Carcinoembryonic Antigen Cell Adhesion Molecule-1 (CEACAM1) in Posterior Uveal Melanoma: Correlation with Clinical and Histological Survival Markers

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PURPOSE. Carcinoembryonic antigen cell adhesion molecule (CEACAM)-1 is a multi-functional protein, with strong predictive value for poor prognosis when found in primary cutaneous melanoma lesions. In this study, the expression of CEACAM1 in uveal melanoma was correlated with clinicopathologic parameters.

METHODS. CEACAM1 expression was immunohistochemically evaluated in 79 primary uveal melanomas and 21 liver metastases of patients who were treated at the Hadassah-Hebrew University Medical Center between the years 1986 and 2006. The findings were correlated with location, cell type, extracellular matrix patterns, tumor size, and metastatic disease.

RESULTS. CEACAM1 was expressed in 45% of the primary tumors compared with 81% of the metastases (Fisher’s exact test, \( P = 0.003 \)). There was no significant association between CEACAM1 and location of the primary tumors. Histologically, CEACAM1 was associated with epithelioid-type tumors (69.6%), but not with spindle-type tumors (25.0%) (Cramer’s \( V = 0.354; P = 0.019 \)). Also it was significantly associated with network extracellular matrix pattern (73.3%), but not with silent pattern (11.8%) (Cramer’s \( V = 0.510; P = 0.004 \)). CEACAM1-positive tumors were not statistically different in size from CEACAM1-negative tumors. The higher frequency of CEACAM1 in patients who ultimately developed metastases (58.8% vs. 41.7%) was not statistically significant (likelihood ratio \( \chi^2 = 2.069; P = 0.1503 \)).

CONCLUSIONS. This report describes CEACAM1 expression in uveal melanoma. Correlation with poor prognostic factors such as epithelioid cell type and networks of extracellular matrix pattern was found, but definitive prognostic conclusions still cannot be deduced. Additional validation studies on the use of CEACAM1 expression as a prognostic marker are warranted. (Invest Ophthalmol Vis Sci. 2011;52:9368–9372) DOI:10.1167/iovs.10-6006

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regulation of CEACAM1 in cutaneous melanoma patients. Indeed, an inverse correlation was observed between prognosis and the presence of CEACAM1 on lymphocytes or with soluble serum CEACAM1 concentration. These studies demonstrate that CEACAM1 provides melanoma with enhanced invasiveness and immune evasion attributes, support the strong clinical association with poor prognosis, and mark CEACAM1 as an attractive target for immunotherapeutic interventions.

The purpose of our study was to determine the expression of CEACAM1 in human uveal melanoma and to correlate it with clinical and histopathological parameters.

METHODS

Uveal Melanoma Patients

Formalin-fixed paraffin-embedded sections of primary posterior uveal melanoma from 79 consecutive patients, who underwent enucleation between 1986 and 2006 at the Department of Ophthalmology of the Hadassah-Hebrew University Medical Center, were investigated. Patients' demographic data, as well as date of diagnosis and enucleation, tumor height, diameter, and location, cell type, and extracellular matrix patterns, and occurrence of first metastasis were recorded from the medical records using data from a 20-year follow-up period. In addition, formalin-fixed paraffin-embedded sections from liver metastases of 21 consecutive uveal melanoma patients were tested. The use of the tumor tissue was approved by the Institutional Review Board (Helsinki committee) for experiments in human subjects.

