with that in benign tumors in RET-mice (Fig. 1A). Immunofluorescence analysis showed that Espin protein was localized in the cytoplasm and lamellipodia (Fig. 1B) and was colocalized with F-actin protein in lamellipodia (Fig. 1B, arrowheads). Disruption of lamellipodia formation and rugged shape were observed in stable clones of Espin-depleted Mel-ret cells (clone E4 in Fig. 1C and D), whereas lamellipodia were organized in the mobile edge of cells in the control clones (clone C6 in Fig. 1C and D). Cortical thick stress fibers were also identified in the
Espin-depleted clone (arrows of clone E4 in Fig. 1D). Statistical analysis further showed about 55% (clones C3 and C6) and about 12% (clones E2 and E4) of lamellipodia formed in control (clones C3 and C6 in Fig. 1D) and Espin-depleted Mel-ret cells (clones E2 and E4 in Fig. 1D), respectively.

Effects of Espin on migration and invasion activities of melanoma cells.

We next performed wound-healing assays and in vitro invasion assays in stable clones of control and Espin-depleted Mel-ret cells. Activities of cell migration in the Espin-depleted clones (clones E2 and E4 in Fig. 2A) were about 77% reduced compared with those in the control cells (clones C3 and C6 in Fig. 2A) in wound-healing assays. Activities of cell invasion in Espin-depleted cells (clones E2 and E4 in Fig. 2B) were also about 80% reduced compared with those in control cells (clones C3 and C6 in Fig. 2B) in invasion assays. Levels of FAK, paxillin, and Rac1 activities, and vinculin expression in Espin-depleted cells (clone E4) were decreased compared with those in control cells (clone C6; Fig. 2C). However,
activity levels of RhoA were comparable in control cells and Espin-depleted cells (Fig. 2C).

**Effects of Espin on metastasis of melanoma cells in vivo**

Because many studies have shown that migration and invasion involve metastasis (15), we then examined the effect of Espin on melanoma metastasis in vivo. GFP-tagged Espin-depleted Mel-ret cells (clone E4; n = 5) and control cells (clone C6; n = 5) were injected into the tail veins of nude mice (Fig. 3). Macroscopic analysis for fluorescence intensity on the surface of the lung showed that the number of metastatic foci in Espin-depleted cells (clone E4) was smaller than that in control cells (clone C6; Fig. 3A). Statistical analysis also showed that the number of GFP-positive metastatic foci on the surface of the lung in Espin-depleted cells (clone E4) was about 25% of that in control cells (clone C6; Fig. 3B). In addition, lung metastasis in Espin-depleted Mel-ret cells (clone E4) and control cells (clone C6) was microscopically confirmed by hematoxylin-eosin (HE) staining (Fig. 3A).

**ESPIN expression in human melanomas**

We finally examined ESPIN protein expression levels in melanoma cell lines and tissues in humans.
Immunoblot analysis showed that ESPIN was expressed in all of the six melanoma cell lines (G361, SK-Mel28, Colo679, HMVII, A375P, and A375M) but not in NHEM (Fig. 4A). Immunohistochemical analysis also showed that ESPIN protein was expressed in more than 80% and 90% of primary melanomas (n = 77) and metastatic melanomas for lymph nodes (n = 23), respectively, whereas no expression of ESPIN was detected in more than 60% of melanocytic nevi (n = 36; Fig. 4B and C). Statistical analysis showed that percentages of ESPIN-positive primary and metastatic melanomas were significantly higher (P < 0.01) than the percentage of ESPIN-positive melanocytic nevi (Fig. 4C).

Discussion

We first demonstrated that the expression of Espin in skin melanomas is definitely higher than that in benign melanocytic tumors in RET-mice. Because our previous study showed that melanomas developed from benign melanocytic tumors in RET-mice (9), the results suggest that increased Espin expression level in melanoma is associated with malignant transformation. We also demonstrated that levels of ESPIN expression in melanoma cell lines and primary and metastatic melanomas are higher than those in NHEM and melanocytic nevi, respectively, in humans. These results suggest that ESPIN could be a potential biomarker for primary and metastatic melanomas.

We then examined the biologic significance of increased Espin expression in melanoma cells. We showed that Espin was colocalized with F-actin in lamellipodia. Lamellipodia are formed when cancer cells migrate on an extracellular matrix (ECM) such as the type I collagen or fibronectin matrix (16, 17). The structure is induced in many biologic processes including cell migration and drives progression of cancer invasion and metastasis via dynamic remodeling of the actin cytoskeleton (18). Because various actin-binding proteins constructing lamellipodia were previously reported to play an important role in cancer invasion (14), we hypothesized that Espin regulates the migration and invasion of melanoma cells. We found localization of Espin at lamellipodia in migrating melanoma cells on the type I collagen matrix as an ECM in addition to decreased migration activity in Espin-depleted melanoma cells with cortical thick stress fibers, which inhibit cell motility (18), in the wound-healing assay. Together with the results showing decreased invasion activity in Espin-depleted melanoma cells, these results suggest that Espin regulates the dynamics of actin polymerization at lamellipodia and is one of the drivers of melanoma cell motility.

Cells in the process of migration must acquire a spatial asymmetry enabling them to turn intracellularly generated forces into net cell body translocation (19). One manifestation of this asymmetry is a polarized morphology, which is clearly distinct between the cell front and cell rear (19). Because central regulators of the cell front are Rho family small GTP-binding proteins (Rho GTPases), including Rac and Rho, they are pivotal regulators of actin and adhesion organization and control the formation of lamellipodia (20). Rac1 is a direct regulator of the organization of lamellipodial structure (8, 16). A previous study showed that Rac1 is downstream of signaling from the complex of paxillin, FAK, and vinculin in focal adhesions and cell–cell contacts (8). FAK is not only a central regulator in focal adhesions but also a critical player in melanoma cell motility via interaction with the ECM and subsequent activation of downstream signals toward a
malignant phenotype (8). Because our results showed decreased levels of FAK, paxillin, and Rac1 activities and vinculin expression in Espin-depleted cells with disruption of lamellipodia, Espin may be associated with lamellipodia formation via regulation of FAK/paxillin/vinculin/Rac1 signaling in melanoma cells. Together with our results showing an approximately 75% decrease of lung metastasis in Espin-depleted cells inoculated in nude mice, the results indicated that Espin is a molecular target for therapy for metastatic melanoma.

In summary, we demonstrated for the first time that Espin/ESPIN is expressed in melanoma and affects metastasis of melanoma by regulation of migration and invasion with modulation of FAK/paxillin/vinculin/Rac1 signaling. Our results indicate that ESPIN is potentially useful for therapy for metastatic melanoma as well as a biomarker for melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Yanagishita, I. Yajima, Y. Matsumoto, M. Kato
Development of methodology: T. Yanagishita, I. Yajima, M. Kumakura
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yanagishita, M. Kumasaka, T. Tsuzuki, M. Kato

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): T. Yanagishita, I. Yajima, Y. Kawamoto, T. Tsuzuki, D. Watanabe, M. Kato

Writing, review, and/or revision of the manuscript: T. Yanagishita, I. Yajima, M. Kumasaka, T. Tsuzuki

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Yanagishita, I. Yajima, M. Kumasaka, T. Tsuzuki

Study supervision: T. Yanagishita, Y. Matsumoto, D. Watanabe, M. Kato

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References


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