

# Receptors for the Liver Synthesized Growth Factors IGF-1 and HGF/SF in Uveal Melanoma: Intercorrelation and Prognostic Implications

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**PURPOSE.** Uveal melanoma disseminates preferentially to the liver. The mechanism for this homing is largely unknown, but growth factors synthesized in the liver may be involved. The present study was undertaken to investigate the possible relationship between cell surface receptors for two such growth factors: the c-Met proto-oncogene, which constitutes the receptor for hepatocyte growth factor/scatter factor (HGF/SF), and the insulin-like growth factor 1 receptor (IGF-1R). Their role as a prognostic factor was also clarified.

**METHODS.** Paraffin-embedded tumor specimens from 132 patients with primary uveal melanoma were analyzed by using well-established specific antibodies against c-Met and IGF-1R. The intercorrelation of receptor expression and association with melanoma-related survival of patients were determined by univariate and multivariate analyses.

**RESULTS.** Whereas the expression of both IGF-1R and c-Met was significantly associated with melanoma-specific mortality by univariate analysis ( $P = 0.004$  and  $P = 0.007$ , respectively) only IGF-1R showed independent prognostic value by multivariate analysis,  $P = 0.004$ . The prognostic value of IGF-1R was stronger than such currently used prognostic parameters as tumor cell type and tumor diameter ( $P = 0.021$  and  $P = 0.026$ , respectively). The expression patterns of the two growth factors receptors were weakly intercorrelated.

**CONCLUSIONS.** In conclusion, the data suggest that the receptors for IGF-1 and HGF/SF may play a role in the spread of uveal melanoma and its affinity to the liver. The strong correlation between IGF-1R expression and melanoma-specific mortality points to the use of IGF-1R as a prognostic tool. (*Invest Ophthalmol Vis Sci.* 2005;46:4372-4375) DOI:10.1167/iov.05-0322

Uveal melanoma is the most common primary intraocular malignant tumor in adults, with an annual incidence of 8.4 to 11.7 cases per million in whites.<sup>1,2</sup> Tumor-related survival has not improved, and some 30% to 50% of patients ultimately succumb to metastatic disease.<sup>3-5</sup> Uveal melanoma behaves very much differently from the more frequently occurring

cutaneous melanoma; specifically, it spreads preferentially to the liver. The reason for this liver-homing is largely unknown, but it is conceivable that hepatic environmental factors may be implicated in the growth, dissemination, and progression of this malignancy.

The insulin-like growth factor (IGF-1) that binds to the IGF-1 receptor (IGF-1R) is mainly produced in the liver. It has been shown to be crucial for tumor transformation, maintenance of malignant phenotype, promotion of cell growth, and prevention of apoptosis.<sup>6-8</sup> A recent study of a limited number of primary uveal melanomas demonstrated that IGF-1R is variably expressed and suggested an association between IGF-1R expression and reduced survival.<sup>9</sup> The hepatocyte growth factor/scatter factor (HGF/SF) is another growth factor produced in the liver and exerts its biological effects through binding to the plasma membrane receptor c-Met. The activation of this receptor by HGF/SF ligand can induce proliferation, motility, adhesion, and invasion of tumor cells. Also, the HGF/SF activation of the c-Met tyrosine kinase has been thought to be one of the key factors influencing the events of tumor progression. Several studies have reported amplification and overexpression of c-Met in cancer cells.<sup>10</sup> In uveal melanoma, Hendrix et al.<sup>11</sup> reported that cells with metastatic phenotype express c-Met.

The present study was designed to clarify the possible relationship between c-Met and IGF-1R expression and their potential role as prognostic predictors in uveal melanoma, by a large number of consecutive cases of primary tumors with complete histopathological and clinical data.

## MATERIAL AND METHODS

### Patients and Tumors

Primary uveal melanoma specimens, fixed in formaldehyde and paraffin embedded, from 152 consecutive patients were available for this study. All patients had undergone enucleation at a date when this treatment was the sole therapeutic option available in Sweden. Twenty lesions were deemed extensively necrotic (defined as >50% of cells necrotic) and excluded from further evaluation, leaving 132 lesions to be immunostained as outlined herein. These lesions were from 55 female and 77 male patients (average age, 63 years; range, 25-85). Although 55 patients succumbed to metastatic uveal melanoma, 26 patients died of causes unrelated to uveal melanoma, and 51 patients were alive at the end of follow-up. The mean follow-up time was 4.8 years (range, 0.08-7.6) for patients who died of disseminated uveal melanoma, 8.2 years (range, 0.4-21.7) for patients who died of other causes, and 12.3 years (range, 1.1-21) for the survivors. All specimens had been independently examined by an experienced ophthalmic pathologist and assessed for tumor location within the uvea, tumor cell type, and largest tumor diameter and tumor height. Briefly, specimens included 11 ciliary body tumors, 53 anterior choroidal tumors, and 65 tumors confined to the posterior choroid. Fifty-five tumors were spindle cell melanoma, 23 tumors were of a mixed cell type, 9 were epithelioid melanoma, and 45 were partially necrotic. The median largest tumor diameter was 12.5 mm (range, 6-22 mm), and the