Immunohistochemistry for the Detection of CEACAM1

Formalin-fixed and paraffin-embedded primary uveal melanoma specimens were cut into 5-μm sections. Paraffin was removed from the sections, which were then rehydrated through a series of graded ethanol and rinsed in Tris-buffered saline (TBS; pH 7.6) with 0.1% Tween-20 added. All antibodies were diluted in antibody diluent with background reducing components (Dako, Glostrup, Denmark). The slides were microwaved at 500 W five times for 2 minutes in 10 mmol/L citrate buffer (pH 6.0). After cooling the slides for 20 minutes, they were washed three times in TBS plus 0.1% Tween-20 for 5 minutes. Non-specific binding was blocked by incubating the slides in 10% normal rabbit serum (Dako) for 30 minutes, followed by incubation with the monoclonal CEACAM1 antibody MRG1 at 8 μg/mL (in-house preparation by GM) in a humid chamber overnight at 4°C. The next morning, the sections were first washed three times in TBS for 5 minutes and then incubated with a 1:40 diluted biotinylated rabbit anti-mouse antibody (Dako) for 40 minutes at room temperature. After additional washes in TBS, sections were incubated with avidin-biotinylated horseradish peroxidase complex (Vectorstain ABC kit; Vector, Burlingame, CA) for 30 minutes. Next, additional washes in TBS were performed. Enzyme reactivity of the alkaline phosphatase complex was visualized using NaphtholAS-bisphosphate as a substrate, and hexazonated new fuchsin was used for simultaneous coupling. Slides were counterstained with Mayer's hemalum diluted 1:1 in distilled water for 10 seconds, blued under running tap water, and mounted (Crystal Mount; Biometra, Foster City, CA). Negative controls were treated the same way, omitting the incubation with the primary antibody. All immunohistochemical samples were graded as being positive or negative for CEACAM1. No measure of staining intensity or percentage of stained cells was recorded for immunohistochemical findings.

Evaluation of the Extracellular Matrix Pattern

Histologic slides of each melanoma case, stained with PAS without hematoxylin counterstain, was viewed at magnification × 100 through a green filter to enhance the contrast and better highlight the PAS-positive material. The presence of PAS-positive patterns was recorded according to previously published guidelines.

Each parameter was categorized as absent or present (anywhere in the section). All histochemically and immunohistochemically stained sections were interpreted without previous knowledge of the clinical outcome of each patient.

Evaluation of Staining Pattern and Statistical Analysis

CEACAM1 staining of melanoma cells was recorded by an observer who was masked to the clinical data at the time of evaluation. Generally, the 79 cases of primary uveal posterior melanoma were divided into positive and negative tumors, as were the 21 metastatic liver sections. For documentation, the slides were examined with a digital camera (Olympus, DP70; Melville, NY) and photographed. The association between CEACAM1 expression and the clinical and histopathological parameters described previously was investigated. Correlations with CEACAM1 were assessed by Cramer's V for categorical factors and Kendall's tau-b for continuous factors. Association with survival was assessed by a Kaplan-Meier survival analysis with melanoma-related mortality as an end point. A stepwise Cox multivariate analysis of the survival was performed to test which of the parameters affects melanoma-related mortality. Statistical significance was assessed using statistical software (SPSS for Windows, version 17; SPSS, Chicago, IL).

Generation of Anti-CEACAM1 mAb MRG1

Balb/c mice were immunized four times with 5 μg recombinant human CEACAM1. Spleen cells of the mouse that exhibited the highest anti-CEACAM1 antibody titer were fused to SP/20 cells. The MRG1 mAb was selected after screening for CEACAM1 binding in flow cytometry and blocking of CEACAM1 function in vitro. For purification of the antibody, confluent hybridoma cells were cultured in 2 L of low protein medium (Biological Industries, Bet Haemek, Israel). The supernatant was collected, centrifuged, filtered to eliminate remnants cells, and passed through a column (Protein G; GE Healthcare Europe GmbH, Munich, Germany). The eluted antibody was dialyzed against PBS overnight and tested for integrity in SDS-PAGE. All experiments were conducted in accordance with the Guiding Principles in the Care and Use of Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cells and Antibodies

Primary cultures of metastatic cutaneous melanoma cells were developed and maintained from surgically removed melanoma lesions as previously described. The cell lines used in this work were 721.221 human EBV-transformed B cell lymphoma, which were transfected with the cDNA of human CEACAM1, CEACAM5, CEACAM6, and CEACAM8 (kindly provided by Ofer Mandelboim, Lautenberg Center for General and Tumor Immunology, BioMedical Research Institute, Hadassah-Hebrew University Medical Center, Jerusalem). The monoclonal antibodies used in this work were MRG1 (see above) and Kat6c (Dako). FITC-conjugated goat anti-mouse antibodies were used as a secondary detection reagent (Jackson ImmunoResearch, West Grove, PA).

