Ezrin as a Prognostic Indicator and Its Relationship to Tumor Characteristics in Uveal Malignant Melanoma

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PURPOSE. Immunohistochemistry was used to investigate whether uveal malignant melanoma expresses ezrin, a protein involved in cell migration and cell recognition by acting as a linker between the plasma membrane and actin cytoskeleton. Also investigated was whether ezrin immunoreactivity correlates with survival prognosis.

METHODS. A monoclonal antibody, 3C12, that reacts with the carboxy-terminal part of ezrin was used in retrospective analysis of a population-based cohort of 167 consecutive choroidal and ciliary body melanomas in eyes enucleated from 1972 through 1981, with a median follow-up of 22 years.

RESULTS. Ezrin immunoreactivity in tumor cells was graded negative in 47 (36%) melanomas, positive in 74 (57%), and strongly positive in 9 (7%). The immunoreactivity tended to be homogenous throughout the tumor, with focal concentrations along the cell surface. Positive reaction was significantly associated with high microvascular density (P < 0.001) and presence of macrophages (P < 0.001), but not with tumor size, cell type, or microvascular loops and networks. The 10-year melanoma-specific survival was significantly associated with ezrin immunoreactivity (P = 0.018). After adjustment by Cox regression for tumor size, cell type, microvascular loops and networks, and microvascular density, a clinically meaningful 0.15 difference in 10-year melanoma-specific survival persisted.

CONCLUSIONS. The presence of ezrin immunoreactivity in uveal malignant melanoma is associated with higher mortality and with two independent high-risk characteristics: microvascular density and number of infiltrating macrophages. Further experimental studies on the interrelationship of these three factors may shed light on the progression of uveal melanoma and perhaps that of other cancers. (Invest Ophthalmol Vis Sci. 2001;42:2442–2449)

Ezrin, radixin, and moesin form the ERM protein family, which mediates interaction between actin microfilaments and cell membranes.1–3 Ezrin is a phylogenetically conserved 80-kDa phosphoprotein isolated from microvilli and previously also called cytovillin.4 It tethers actin filaments to the plasma membrane on one hand and anchors membrane proteins at specific sites on the other, thus enabling cells to maintain specialized functions in defined surface environments.5,6 Blocking the function of ezrin and other ERM proteins prevents formation of microvilli and other membrane protrusions.7–9 Ezrin is regulated by growth-factor-induced phosphorylation.10–12 It is widely expressed by epithelial cells, but also by several nonepithelial cell types.1

Because ezrin is a protein that participates in cell migration and cell recognition by the immune system, it may have an impact on tumor progression and development of metastasis.9,13–15 In cell culture, transformation of fibroblasts is associated with increased expression, hyperphosphorylation, and redistribution of ezrin.16 In surgical specimens epithelial tumors, renal cell adenocarcinoma, and stromal cells of capillary hemangioblastoma express ezrin detectable in immunohistochemistry.16–17 The expression of ezrin in cell lines of endometrial, pancreatic, and colorectal carcinoma has suggested that it has a role as a modulator of tumor cell morphology, migration, and invasion.9,14,15 A closely related protein, merlin, is a tumor suppressor protein inactivated in schwannomas and meningiomas.18

When charting the presence of ezrin in the human retinal pigment epithelium19 we observed that a subset of uveal malignant melanomas were immunoreactive with monoclonal antibody (mAb) 3C12 to ezrin. Given the potential pathogenic role of ezrin in malignant cells, we undertook a study to investigate in a larger, population-based data set whether uveal melanomas express ezrin, whether ezrin immunoreactivity is correlated with prognosis, and which tumor characteristics are associated with the presence of ezrin immunoreactivity. To our knowledge, the present report is the first clinicopathologic study that links ezrin to tumor-specific survival.

MATERIALS AND METHODS

The primary goal was to determine whether the presence of ezrin in uveal melanoma is associated with prognosis. The secondary purpose was to chart association between ezrin immunoreactivity and other tumor characteristics—in particular, microvascular factors and cell type.