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median tumor height was 7.3 mm (range, 2–17 mm). The study conformed to the tenets of the Declaration of Helsinki and was approved by the ethics committee of the Karolinska Institutet.

### Antibodies

A rabbit polyclonal antibody directed to the human IGF-1R (N-20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and a mouse monoclonal antibody directed to human c-Met (NCL-cMET) was provided by Immunkemi (Novocastra Ltd., Newcastle-upon-Tyne, UK).

### Immunostaining Protocol

Immunostaining of tissue sections was performed by using the standard avidin-biotin complex technique (ABC complex; Vector Laboratories, Burlingame, CA). Briefly, 4- $\mu$ m tissue sections were cut from each of the selected 132 paraffin-embedded tumor specimens. Tissue sections were deparaffinized and rehydrated. The samples were bleached with hydrogen peroxide-disodiumhydrogenphosphate at room temperature—IGF-1R samples overnight and c-Met 3 hours only, because they were subjected to antigen retrieval according to the manufacturer's instructions.

After antigen retrieval (c-Met) tissue sections were rinsed in Tris-buffered saline (TBS, pH 7.6) and incubated with blocking serum (1% bovine serum albumin) for 20 minutes at room temperature followed by an overnight incubation at 8°C with an excess of anti c-Met antibody or anti-IGF-1R antibody. Biotinylated anti-mouse monoclonal IgG and anti-rabbit polyclonal IgG antibodies were added for c-Met and IGF-1R, respectively, and incubation was continued for an additional 30 minutes at room temperature, followed by application of the ABC complex. The peroxidase reaction was developed for 6 minutes at room temperature with 0.6 mg/mL 3'3'-diaminobenzidine tetrahydrochloride (DAB) with 0.03% hydrogen peroxide. Counterstaining was performed with Mayer's hematoxylin. TBS was used for rinsing between the different steps. Appropriate positive and negative controls were included.

