Overexpression of Epidermal Growth Factor Receptor Restricted to Macrophages in Uveal Melanoma

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Objective: To determine whether expression of the epidermal growth factor receptor (EGFR) is of prognostic value in uveal melanoma.

Methods: Thirty consecutive patients treated for primary posterior uveal melanoma by enucleation or local resection were studied. Tumors were examined for EGFR and CD68 expression by immunohistochemistry on formalin-fixed, paraffin-embedded sections. Extracted DNA from paired frozen tumor and blood samples was examined for loss of heterozygosity on chromosome 3 using polymerase chain reaction–based microsatellite analysis. Immunoreactivity for EGFR was correlated with clinical-pathological, chromosome 3, and follow-up data.

Results: Immunoreactivity for EGFR was observed in 7 (23%) of 30 uveal melanomas, but was restricted to solitary or small groups of cells with macrophage-like morphology. Immunoreactive cells were confirmed as macrophages using an antibody to the macrophage marker CD68. Chromosome 3 loss, epithelioid cells, and microvascular loops were detected in 17 (57%), 22 (73%) and 19 (63%) of the 30 tumors, respectively. Metastatic disease was detected in 5 patients (17%). No correlation was found between any of these variables and EGFR positivity.

Conclusions: The absence of EGFR immunoreactivity in tumor cells does not support the use of EGFR expression as a prognostic indicator in patients with uveal melanoma. Future EGFR studies in uveal melanoma should be interpreted with caution in view of our findings that tumor-associated macrophages can express this receptor.


THE EPIDERMAL growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, is of fundamental importance in the regulation of epithelial differentiation and proliferation. The interactions of this receptor are complex. Epidermal growth factor receptor can bind at least 6 growth factors, including EGF, transforming growth factor α, amphiregulin, heparin-binding EGF, betacellulin, and epiregulin. Binding of EGF results in receptor down-regulation, whereas transforming growth factor α prolongs EGFR expression and signalling. The EGFR appears to play a role in malignant transformation and may be a useful target for selective antitumor therapy. Overexpression of EGFR has been detected in many solid human tumors, including cancers of the head and neck, breast, lung, and bladder, and has been associated with poor prognosis, most convincingly in squamous cell carcinoma of the head and neck. Increased immunoreactivity for EGFR has also been reported in cutaneous benign and malignant cutaneous lesions of melanocytic origin.

Uveal melanoma is the most common primary intraocular malignant neoplasm in adults. The mortality rate for patients with large tumors is high because of the propensity for these tumors to metastasize to the liver. After clinical diagnosis of hepatic metastases, life expectancy is extremely poor, with a median survival time of only 7 months. Recently, in a nude mouse model, expression of EGFR was correlated with the metastatic potential of uveal melanoma cell lines and an increased capacity for these tumor cells to localize in the liver. These findings suggested that EGFR expression may be a valuable indicator of metastatic potential in uncultured uveal melanoma.

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PATIENTS, MATERIALS, AND METHODS

PATIENTS AND SPECIMENS

Thirty consecutive patients treated for choroidal and/or ciliary body melanoma by enucleation or local resection formed the basis of this study. Informed consent was obtained from all patients to collect tumor and blood samples for experimental purposes. Part of each tumor was snap frozen in liquid nitrogen and the remainder fixed with formalin and embedded in paraffin. Histological features, including cell type (spindle, epithelioid, or mixed) and the presence or absence of microvascular loops, were determined from paraffin-embedded sections of tumor stained with hematoxylin-eosin and periodic acid–Schiff. Clinical details and follow-up data were obtained from our ocular oncology database, linked with the Office of National Statistics (Southport, England) to ensure accurate mortality data.

IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded tumor tissue was examined for EGFR expression by immunohistochemistry, using a monoclonal anti-EGFR antibody (clone EGFR 113; Novacastra Laboratories Ltd, Newcastle upon Tyne, England; diluted 1:75) and a polyclonal anti-EGFR antibody (1005; Santa Cruz Biotechnology Inc, Santa Cruz, Calif; diluted 1:100). Sections were obtained from 2 tumor blocks for each patient. The titers of the primary antibody used was the serial dilution that gave intense membranous staining with negligible background in formalin-fixed, paraffin-embedded human placenta, which overexpresses EGFR.15 Tumor sections (4 µm thick) were de-waxed in xylene, then hydrated through graded alcohols to water. Subsequently, sections were submerged in 0.01-mol/L citric acid (pH, 6.0), microwaved for 18 minutes, and left to stand in this solution for an additional 15 minutes. Sections were then washed in Tris-buffered saline solution (TBS; pH 7.6) for 5 minutes and blocked using normal horse serum (Vector Laboratories Ltd, Peterborough, England). Blocking serum was removed and the sections were incubated overnight with the primary antibody at 4°C in a moist chamber. After washing in TBS, sections were incubated with a biotinylated secondary antibody (Vector Laboratories Ltd) for 30 minutes, then washed twice in TBS, and incubated with streptavidin-biotin–alkaline phosphatase complex (Dako Ltd, High Wycombe, England) for 30 minutes, all at room temperature. Sections were washed in TBS, and alkaline phosphatase–fast red substrate solution (Vector Red substrate and levamisole [Vector Laboratories Ltd] with 5 mg of fast red TR salt [Merck Ltd, Lutterworth, England] in 5 mL of TBS [pH, 8.2]) was applied for 25 minutes. Sections were washed in running tap water for 5 minutes, counterstained with hematoxylin, and mounted with an aqueous mounting medium (Glycergel; Dako Ltd). Human placenta sections were included as positive controls, and sections with replacement of the primary antibodies with an isotype control antibody or blocking peptide served as negative controls. A bleaching protocol16 with 3,3′-diaminobenzidine tetrahydrochloride as chromogen (which is resistant to hydrogen peroxide) was used for 4 sections that were heavily pigmented. Melanin was bleached by overnight incubation of sections in 1% (weight-volume ratio) disodium hydrogen phosphate solution containing 3% hydrogen peroxide. Confirmation of the identity of cells with macrophage-like morphology was determined using an antibody to the macrophage marker CD68 (Dako Ltd), as described for the anti-EGFR antibody. Dual labeling with the polyclonal anti-EGFR and monoclonal anti-CD68 antibodies was performed using an alkaline phosphatase technique. Red and blue chromogens were used for EGFR and CD68, respectively (Vector Red and Vector Blue; Vector Laboratories Ltd).

MICROSATELLITE ANALYSIS

The DNA was extracted from blood and frozen tumor sections using standard procedures.17 One frozen section of each tumor was stained with hematoxylin-eosin to confirm the histological features and that the specimen was composed of at least 90% tumor cells. Chromosome 3 alterations were detected by polymerase chain reaction–based microsatellite analysis,18 using the following 10 microsatellite markers on the p and q arms of chromosome 3 (Research Genetics, Huntsville, Ala): D3S1283 (3p26.1-3p25.2), D3S1283 (3p25-3p24.2), D3S1619 (3p24.2-3p22), D3S1029 (3p21.3-3p21.2), D3S1210 (3p14.1-3p12), D3S1271 (3cen-q13), D3S1589 (3q21), D3S1605 (3q23.1-3q23.2), D3S1580 (3q27), and D3S1311 (3q27-qter). Loss of heterozygosity (LOH) was recorded for informative markers if the intensity of a tumor allele was reduced by at least 30% relative to normal DNA.

RESULTS

The posterior uveal melanomas studied consisted of 8 spindle cell and 22 mixed/epithelioid cell tumors. The largest basal diameter of these tumors ranged from 10 to 20 mm (mean, 15.8 mm), and 4 tumors had ciliary body involvement. Microvascular loops were detected in 19 tumors (63%). Chromosome 3 alterations were identified in 17 tumors (57%). Metastases were detected in 5 patients (17%) within 8 to 21 months of primary treatment (maximum patient follow-up, 27 months); 3 of these patients have died.

