Expression of p53, bcl-2, E-Cadherin, Matrix Metalloproteinase-9, and Tissue Inhibitor of Metalloproteinases-1 in Paired Primary Tumors and Brain Metastasis

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ABSTRACT

The objectives of this study were to: (a) characterize the immunohistochemical expression of p53, bcl-2, E-cadherin (EC), matrix metalloproteinase-9 (MMP-9), and tissue inhibitor of metalloproteinases-1 (TIMP-1) in brain metastases; (b) compare immunohistochemical (IHC) expression of brain metastases with their primary tumors; and (c) assess the prognostic value of expression of these markers. Tumors from 35 patients with brain metastasis were studied for IHC expression of p53, bcl-2, EC, MMP-9, and TIMP-1. In 17 cases, primary tumors were also available for study. In brain metastases, p53 was positive in 91% of cases, intermediate in 9%, MMP-9 was positive in all cases, TIMP-1 was intermediate in 6% and negative in 94% of cases, EC expression was positive in 86% of cases and intermediate in 14%, and bcl-2 was variable. All primary tumors were positive for p53 and MMP-9, 3% were intermediate for TIMP-1 and 97% were negative, 65% were positive for EC and 35% were intermediate, whereas bcl-2 expression was variable. Neither p53, bcl-2, TIMP-1, or EC staining correlated with overall survival or survival with brain metastases. No assessment of survival differences could be made for MMP-9 because of its overexpression in brain metastases. The high expression of EC may indicate the importance of adherence at late stages of metastasis but requires further study.

INTRODUCTION

The metastatic process includes many steps: detachment from the primary tumor site, response to chemotactic factors, invasion and degradation of the ECM, angiogenesis, and successful growth within the target organ (1, 2, 3). Despite our growing understanding of tumor progression, the molecular mechanisms of brain metastasis remain poorly understood. In part, this is because the brain is physiologically distinct from other organs by virtue of the blood-brain barrier, autoregulation of blood flow, lack of lymphatic drainage, and lack of regenerative capacity of injured neurons (1, 3, 4). To metastasize to the CNS, tumor cells must adhere to the brain microvasculature, penetrate the blood-brain barrier, and grow within the brain parenchyma. Whether the pathways used are unique to the brain or common to a universal metastatic process is unknown.

At the cellular level, the balance of proliferation and apoptosis contributes to tumor progression and metastasis. The tumor suppressor gene p53 controls the G1-S cell cycle checkpoint. Nuclear accumulation of p53 confers a more aggressive phenotype in many cancers and is seen more frequently in the metastatic state (5). bcl-2 is an oncogene that protects cells from apoptosis, allowing genetically damaged cells to continue to replicate; its deregulation occurs as tissues become more dysplastic (6). EC is a calcium-dependent transmembrane glycoprotein that allows cell-cell adhesion and invasion-suppression in epithelial cells. Its expression is often lost during tumor progression (7, 8). MMP-9 degrades many elements of the ECM (specifically, gelatins, collagen types II, III, and IV, and the α chain of collagen type I), allowing tumor invasion into distant sites. Several studies have shown a correlation between increased MMP-9 expression and increased metastatic potential (9, 10, 11). TIMP-1 binds MMP-9, blocking endogenous enzymatic activity. The overexpression of MMPs is thought to shift the balance of tumor cells to a more invasive phenotype, whereas native TIMP-1 inhibits in vivo metastasis in animal models (12).

To evaluate the factors that allow tumor cells to dissemi-

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2 The abbreviations used are: ECM, extracellular matrix; CNS, central nervous system; EC, E-cadherin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; IHC, immunohistochemistry.
nate to the brain, primary tumors and matched brain metastases were studied for IHC expression of p53, bcl-2, EC, MMP-9, and TIMP-1. These oncoproteins were selected for study because of their established role in the metastatic process in other organ sites. The objectives of this study were to: (a) characterize the IHC expression of p53, bcl-2, EC, MMP-9, and TIMP-1 in brain metastases; (b) investigate the role of these oncoproteins in the development of brain metastasis by comparing IHC expression of brain metastases with their primary tumors; and (c) assess the prognostic value of IHC expression of these oncoproteins.

MATERIALS AND METHODS

Tissue Retrieval and Patient Characteristics. Archival tissues from 35 patients with single brain metastases who underwent surgical resection or needle biopsy, followed by whole-brain radiotherapy (13), were used. Where possible, the matched primary tumors (17 of 35) were also collected. Blinded chart reviews were performed for the following prognostic variables: age, sex, histological type, time from initial diagnosis to death, time from brain metastasis diagnosis to death, brain recurrence, extraneural recurrence, and cause of death. Table 1 describes patient characteristics, whereas Table 2 describes the histological subtype of the brain metastases.