Flow Cytometry

Flow cytometry analysis was performed as previously described.

RESULTS

Specificity of MRG1 mAb

Monoclonal antibody MRG1 was raised against human recombinant CEACAM1 as described above. The specificity of this mAb was tested in flow cytometry using a system of transfectants based on the CEACAM-negative 721.221 cell line, as described in Methods. The pan anti-CEACAM mAb Kat6c ensured that all transfectants expressed the relevant protein.
The MRG1 strongly stained the 721.221/CEACAM1 cells, with extremely mild cross-reactivity with 721.221/CEACAM5. It should be noted that the CEACAM5 was expressed more than fivefold stronger than CEACAM1. Further, it was previously reported that CEACAM5 (CEA) is hardly expressed by uveal melanoma tumors. There was no cross-reactivity with the rest of the CEACAM proteins (Fig. 1). These results indicate that MRG1 is specific for CEACAM1 and suitable for this study.

Patient Characteristics

There were 45 female patients and 34 male patients. Mean age at diagnosis was 60.4 years (age range, 19–93 years). Thirty-six of the tumors were CEACAM1-positive (45.6%) whereas 43 were CEACAM1-negative (54.4%) (Fig. 2). There was no significant association between expression of CEACAM1 and sex (Cramer’s V = 0.077, P = 0.493). The mean age (± SD) of patients with CEACAM1-positive tumors was 59.6 ± 17.8 years and the mean age of patients with CEACAM1-negative tumors was 61.2 ± 16.8 years.

Correlation of CEACAM1 Expression with Tumor Location

The location of the primary tumors included the choroid (n = 41; 53%), ciliochoroid (n = 33; 42%), and the ciliary body only (n = 4; 5%). CEACAM1 was found in 15 of the choroidal tumors (36.6%), and 26 were CEACAM1-negative (63.4%). A higher percentage of CEACAM1-positive tumors were observed among the ciliochoroidal tumors, of which 18 were CEACAM1-positive (54.6%), whereas 15 were CEACAM1-negative (45.4%). The ciliary body tumors comprised a small subgroup (n = 4), of which three were CEACAM1-positive. No statistically significant correlation was demonstrated between CEACAM1 expression and ciliary body involvement (Cramer’s V = 0.242; P = 0.200) (Table 1).

Correlation of CEACAM1 Expression with Cell Type

The majority of the tumors were of the mixed cell type (n = 38; 49.4%), 23 were of the epithelioid cell type (29.9%), and 16 were of the spindle cell type (20.8%) (Table 1). Of the epithelioid-type tumors, 16 were CEACAM1-positive (69.6%), whereas seven were CEACAM1-negative (30.4%). Of the mixed type tumors, 16 were CEACAM1-positive (42.1%) and 22 were CEACAM1-negative (57.9%). Of the spindle cell type, four were CEACAM1-positive (25.0%), and 12 were CEACAM1-negative (75.0%). CEACAM1 expression significantly correlated with the epithelioid cell type (Cramer’s V = 0.354; P = 0.019).

Correlation of CEACAM1 Expression with the Vasculogenic Mimicry Extracellular Matrix Patterns

The silent pattern was observed in 18 tumors whereas the network pattern was demonstrated in 15 tumors. Among the tumors that exhibited the silent pattern, only two (11.1%) were CEACAM1-positive, whereas 16 (88.9%) were CEACAM1-negative. In contrast, 11 specimens (73.3%) of the tumors that exhibited the network pattern were CEACAM1-positive, compared with four (26.7%) that were CEACAM1-negative (Table 1). There was a highly significant correlation between CEACAM1 expression and the presence of worse vasculogenic mimicry patterns (Cramer’s V = 0.510; P = 0.004).