Study Population and Exclusion Criteria

A previously validated, consecutive sample of 167 patients who had undergone enucleation for choroidal or ciliary body melanoma between 1972 and 1981 was analyzed.20,21 The patients were ascertained from diaries of the Ophthalmic Pathology Laboratory, Helsinki University Central Hospital. During the study period, enucleation was the standard treatment for all but the smallest uveal melanomas, and all tumors in enucleated eyes in the district were submitted to this laboratory, making the present series essentially unselected and representative of all uveal malignant melanomas treated. The Declaration of Helsinki was adhered to throughout the study.

Follow-up data for this cohort of patients was updated to December 1999 by previously described routines.20 Complete data, with a median follow-up time of 22 years (range, 18–26 years) for patients still alive, was available in 166 patients. Altogether, 50 (63%) of 80 deaths due to uveal melanoma, and all 9 (100%) deaths due to other cancers were reconfirmed by immunohistochemistry.20 In addition, 14 melanoma-related deaths had been confirmed by fine-needle aspiration biopsy.
Assessment of Ezrin Immunoreactivity

Melanomas that were more than 50% necrotic (15 tumors) or in which less than 50% of the original tumor remained (16 tumors) were excluded from the analysis. Two blocks could not be relocated, leaving 134 patients to immunohistochemistry (inclusion rate, 80%). The paraffin blocks were cut at 5 μm and the slides were randomly coded by an outside technician. The code was broken only after immunohistochemical and survival data were ready for analysis, all investigators being masked to the outcome of individual patients until that time. Sections were mounted on chromium-gelatin–coated glass slides (0.05 g potassium chromium[III]sulfate dodecahydrate and 0.5 g gelatin in 100 ml distilled water).

Immunostaining for ezrin was performed by the avidin-biotinylated peroxidase complex method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) as described previously in detail.22 The primary murine IgG mAb to human ezrin (clone 3C12, ascites, diluted 1:1000) was used after antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes at 95°C. This mAb, raised against the carboxyl-terminal part of ezrin (amino acids 362-585), detects a single 80-kDa band compatible with ezrin in immunoblot analysis and does not cross-react with moesin and radixin.17 An unrelated murine IgG mAb (Clone X63, ascites, diluted 1:350; American Type Culture Collection, Manassas, VA) was used as a negative control.

To enable evaluation of immunoreaction in pigmented tumors, the peroxidase reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride and, regardless of the grade of pigmentation, melanin was then bleached with 3.0% (vol/vol) hydrogen peroxide and 1.0% (wt/vol) disodium hydrogen phosphate, as described previously.23 This sequence obviated any problems of altered antigenicity that may occur if bleaching is done before immunostaining.

Assessment of Microvascular Factors

Closed microvascular loops and microvascular networks, consisting of at least three back-to-back loops, were identified according to Folberg et al.,25,26 from sections bleached with potassium permanganate and oxalic acid and stained with periodic acid-Schiff without counterstain, as described previously. Sections were viewed under a green filter (Wratten No. 58; Eastman Kodak, Rochester, NY).

Microvessels were identified with the monoclonal antibody QBEND/10 to the CD34 epitope of endothelial cells (lot 121202, diluted 1:25; Novocastra Laboratories, Newcastle-upon-Tyne, UK). Microvascular density (MVD) was determined from the most highly vascularized area, by using an eyepiece with an etched graticule corresponding to 0.313 mm² (WK 10×/20L-H; Olympus, Tokyo, Japan).21 Any immunolabeled channel, separate from an adjacent one and totally inside the graticule or touching its top or left border, was counted as a microvessel.21,27