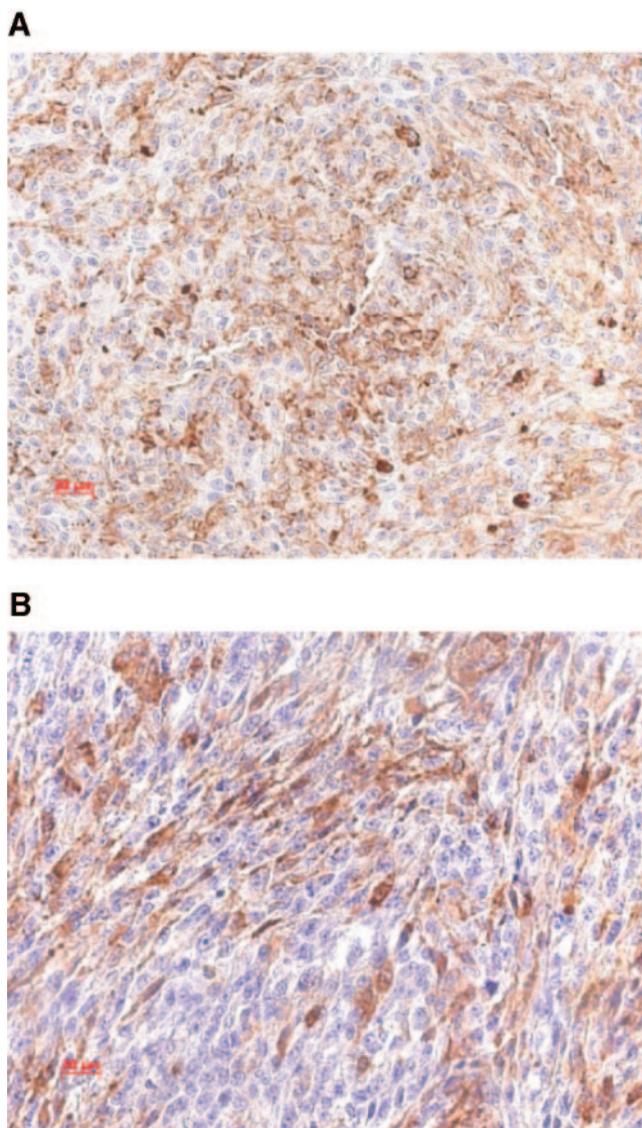
### Staining Assessment

All stained cells were considered positive, irrespective of staining intensity. The immunoreactivity was differentiated from melanin pigment, as reported previously.<sup>12</sup> Specifically, the dark brown, finely granular appearance of the immune-reaction product was separated from the coarse granular appearance of the melanin pigment. We scored the results of c-Met and IGF-1R immunoreaction as *negative* when no staining was present, *low* when less than 10% of cells were stained, *moderate* when 10% to 50% of cells were stained, and *high* when more than 50% of cells were immunoreactive. At a later stage, and without knowledge of the initial result, the same observer (ME) repeated the assessment for a random sample of slides from 30 uveal melanoma specimens. These slides were also independently assessed by an experienced ophthalmic pathologist (SS) using the same grading system. The interobserver reproducibility according to the  $\kappa$ -test was 0.73 (95% CI, 0.54–0.92), and the intraobserver reproducibility was 0.69 (95% CI, 0.49–0.89). Both observers were masked to results from earlier assessments and to survival data.

To verify that epitopes would withstand any inadvertently prolonged exposure to formaldehyde, small pieces (approximately 2 mm<sup>3</sup>) of fresh uveal melanoma tissue was fixed in 10% formaldehyde for periods of 1, 2, 4, 8, 16, and 32 days, respectively, and paraffin embedded. Sections were then cut from each paraffin block and immunostained for IGF-1R or c-Met as just described. The tumor cells retained their staining intensity and pattern from days 1 through 32.

### Statistical Analysis

Survival data without loss to follow-up were obtained for all patients from the Swedish National Causes of Death registry. The survival time from the date of enucleation to death or to the end of 1992 was considered censored if the patient was alive at the end of the study or



**FIGURE 1.** (A) Immunostaining for c-Met protein in uveal melanoma showing a *brown* reaction product confined to the cytoplasm of more than 50% of tumor cells, scored as a high proportion of immunoreactive cells. (B) Immunostaining for IGF-1R in uveal melanoma showing a *brown* reaction product confined to the cytoplasm of 10% to 50% of tumor cells, scored as a moderate proportion of immunoreactive cells.

if the patient had died of any cause that was not melanoma related. Kaplan-Meier survival curves were plotted for each semiquantitatively assessed group of immunoreaction of c-Met and IGF-1R. Univariate and multivariate Cox regression models were used to assess the prognostic values of the covariates. The significance level was set at 0.05. All calculations were performed on computer (Statistica, ver. 5.5; Statsoft Inc., Tulsa, OK; MedCalc software; MedCalc Inc., Mariakerke, Belgium).

### RESULTS

The expression of c-Met and IGF-1R was analyzed on paraffin-embedded slides, with antibodies that have been shown to be specific and sensitive.<sup>9,13,14</sup>

A distinct immunoreactivity confined to the plasma membrane and cytoplasm in a variable percentage of tumor cells was present in 114 (86%) of 132 slides immunostained for the c-Met receptor (Fig. 1A) and in 97 (73%) of 132 slides immu-

TABLE 1. Univariate Cox Regression for Covariates of Potential Prognostic Value

| Covariate          | Scoring or Unit                                   | Wald  | P      | Hazard Ratio (95% CI) |
|--------------------|---|-------|--------|-----------------------|
| IGF-1 R expression | Neg.; <10%; 10%-50%; >50% stained cells           | 8.27  | 0.004  | 1.49 (1.29-1.96)      |
| Tumor cell type    | Spindle, mixed, epithelioid, partially necrotic   | 11.46 | <0.001 | 1.41 (1.15-1.73)      |
| Tumor diameter     | Millimeters                                       | 13.91 | <0.001 | 1.18 (1.08-1.30)      |
| c-Met expression   | Neg.; <10%; 10%-50%; >50% stained cells           | 7.36  | 0.007  | 1.46 (1.11-1.93)      |
| Tumor location     | Posterior choroid, anterior choroid, ciliary body | 0.31  | 0.580  | 0.88 (0.57-1.36)      |
| Patient's age      | Years   | 3.91  | 0.048  | 1.02 (0.99-1.05)      |
| Patient's sex      | Male, female                                      | 1.20  | 0.274  | 0.73 (0.42-1.36)      |

nostained for IGF-1R (Fig. 1B). In slides featuring c-Met-immunopositive tumor cells, 39 (30%) showed less than 10% immunopositive cells, 36 (27%) slides had 10% to 50% immunoreactive cells and 39 (30%) contained more than 50% immunopositive tumor cells. In slides with IGF-1R-immunopositive cells 55 (42%) showed less than 10% immunopositive cells, 29 (22%) slides had 10% to 50% immunoreactive cells, and 13 (10%) contained more than 50% immunopositive tumor cells.