Correlation of CEACAM1 Expression with Tumor Height and Diameter

The mean height (± SD) for CEACAM1-positive tumors was 8.91 ± 2.94 mm, and 9.91 ± 3.89 mm for CEACAM1-negative tumors. This difference was not statistically significant (Kendall’s tau-b = −0.144; P = 0.189) (Table 2). The mean largest basal diameter (LBD) for CEACAM1-positive tumors was 14.45 ± 4.29 mm compared with 14.05 ± 5.37 mm for CEACAM1-negative tumors. This difference was not statistically significant (Kendall’s tau-b = 0.003; P = 0.979).
Correlation of CEACAM1 Expression with the Presence of Liver Metastasis

Thirty-four patients developed liver metastasis, and 36 patients were not reported to have developed metastasis, during their follow-up period. No information was available on the remaining nine patients. Among the patients who developed metastasis, 20 of the tumors were CEACAM1-positive (58.8%), whereas 14 were CEACAM1-negative (41.2%). In the patients who did not develop metastasis, 15 of the tumors were CEACAM1-positive (41.7%) and 21 of the tumors were CEACAM1-negative (58.3%). This correlation between the development of metastasis and the presence of CEACAM1 was not statistically significant (likelihood ratio $\chi^2 = 2.069; P = 0.1503$).

Correlation of CEACAM1 Expression with Survival

Kaplan-Meier survival analysis by CEACAM1 positivity showed that there was no difference in survival between patients with CEACAM1-positive tumors compared with those with CEACAM1-negative tumors (log rank $P = 0.236$). Moreover, in a stepwise Cox multivariate analysis of the survival time with melanoma-related mortality as an end point, with the following variables: sex, age at diagnosis, tumor location (ciliary body involvement, choroidal), cell type (spindle, mixed, epithelioid), vasculogenic mimicry extracellular matrix pattern (present, absent), tumor height, tumor largest basal diameter (LBD), and CEACAM1 (positive, negative), the only significant variable was a larger basal diameter ($P = 0.023$).

CEACAM1 Expression in Liver Metastatic Lesions

Sections of 21 liver metastases were evaluated for CEACAM1 expression. Remarkably, 17 were found to positively express CEACAM1 (80.9%), which is significantly higher than the 45% expression in primary uveal melanoma tumors (Fisher’s exact test, $P = 0.003$). This high rate is similar to the CEACAM1 expression rate in metastatic lesions of cutaneous melanoma. Indeed, analysis of 58 metastatic lesions of cutaneous melanoma cells showed that 46 specimens (79.3%) were positively stained for CEACAM1 expression (unpublished results).

**TABLE 2. Association with CEACAM1—Categorical Factors**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CEACAM1 (+), $n$ (%)</th>
<th>CEACAM1 (−), $n$ (%)</th>
<th>Cramer’s $V$</th>
<th>$P$</th>
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</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>14 (41.2)</td>
<td>20 (58.8)</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>22 (48.9)</td>
<td>23 (51.2)</td>
<td></td>
</tr>
<tr>
<td>VM</td>
<td>(+)</td>
<td>29 (56.9)</td>
<td>22 (43.1)</td>
<td>0.391</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>4 (16.7)</td>
<td>20 (83.3)</td>
<td></td>
</tr>
<tr>
<td>Cell Type</td>
<td>Epithelioid</td>
<td>16 (69.6)</td>
<td>7 (30.4)</td>
<td>0.354</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>16 (42.1)</td>
<td>22 (57.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spindle</td>
<td>4 (25.0)</td>
<td>12 (75.0)</td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>(+)</td>
<td>21 (56.8)</td>
<td>16 (43.2)</td>
<td>0.236</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>15 (36.6)</td>
<td>26 (63.4)</td>
<td></td>
</tr>
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</table>

From the data presented here, it may be seen that there is a positive association between CEACAM1 and vasculogenic mimicry extracellular matrix patterns and between CEACAM1 and cell type (specifically with epithelioid cell type, which is the cell type with the poorest prognosis). +, positive; −, negative; F, female; M, male; VM, vasculogenic mimicry extracellular matrix patterns; CB, tumors involving the ciliary body (including ciliochoroidal tumors).