A set of uveal melanomas not included in the study was initially immunostained with mAb 3C12. They were graded semiquantitatively into three groups under a double-headed microscope by two investigators: negative (no or only a few convincingly immunopositive tumor cells; Figs. 1A, 1B), positive (faint to moderate, easily recognizable positive granular immunoreaction in the majority of tumor cells; Figs. 1C, 1D), and strongly positive (stronger than average granular immunoreaction in the majority of tumor cells; Figs. 1E, 1F). The retina and choroid showed variable background. Neoplastic cells were graded immunopositive only if the reaction intensity was indisputably stronger than the background. Conjunctival epithelium and retinal pigment epithelium acted as an internal positive control for ezrin.19,24
Statistical Analysis

All analyses were performed on computer (PC-90; BMDP Statistical Software, Cork, Ireland; and StatXact-3; Cytel Software, Cambridge, MA). The Fisher exact test and χ² test were used to compare proportions in unordered contingency tables. Kruskal-Wallis and Jonckheere-Terpstra tests were used to compare proportions in singly and doubly ordered contingency tables, respectively. A weighted κ statistic was used to estimate chance-corrected interobserver agreement. All tests were two-tailed and used exact probability distributions.

Univariate analysis of survival was based on the Kaplan-Meier product-limit method, and melanoma-specific survival was compared with the Mantel-Cox test, which gives equal weight to the entire survival curve. Because follow-up times were very long, making it possible that early and late mortality may depend on a different set of variables, survival was alternatively compared with the Breslow test, which emphasizes differences in early survival. Trend tests were used if the categories analyzed were ordered. Patients judged to die of other causes were censored at time of death. To guard against the possibility that these patients were more or less likely to have progression than other patients, all-cause mortality was also analyzed. Because all-cause mortality after extended follow-up always approaches 100%, the Breslow test was the primary statistic in comparing all-cause mortality. Equality of follow-up was ascertained by comparing Kaplan-Meier curves with reverse censoring. Power analysis by computer simulation showed that the present study had an 80% chance of detecting a 0.25 difference in 10-year survival as significant.

Immunoreactivity with mAb 3C12 to ezrin was primarily analyzed as a three-category variable as planned a priori (negative, positive, strongly positive). After the data had suggested that difference in the intensity of positive immunoreaction may be due to technical factors, the variable was collapsed in two categories (negative, positive) as a secondary post hoc analysis. Cell type was analyzed according to the presence or absence of epithelioid cells, and tumor location according to the presence or absence of ciliary body involvement. Microvascular patterns were analyzed as a three-category variable that considered networks to be an advanced stage of loops (no loops, loops without networks, networks) formed of at least three back-to-back loops. The number of tumor-infiltrating macrophages was graded from sections immunostained with mAb PG-M1 (Dakopatts, Glostrup, Denmark) to the CD68 epitope and analyzed in three categories (few, moderate, many). Some sections were also studied with mAb KP1 to CD68 epitope (Dakopatts).

Cox proportional hazards regression was used to adjust the survival for the effect of previously identified independent predictors of prognosis. The tumor's largest basal diameter (LBD) and MVD were analyzed as a three-category variable, was not significantly associated with survival. The presence of immunoreactivity with mAb 3C12, analyzed as a three-category variable, was not significantly associated with LBD (Fig. 3A), presence of epithelioid cells, or presence of microvascular loops and networks (Table 1). In contrast, melanomas with high MVD (Fig. 3B; P = 0.0004, Jonckheere-Terpstra test), high number of tumor-infiltrating macrophages (P = 0.0001), and heavy pigmentation (P = 0.026) were significantly more often labeled with mAb 3C12 to ezrin than were tumors that did not show these characteristics. The results are comparable when presence of ezrin is analyzed as a two-category variable (Table 1), except that association with the degree of pigmentation loses statistical significance (P = 0.13, Kruskal-Wallis test).

Univariate Analysis of Survival

At the end of the follow-up, 37 (22%) of 167 patients were alive without evidence of recurrent melanoma, 80 (48%) had died of metastatic uveal melanoma, 49 (29%) had died of other causes, and 1 (1%) had died of unknown cause.