Immunoreactivity for EGFR, of similar intensity to that in placenta (positive control; Figure, A), was observed in 7 (23%) of 30 uveal melanomas. The proportion of EGFR-positive tumors associated with particular clinical and histological variables was as follows: 3 (38%) of 8 spindle cell and 4 (18%) of 22 mixed/epithelioid cell tumors; 3 (16%) of 19 tumors with microvascular loops...
and 4 (36%) of 11 without microvascular loops; 3 (18%) of 17 tumors with LOH and 4 (31%) of 13 without LOH on chromosome 3; 2 (22%) of 9 tumors with a basal tumor diameter of less than 15 mm and 5 (24%) of 21 with a basal tumor diameter of at least 15 mm; and 1 (25%) of 4 tumors with ciliary body involvement and 6 (23%) of 26 without ciliary body involvement. There was no statistically significant correlation between detection of EGFR and tumor cell type, presence of microvascular loops, chromosome 3 LOH, tumor size, or ciliary body involvement (Fisher exact test). Only 1 (20%) of 5 tumors from patients with metastatic disease showed EGFR immunoreactivity, whereas all 5 tumors showed chromosome 3 LOH.

Immunoreactivity for EGFR was restricted to solitary or small groups of cells with morphology typical of macrophages rather than tumor cells (Figure, B). Immunoactive cells were confirmed as macrophages using an antibody to the macrophage marker CD68 on a section adjacent to that used for EGFR detection (Figure, C) and by dual labeling, which demonstrated colocalization of CD68 on EGFR-immunoreactive cells (not illustrated). More cells were reactive with anti-CD68 than with anti-EGFR antibody.

**COMMENT**

Overexpression of EGFR has been found in a number of solid tumor types and has been associated with poor prognosis. Recently, expression of EGFR was correlated with the hepatic spread of uveal melanoma cell lines in a nude mouse model, raising the possibility that EGFR expression may be a valuable indicator of metastatic potential in uncultured uveal melanoma. Expression of EGFR, however, had not been described in uncultured uveal melanoma, and the tumors from which the cell lines were derived were not tested. In this study, we examined the expression of EGFR in 30 uncultured primary posterior uveal melanomas. Expression of EGFR was detected in 7 tumors; however, the immunoreactive cells had morphologic features resembling macrophages rather than tumor cells and were confirmed as macrophages using an anti-CD68 antibody.

This lack of detection of EGFR on uncultured tumor cells apparently conflicts with previous findings on cell lines. The extent to which the cell lines used by Ma and Niederkorn represent the tumors from which they were derived, however, is not known. Cultured cells can diverge significantly from the original tumor, eg, by accumulation of genomic alterations, and expression of EGFR can be modulated during culture. Alternatively, a small proportion of uveal melanomas may overexpress EGFR, and these tumors may be more readily established as cell lines. In breast cancer, for example, the success rate of establishing cell lines is low, but tumors overexpressing the growth factor receptor HER-2/neu (ErbB2) are more likely to develop into continuous cell lines. High concentrations of EGF assist establishment of cell lines of uveal melanoma. However, the proportion of cells expressing EGFR was less than 6% in 4 of the 7 cell lines studied by Ma and Niederkorn. As the regulation of EGFR is complex, it is possible that prolonged culture in growth factor–containing media may have affected receptor expression. Cell lines composed purely of spindle cells had a higher percentage of EGFR-positive cells (5.3%-20.3%) than the epithelioid cell lines (1.7%-2.5%), and the incidence of metastasis in nude mice was lowest with the epithelioid cell lines. This is the opposite of what might be expected from human studies of tumor cell type and prognosis in patients with uveal melanoma.
It cannot be ruled out that occasional uncultured uveal melanoma tumor cells express EGFR, as the proportion of cells expressing EGFR was low in some of the cell lines studied by Ma and Niederkorn. In our study, to verify the lack of tumor cell immunoreactivity with the monoclonal anti-EGFR antibody, we analyzed sections from a second block of each tumor using an additional polyclonal anti-EGFR antibody. However, tumor cells expressing EGFR were still not detected.