IHC. Sections of paraffin-embedded tissues were studied by IHC using monoclonal antibodies to p53 (Clone DO-7; Dako), bcl-2 (Clone 124; Dako), EC (Transduction Labs), MMP-9 (Oncogene Research Products), and TIMP-1 (Chemicon International; MAB 3301). Using methods described previously (14), 8-μm sections were treated using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC kit; Vector Labs) with the following modifications. Endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 30 min. Sections were blocked with 20% horse serum for 1 h to prevent nonspecific binding. Primary antibody at a 1:50 dilution (for p53, bcl-2, and EC) and 1:100 dilution (MMP-9 and TIMP-1) was applied overnight at 4°C, with appropriate negative and positive controls. Secondary biotinylated antibody at 1:200 was applied for 1 h to visualize bound antibody. ABC reaction was performed for 1 h at room temperature. The peroxidase activity was developed by incubation in 0.05% 3,3’-diaminobenzidine for 5 min, and slides were counterstained with hematoxylin.

Specimen Analysis. Five high power fields (0.148 mm2) were assessed per slide, and the ratio of immunoreactive:total tumor cells was measured by a digital image analysis system (Alpha Station 250 4/266; Digital Equipment Company) for p53, bcl-2, and MMP-9. Because EC stained both cell membrane and cytoplasm, cell borders were indistinct, and digital image analysis was inaccurate; therefore, microscopic examination was performed by two independent investigators (TIMP-1 was also evaluated by two independent investigators). The percentage of immunoreactive cells to total tumor cells was evaluated and is defined in Table 3 as reported previously (6, 8, 15, 16, 17).

Tumor cells were considered positive for p53 (nuclear staining) and bcl-2 (cytoplasmic staining) if >50% showed strong reactivity, intermediate if between 5 and 50% were immunoreactive, and negative if <5% were immunoreactive. EC staining was considered positive if >90% of cells retained cytoplasmic and/or membranous immunoreactivity, intermediate if 10–90% were immunoreactive, and negative if <10% were immunoreactive. All cells showed either cytoplasmic or cytoplasmic/membranous staining, and both patterns were considered positive. MMP-9 and TIMP-1 staining were considered positive if >75% cells had cytoplasmic staining; intermediate if 25–75% of cells were immunoreactive, and negative if <25% cells were immunoreactive. Necrotic areas were not scored.

Statistical Analysis. χ2 analysis was used for univariate categorical analysis. In relation to p53, bcl-2, EC, and TIMP-1 expression, survival curves were calculated using the Kaplan/Meier method and were compared using the log-rank test. Over-all survival from the diagnosis of brain metastasis was calculated from the date of metastatic diagnosis until the date of death. P < 0.01 was considered significant.

RESULTS

Pathological and Clinical Characteristics. The 35 patients had a median age of 59 years (range, 44–74) with 22 males and 13 females (Table 1). Histological subtypes are

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Table 1  Demographic data

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n = 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age (yr)</td>
<td>59</td>
</tr>
<tr>
<td>Age range (yr)</td>
<td>44–74</td>
</tr>
<tr>
<td>Sex: Male/Female</td>
<td>22/13</td>
</tr>
<tr>
<td>Average TTD from 10 Dx (mo)</td>
<td>21.5</td>
</tr>
<tr>
<td>Average TTD from Met (mo)</td>
<td>8.8</td>
</tr>
<tr>
<td>COD: CNS/non-CNS</td>
<td>15/20</td>
</tr>
</tbody>
</table>

“TTD, time to death; 10 Dx, primary diagnosis; Met, brain metastasis; COD, cause of death.

Table 2  Primary site of tumors metastasizing to the brain

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung carcinoma</td>
<td>9</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Large cell</td>
<td>1</td>
</tr>
<tr>
<td>Squamous</td>
<td>3</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>1</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1</td>
</tr>
<tr>
<td>Rectal carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Uterine adenoacanthoma</td>
<td>1</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 3  Category definitions of immunoreactive cells to total tumor cells

<table>
<thead>
<tr>
<th>Stain (Ref.)</th>
<th>Positive</th>
<th>Intermediate</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (14, 15)</td>
<td>&gt;50%</td>
<td>5–50%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>bcl-2 (6)</td>
<td>&gt;50%</td>
<td>5–50%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>EC (16)</td>
<td>&gt;90%</td>
<td>10–90%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>MMP-9 (11)</td>
<td>&gt;75%</td>
<td>25–75%</td>
<td>&lt;25%</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>&gt;75%</td>
<td>25–75%</td>
<td>&lt;25%</td>
</tr>
</tbody>
</table>
described in Table 2. Twenty-four patients underwent craniotomy and tumor resection, and 11 had stereotactic biopsy. All patients had 3600 cGy of external beam radiation to the brain, 21 had radiotherapy to their primary tumor, and 12 patients received at least one form of chemotherapy. Average time from the primary tumor diagnosis to death was 21 months, whereas the average time from the diagnosis of the brain metastasis to death was 8.8 months. The cause of death was attributed to the brain metastasis in 15 of 35 cases and to non-CNS causes in 20 of 35 cases.