The 10-year cumulative melanoma-specific probability of survival was 0.84 for melanomas not immunoreactive with mAb 3C12, 0.43 for tumors that were immunoreactive, and 0.63 for tumors that were strongly immunoreactive (Fig. 3C; P = 0.018 and P = 0.010, Mantel-Cox and Breslow tests for trend, respectively). Pairwise comparisons showed this difference to be due to higher mortality of patients with ezrin-immunopositive tumors compared with patients with immunonegative ones (P = 0.0016 and P = 0.001, Mantel-Cox and Breslow tests with Bonferroni correction, respectively). No difference was found between negative and strongly immunopositive melanomas (P = 0.62 and P = 0.60) or between immunopositive and strongly positive melanomas (P = 0.62 and P = 0.58). The 10-year cumulative all-cause survival also differed significantly among the three groups (0.64 for negative, 0.32 for positive, and 0.56 for strongly positive melanomas, P = 0.028 and P = 0.061, Breslow and Mantel-Cox tests for trend, respectively).

Because a difference in survival between immunopositive and strongly immunopositive melanomas was not observed and the group with strong immunostaining was small compared with the other two categories, the two groups with

Pattern of Ezrin Immunoreactivity

A granular, diffuse intracytoplasmic immunoreaction which frequently concentrated focally along the cell surface was obtained with mAb 3C12 in uveal melanoma cells (Figs. 1D, 1F). The two investigators agreed on the grade of immunoreactivity in 82 (65%) of the 130 specimens (95% confidence interval [CI], 54–71). The interobserver agreement (weighted κ) was 0.50 (95% CI 0.36–0.63). After consensus, 47 (36%, 95% CI 28–45) melanomas were classified as negative, 74 (57%, 95% CI 48–66) as positive, and 9 (7%, 95% CI 3–13) as strongly positive for ezrin. Some discrepancies were due to presence of large numbers of immunonegative lymphocytes (Figs. 2A, 2B). A two-category discrepancy occurred only once.

The labeling intensity ranged from negative to strongly positive (Figs. 1A–F). When positive, the immunoreaction tended to be homogeneous throughout the tumor (Figs. 1D, 1F). However, in 4 of the 130 melanomas (3%, 95% CI 1–8) areas of weakly and strongly immunopositive tumor cells were found adjacent to each other (Figs. 2C, 2D). Adjacent sections immunostained with mAb PG-M1 and KP1 suggested that putative macrophages were typically not labeled by mAb 3C12 (Figs. 2E, 2F).

Association between Ezrin Immunoreactivity and Other Tumor Characteristics

The presence of immunoreactivity with mAb 3C12, analyzed as a three-category variable, was not significantly associated with LBD (Fig. 3A), presence of epithelioid cells, or presence of microvascular loops and networks (Table 1). In contrast, melanomas with high MVD (Fig. 3B; P = 0.0004, Jonckheere-Terpstra test), high number of tumor-infiltrating macrophages (P = 0.0001), and heavy pigmentation (P = 0.026) were significantly more often labeled with mAb 3C12 to ezrin than were tumors that did not show these characteristics. The results are comparable when presence of ezrin is analyzed as a two-category variable (Table 1), except that association with the degree of pigmentation loses statistical significance (P = 0.13, Kruskal-Wallis test).
positive immunoreactivity were combined. In this post hoc analysis, the 10-year cumulative melanoma-specific probabilities of survival were 0.84 and 0.45 for melanomas unreactive and reactive with mAb 3C12, respectively (Fig. 3D; \( P \leq 0.0014 \) and \( P \leq 0.0005 \), Mantel-Cox and Breslow tests, respectively), and probabilities of all-cause survival were 0.64 and 0.34, respectively (Fig. 3E; \( P \leq 0.004 \) and \( P \leq 0.024 \), Breslow and Mantel-Cox tests, respectively).