DISCUSSION

Despite constant improvements in the diagnosis and treatment of primary uveal melanoma, a corresponding decrease in metastatic death has not been documented.29 Cell adhesion molecules play a key role in tumor invasion and metastasis.30 It has been postulated that changes in cell-cell and cell-matrix interactions account for the ability of cancer cells to cross tissue boundaries and to disseminate to distant sites. The loss of cell-cell binding that closely correlates with differentiation and the invasive potential of malignant tumors is accompanied by altered expression of cell adhesion molecules.31

The purpose of this study was to examine the expression of CEACAM1 in uveal melanoma using immunohistochemistry. The lack of effect, and only a larger study can answer this question. To our knowledge, this is the first study to analyze the expression of CEACAM1 in uveal melanoma. A cohort of 79 patients was tested for whom biological and clinical data were available. This cohort provided a unique platform to study the involvement of CEACAM1 in the biology of uveal melanoma. Nine (11%) of the patients were lost to follow-up and thus no information could be obtained regarding the presence or absence of metastasis, which may have influenced our analyses. The lack of significant correlation in this limited study between

**TABLE 2. Association with CEACAM1—Continuous Factors**

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>CEACAM1</th>
<th>Largest Basal Diameter</th>
<th>Height (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEACAM1</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Valid, $n$</td>
<td>30</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>Mean</td>
<td>61.23</td>
<td>59.61</td>
<td>14.05</td>
</tr>
<tr>
<td>SD</td>
<td>17.76</td>
<td>17.76</td>
<td>5.37</td>
</tr>
<tr>
<td>Minimum</td>
<td>26.00</td>
<td>19.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Median</td>
<td>68.00</td>
<td>61.00</td>
<td>14.90</td>
</tr>
<tr>
<td>Maximum</td>
<td>83.00</td>
<td>93.00</td>
<td>20.50</td>
</tr>
<tr>
<td>$t$ Test, $P$ value</td>
<td>0.572</td>
<td>0.577</td>
<td>0.474</td>
</tr>
<tr>
<td>Kendall’s tau-b</td>
<td>−0.052 ($P = 0.628$)</td>
<td>0.003 ($P = 0.979$)</td>
<td>−0.144 ($P = 0.189$)</td>
</tr>
</tbody>
</table>

No significant association between CEACAM1 and the continuous prognostic factors was found.
CEACAM1 and survival could also be speculated to be due to the development of subclinical micrometastasis that was not detected in some patients.

Interestingly, CEACAM1 expression frequency reported here for primary and metastatic lesions is similar to its frequency in the corresponding lesions of cutaneous melanoma. This again suggests that CEACAM1 may act as melanoma-promoting in uveal melanoma by changing cell-cell and cell-matrix interactions and thus facilitating tumor spread to distant organs. Also, an angiogenic potential was previously described for CEACAM1 and an additive angiogenic effect to VEGF was found, again suggesting that CEACAM1 may play a role in sustaining primary tumor and metastasis growth. Could CEACAM1 act as a biomarker in uveal melanoma patients? Additional validation studies are needed to study the levels of soluble CEACAM1 in the sera of patients and correlate it with clinical, pathologic, and survival outcomes. Indeed, it was recently shown that serum CEACAM1 levels have prognostic predictive value in cutaneous melanoma patients. More needs to be revealed with regard to the pathogenic role of CEACAM1 in uveal melanoma to better identify factors promoting tumor development and dissemination.

References