Although long-term survival data are not yet available, detectable metastases developed in 5 of the patients studied, and 3 of these have died. Tumor cells showing EGFR immunoreactivity were not detected in the uveal melanomas from these patients, and only 1 lethal tumor showed EGFR immunoreactivity in macrophages. The lack of detection of EGFR in tumor cells from these patients suggests that expression of this receptor is not a sensitive prognostic indicator in posterior uveal melanoma. Furthermore, it is expected that many other patients in our study will ultimately die of metastatic disease. Microvascular loops and chromosome 3 LOH, which are strongly associated with a poor prognosis, were detected in a high proportion of the tumors. Indeed, chromosome 3 LOH was detected in 17 (57%) of 30 tumors, a prevalence similar to that reported by other groups, and all of the tumors from which metastases were detected showed this alteration, supporting previous findings that chromosome 3 loss is a sensitive prognostic indicator.

Macrophages immunoreactive for EGFR have been reported in other lesions and may be involved in their pathogenesis. It appears that macrophages may influence angiogenesis, a process that is critical for tumor growth and metastasis. Macrophage infiltration in breast tumors and gliomas has been associated with angiogenesis and poor prognosis, and anti-EGFR antibody has been shown to inhibit angiogenesis in a model of transitional cell carcinoma of the bladder. Activated macrophages may play a role in the neovascularization of cutaneous melanoma, as a correlation between increased macrophase index, malignancy, and high vascular grade has been reported in these tumors. In our study, microvascular density was not determined, but other workers, using endothelial cell markers, previously have reported this variable to be of prognostic significance in uveal melanoma.

We are now performing further studies of macrophage and microvascular patterns to determine the relationship between these variables in uveal melanoma. The presence of microvascular loops, also an indicator of poor prognosis in uveal melanoma, was assessed in our study. There was no correlation between the presence of EGFR-positive macrophages and microvascular looplike structures; however, it has been shown that endothelial cells do not line these periodic acid–Schiff–positive structures. In summary, the absence of EGFR immunoreactivity in tumor cells does not support the use of EGFR expression as a prognostic indicator in patients with uveal melanoma. In view of our findings that EGFR-immunoreactive cells in uveal melanoma were macrophages, we suggest that EGFR studies in uveal melanoma be interpreted with caution.

Accepted for publication August 5, 2000.
This study was supported by a grant from the University of Liverpool Research Development Fund, Liverpool, England.

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REFERENCES

Congratulations to the winner of our November quiz, Dr Venkatasubramaniam, of Bangalore, India. The correct answer to our November challenge was mycosis fungoides. For a complete discussion of this case, see the Clinicopathologic Report section in the December ARCHIVES (Lois N, Hiscott PS, Nash J, Wong D. Immunophenotypic shift in a case of mycosis fungoides with vitreous invasion. Arch Ophthalmol. 2000;118:1692-1694).

Figure 1. Fundus photograph. A, Right eye shows no abnormalities. B, Left eye shows haziness due to diffuse vitreous opacities.

Be sure to visit the Archives of Ophthalmology World Wide Web site (http://www.archophthalmol.com) and try your hand at our Clinical Challenge Interactive Quiz. We invite visitors to make a diagnosis based on selected information from a case report or other feature scheduled to be published in the following month’s print edition of the ARCHIVES. The first visitor to e-mail our Web editors with the correct answer will be recognized in the print journal and on our Web site and will also receive a free copy of the book One Hundred Years of JAMA Landmark Articles.