By univariate analysis, c-Met immunoreactivity, IGF-1R immunoreactivity, largest tumor diameter, and tumor cell type were all significantly associated with survival (Table 1). Kaplan-Meier curves indicated that a high expression of both c-Met and IGF-1R is associated with a poor prognosis. For each group containing a higher proportion of cells immunoreactive for each of these growth factors, melanoma-related survival was gradually reduced (Figs. 2, 3). In the groups with melanomas displaying low c-Met and IGF-1R expression, 19 (33%) of 57 and 31 (34%) of 90 patients, respectively, died of metastatic disease. In contrast, patients in groups with high c-Met and IGF-1R expression fared worse; 36 (48%) of 75 and 24 (57%) of 42 patients, respectively, died of disseminated uveal melanoma.

All parameters were then entered into a multivariate Cox regression model, but only the largest tumor diameter, tumor cell type, and IGF-1R immunoreactivity showed independent statistical significance (Table 2), suggesting some degree of confounding. The best fit (highest Wald score) for the model was obtained by IGF-1R immunoreactivity.

The potential association between expression of growth factor c-Met and IGF-1R was tested by the Kendall  $\tau$ , resulting in  $\tau = 0.22$ , suggesting a weak, but statistically significant intercorrelation ( $\chi^2 = 19.0$ ;  $P = 0.024$ ).

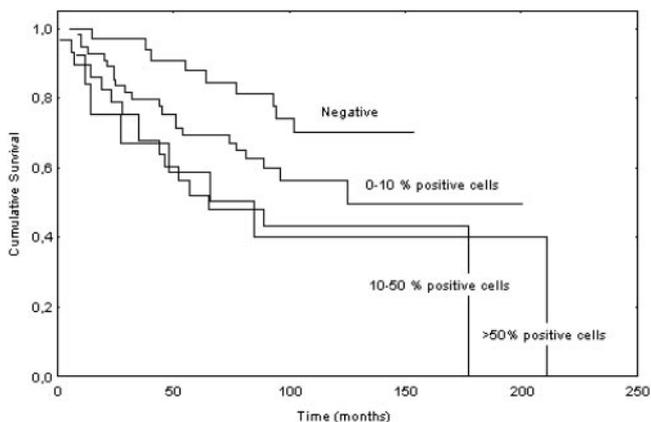


FIGURE 2. Kaplan-Meier survival curves for uveal melanoma cells showing negative (0%) and a low (<10%,  $P = 0.0692$ ), moderate (10%-50%,  $P = 0.00699$ ), and high (>50%,  $P = 0.0298$ ) proportion of tumor cells with immunoreactivity for IGF-1R protein.

## DISCUSSION

Receptor tyrosine kinases (RTK) play a crucial role in signal transduction of such functions as cellular proliferation, apoptosis, and differentiation. Examples of RTKs expressed by normal melanocytes are those encoded by the proto-oncogene *MET* and the *IGF-1R* gene.<sup>15</sup>

In the past few years, reports have been accumulating that consistently show that downregulation of the IGF-1R causes apoptosis and growth inhibition of cancer cells.<sup>9,16-18</sup>

Overexpression of c-Met has been described in a multitude of malignant human neoplasms.<sup>19-22</sup>

Uveal melanoma spread preferentially to the liver, and an altered IGF-1R and c-Met expression in uveal melanoma may act to enhance cell growth and tumor progression. In this study, the c-Met and IGF-1R immunoreactivity in 132 cases of paraffin-embedded histopathologically and clinically well-characterized primary uveal melanomas were investigated. Both RTKs were variably expressed, and a high expression was associated with a decreased survival in this disease. Uveal melanoma is simple to analyze by immunohistochemistry, because the tumor tissue is solid and homogenous. Scattered macrophages occur but are usually confined to the peripheral part of the tumor. They usually represent less than 10% of cells within the core of the tumors.<sup>9,23</sup> Furthermore, the macrophages were easily detected by their morphologic appearance and by immunoreactivity to CD68 antibodies<sup>9</sup> and did not cause any problem in scoring the melanoma cell immunoreactivity to IGF-1R and c-Met.