**Multivariate Analysis of Melanoma-Specific Survival**

Immunoreactivity with mAb 3C12, whether classified in three or two categories, fulfilled the assumption of proportional hazards (\( P = 0.34 \) and \( P = 0.11 \), respectively). The two-category variable was used in multivariate modeling. By univariate Cox regression, immunoreactivity for ezrin was significantly associated with melanoma-specific mortality (Table 2; \( P = 0.016 \), likelihood ratio test). Ciliary body involvement, LBD, degree of pigmentation, presence of epithelioid cells, high number of macrophages, presence of microvascular loops and networks, and MVD were also significantly associated with an increased risk of melanoma-related death (Table 2).

Multivariate Cox regression was used to adjust the survival difference between mAb 3C12 immunonegative and immunopositive melanomas for the effect of previously identified independent predictors of prognosis (Table 2). These included the presence of epithelioid cells (\( P = 0.010 \); hazard ratio [HR] 2.05), large tumor size (\( P = 0.002 \); HR 1.13 for each millimeter increase in tumor diameter), high MVD (\( P = 0.0014 \); HR 1.27 for each unit increase in square-root–transformed vessel count), and the presence of microvascular loops and networks (\( P = 0.054 \); HR 1.13 for each millimeter increase in tumor diameter), as well as the number of macrophages modeled as a stratification variable. After adjustment, the predicted survival difference of patients with immunonegative and immunopositive melanomas at 10 years was 0.15 (0.72 vs. 0.57; \( P = 0.093 \), HR 1.70; Fig. 3F).

**DISCUSSION**

In the present series, two thirds of choroidal and ciliary body malignant melanomas were immunoreactive with mAb 3C12 to ezrin, an actin-plasma membrane protein linker implicated in cell motility, intracellular signaling, intercellular recognition, and cell adhesion—all phenomena that are involved in cancer progression.\(^3,8,9,14\) Ezrin also redistributes cell adhesion molecules recognized by natural killer cells.\(^13\) Typically, diffuse granular cytoplasmic immunoreaction was observed in most of the uveal melanomas cells, often with focal concentrations along the cell surface, and only rarely did a tumor contain a mixture of areas distinctly negative and positive for ezrin. The latter may represent clones of cells that have, for example, different migratory properties, but the function and activity of ezrin is unsolved both in vitro and in vivo in uveal melanoma. The diffuse cytoplasmic immunoreactivity in melanoma differs from the typical reactivity of epithelial cells in which ezrin is predominantly segregated under the plasma membrane. However, after dephosphorylation, ezrin is redistributed through-
out the cytoplasm. Diffuse ezrin immunoreactivity is found, for example, in reactive retinal pigment epithelial cells, even though it is normally confined to their microvilli and apical cytoplasm.

As regards other high-risk characteristics of uveal melanoma, immunoreactivity for ezrin was significantly associated with high MVD, measured from the most vascularized area of the section or “hot spot.” Even though MVD is only a rough index of tumor vascularity, high MVD is convincingly associated with poorer prognosis in uveal melanoma.

FIGURE 3. Association between immunoreactivity with mAb 3C12 to ezrin, tumor characteristics, and survival among 130 patients with choroidal and ciliary body malignant melanoma. Immunoreactivity for ezrin was not associated with tumor LBD (in millimeters; A), but was significantly associated with globally highest MVD (B), obtained with mAb QBEND/10 to the CD34 epitope (Jonckheere-Terpstra test). Melanoma-specific mortality differed among patients with ezrin immunonegative, immunopositive, and strongly immunopositive uveal melanomas (C). The difference in melanoma-specific (D) and all-cause mortality (E) was maintained if immunopositive tumors were pooled into one group for statistical analysis. Mantel-Cox test (C, D) and Breslow test (E). An adjusted survival curve predicted a trend for higher melanoma-specific mortality among patients with ezrin-immunopositive tumors even when four high-risk indicators, tumor LBD, presence of epithelioid cells, presence of microvascular loops and networks, and MVD, were controlled by Cox multivariate regression (F; likelihood ratio test). Crossbars: upper and lower 95% CIs; ticks: censored observations; numbers: patients at risk, by Kaplan-Meier survival curve (C–E).
been proposed that uveal melanoma cells may directly contribute to vasculogenesis and generate patterned tumor microvessels—in particular, microvascular loops and networks.