Sections of some specimens showed no immunostaining for c-Met or IGF-1R. Because the fixation time of archival tissue cannot be controlled retrospectively, we verified the robustness of epitopes to any inadvertently prolonged exposure to formaldehyde. Small pieces of tumor tissue were shown to

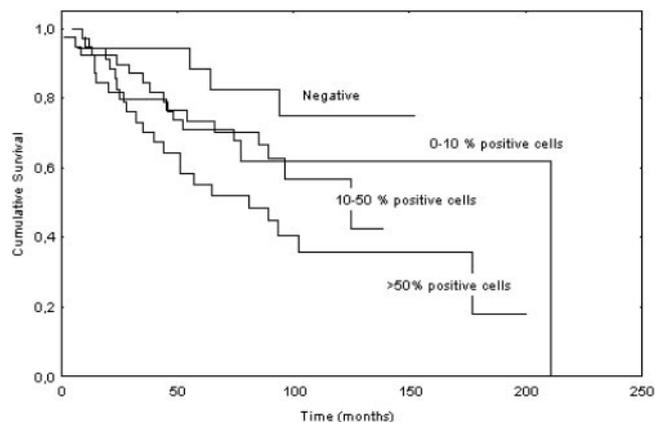


FIGURE 3. Kaplan-Meier survival curves for uveal melanoma cells showing negative (0%) and a low (<10%,  $P = 0.261$ ), moderate (10%-50%,  $P = 0.153$ ), and high (>50%,  $P = 0.01$ ) proportion of tumor cells with immunoreactivity for c-Met protein.

TABLE 2. Multivariate Cox Regression for Covariates of Potential Prognostic Value

| Covariate          | Scoring or Unit                                   | Wald | P     | Hazard Ratio (95% CI) |
|--------------------|---|------|-------|-----------------------|
| IGF-1 R expression | Neg.; <10%; 10%-50%; >50% stained cells           | 8.30 | 0.004 | 1.57 (1.25-1.89)      |
| Tumor cell type    | Spindle, mixed, epithelioid, partially necrotic   | 5.30 | 0.021 | 1.30 (1.08-1.52)      |
| Tumor diameter     | Millimeter  | 4.96 | 0.026 | 1.13 (1.03-1.23)      |
| c-Met expression   | Neg.; <10%; 10%-50%; >50% stained cells           | 1.12 | 0.290 | 1.18 (0.86-1.50)      |
| Tumor location     | Posterior choroid, anterior choroid, ciliary body | 0.11 | 0.735 | 0.93 (0.49-1.37)      |
| Patient's age      | Years   | 0.10 | 0.748 | 1.00 (0.98-1.02)      |
| Patient's sex      | Male, Female                                      | 0.05 | 0.822 | 0.93 (0.31-1.55)      |

retain their immunostaining characteristics for c-Met and IGF-1R through 32 days of fixation. Therefore, the absence of immunostaining in a subset of specimens is unlikely to have been caused by overfixation. Negative immunostaining may have been caused by a protein content that was less than detection level.

The expression of c-Met has recently been suggested as a prognostic factor in cutaneous malignant melanoma.<sup>10</sup> In 1998 Hendrix et al.<sup>11</sup> were the first to demonstrate the expression of c-Met in uveal melanoma cells. Furthermore, they found a correlation between keratin and c-Met expression by the interconverted uveal melanoma cells and their ability to respond to HGF/SF and invade. In agreement with the study by Hendrix et al.<sup>11</sup> we found that c-Met is expressed in uveal melanoma. Moreover, we were able to show that c-Met is a statistically significant prognostic factor in a univariate model. In the group with melanomas displaying low c-Met ( $P = 0.261$ ; Fig. 3), 33% of the patients died of metastatic disease in contrast to patients in the group with high c-Met ( $P = 0.01$ ; Fig. 3), where 48% died of disseminated uveal melanoma.

We have formerly shown on a small sample of uveal melanomas that the expression of IGF-1R may be an important prognostic factor.<sup>9</sup> The present study is the first to define the IGF-1R as a strong prognostic factor in uveal melanoma in a large number of cases. Our results demonstrate a strongly significant association between the expression pattern of IGF-1R and the progression of uveal melanoma, as well as the overall survival of patients with uveal melanoma. In this study, IGF-1R was an even stronger prognostic factor than tumor diameter in the multivariate analysis. Thirty-four percent of patients with a low expression of IGF-1R ( $P = 0.0692$ , Fig. 2) in the tumors died of metastatic disease compared with 57% in the group with high IGF-1R expression ( $P = 0.0298$ , Fig. 2).

The present study points to the possibility of using IGF-1R and c-Met as predictive factors in the clinical management of uveal melanoma. The significant and independent association of high IGF-1R expression with a poor prognosis suggests that IGF-1R may play a role in the molecular mechanisms leading to tumor dissemination and/or progression in uveal melanoma.

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