Aggressive uveal melanoma cells cultured in collagen gels form tubular networks that evolve into channels and sinusoids resembling loops and networks typically seen in enucleated eyes with uveal melanomas.

Aggressive uveal melanoma cells also contract collagen gels, which is considered a prerequisite for tubule formation. Cytochalasin inhibits this contraction, indicating that it depends on actin microfilaments. Overproduction of ezrin within other cell types cultured in collagen gels such as kidney epithelial cells induces production of long tubules, and ezrin mutants inhibit this actin-mediated tubulogenesis. At the tumor level, we did not observe a statistically significant association between ezrin immunoreactivity and the presence of microvascular loops and networks, however.

We have recently found that two thirds of surgically removed uveal melanomas contain moderate to high numbers of tumor-infiltrating macrophages, which are associated with the presence of epithelioid cells, high MVD, and melanoma-specific mortality. The presence of ezrin immunoreactivity in uveal melanoma cells was also associated with the number of infiltrating macrophages. Activated macrophages are one source of hepatocyte growth factor (HGF), a growth factor that is postulated to be associated with angiogenesis and cell migration. Indeed, HGF induces migration of uveal melanoma cells, if they have the transmembrane proto-oncogene c-met, a receptor of HGF. This proto-oncogene possesses intrinsic tyrosine kinase activity and phosphorylates ezrin, a downstream target of HGF. Also in the kidney epithelial cells mentioned earlier, ezrin mediates the HGF-triggered actin cytoskeleton dynamics that are required for tubulogenesis.

Regarding metastasis, the association of ezrin immunoreactivity with increased melanoma-specific mortality was statistically significant by univariate analysis. Categorization of ezrin immunoreactivity in three groups offered no advantage compared with post hoc categorization in two groups, probably because immunohistochemistry is not a quantitative method. The difference in survival between patients who had an ezrin-immunoreactive melanoma and patients with ezrin-negative melanoma was 0.39. The difference narrowed to 0.15 after adjustment for tumor LBD, presence of epithelioid cells, presence of microvascular loops and networks, and MVD, which are strong independent indicators of high tumor-specific mortality in uveal melanoma.

The magnitude of the adjusted difference is clinically and biologically significant, considering that the effect of the strongest previously known prognostic indicators has been eliminated by multivariate analysis. We did not have adequate statistical power to detect a difference of this size as statistically significant in multivariate analysis, and our results are consistent with previously known prognostic indicators.
tent with the theory that patients who have an ezrin-immunopositive uveal melanoma have a moderately poorer prognosis than those in whom tumors are not immunoreactive for ezrin, given an otherwise identical tumor. Moreover, immunohistochemical assessment of ezrin is probably an imperfect reflection of true ezrin activity, which decreases the ability to show the magnitude of the association.

References


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<th>Variable</th>
<th>Regression Coefficient (± SE)</th>
<th>Likelihood Ratio*</th>
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<th>Hazard Ratio (95% CI)</th>
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<td>Age</td>
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<td>1.078 ± 0.256</td>
<td>17.2</td>
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<td>Largest basal tumor diameter</td>
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<td>10.9</td>
<td>0.0010</td>
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<td>Tumor height</td>
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<td>0.0014</td>
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<td>16.2</td>
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<td>0.536 ± 0.325</td>
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<td>0.087</td>
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*χ² test, two-sided.
†Number of macrophages did not fulfill the assumption of proportional hazards.
‡Categories: no, 0; yes, 1.
§Categories: weak, 0; medium, 1; strong, 2.
¶Categories: no loops, 0; loops without networks, 1; networks, 2.
§Square-root-transformed single globally highest vessel count/0.313 mm² area, antibody QBEND10 to the CD34 epitope.
#Categories: negative, 0; positive, 1; strongly positive, 2.
**Categories: negative, 0; positive, 1.
††Model stratified by the number of macrophages.