Brain Metastases Results. Brain metastases were positive for p53 overexpression in 91% (32 of 35) cases and intermediate in 9% (3 of 35; \( P < 0.0001 \)), with a mean value of 79.9% immunoreactive cells (Table 4). bcl-2 staining was variable with 29% (10 of 35) positive, 23% (8 of 35) intermediate, and 48% (17 of 35) negative (\( P \), not significant) with a mean value of 27.1%. EC expression was positive in 86% (30 of 35) patients, whereas 14% (5 of 35) showed intermediate expression (\( P < 0.0001 \)), with a mean value of 91.9%. MMP-9 was positive in all brain metastases (100%; 35 of 35; \( P < 0.0001 \)) with a mean value of 95.1%. TIMP-1 was intermediate in 3% (1 of 35) and negative in 97% (34 of 35; \( P < 0.0001 \)) with a mean value of 2.1%. In comparison to surrounding brain parenchyma, MMP-9, p53, and EC expression were much higher in tumor tissue. Adjacent reactive astrocyte and mononuclear cells showed modest immunoreactivity for p53, bcl-2, EC, MMP-9 (Fig. 1), and TIMP-1 (not shown).

Primary Tumors. All primary tumors (100%; 17 of 17) were positive for p53 (\( P < 0.0001 \)) with a mean value of 89.7% immunoreactive cells (Table 5). bcl-2 expression was variable (24%; 4 of 17) positive, 35% (6 of 17) intermediate, and 41% (7 of 17) negative (\( P \), not significant) with a mean value of 29.8%. Primary tumors were positive for EC in 65% (11 of 17) cases and intermediate in 35% (6 of 17) with a mean value of 95.9%. MMP-9 expression was high in all primary tumors studied 100% (17 of 17; \( P < 0.0001 \)) with a mean value of 93.2%. TIMP-1 was intermediate in 6% (1 of 17) and negative in 94% (16 of 17; \( P < 0.0001 \)) with a mean value of 2.4%.

Survival Data. There was no difference in survival from the time of diagnosis of the primary tumor or the brain metastasis to death for p53, EC, or TIMP-1 expression (Table 6). When analyzed in comparison to mean time to death, the data

Table 4  Immunostaining of brain metastasis

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Positive</th>
<th>Intermediate</th>
<th>Negative</th>
<th>( P^* )</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>91% (32/35)</td>
<td>9% (3/35)</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>79.9%</td>
</tr>
<tr>
<td>bcl-2</td>
<td>29% (10/35)</td>
<td>23% (8/35)</td>
<td>48% (17/35)</td>
<td>NS</td>
<td>27.1%</td>
</tr>
<tr>
<td>EC</td>
<td>86% (30/35)</td>
<td>14% (5/35)</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>91.3%</td>
</tr>
<tr>
<td>MMP-9</td>
<td>100% (35/35)</td>
<td>0</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>95.1%</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0</td>
<td>3% (1/35)</td>
<td>97% (34/35)</td>
<td>&lt;0.0001</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

*For testing for equal distribution among the three categories. NS, not significant.

Fig. 1  A, p53 immunostaining of a lung adenocarcinoma metastatic to the brain demonstrating strong nuclear immunoreactivity of tumor cells and slight immunoreactivity of adjacent inflammatory cells and astrocytes. B, bcl-2 immunostaining showing a lack of immunoreactivity of tumor cells but modest immunoreactivity of adjacent inflammatory cells and astrocytes; C, E-cadherin immunostaining showing strong nuclear, cytoplasmic, and membranous immunoreactivity of tumor. D, MMP-9 immunostaining showing strong cytoplasmic immunoreactivity, with slight immunoreactivity of adjacent inflammatory cells and astrocytes. (TIMP-1 expression not shown).
suggest a significant improvement in survival in some patients with respect to the bcl-2 expression. Mean time to death was highest for positively staining tumors, intermediate for negatively staining tumors, and lowest for intermediately staining tumors (Table 6), a perplexing finding. When reanalyzed using two categories, positive (>50% immunoreactive cells) versus nonpositive (<50%), there was a trend toward a significant difference in survival from both the time of metastatic diagnosis to death \( (P = 0.096) \) and primary diagnosis to death \( (P = 0.041; \text{data not shown}) \). Because the majority of brain metastases and primary tumors overexpressed MMP-9, it was not possible to compare patient survival curves with regard to this variable.

**DISCUSSION**

This report describes the IHC expression of p53, bcl-2, EC, MMP-9, and TIMP-1 in a series of 35 brain metastases. To our knowledge, this represents the largest published series of brain metastases studied for the IHC expression of these oncoproteins.

Our study demonstrates a striking pattern of MMP-9 overexpression by IHC in all brain metastases studied. Twenty-four of the 35 patients studied had non-small cell lung cancer, but regardless of tumor type, all brain metastases showed overexpression of MMP-9. Even with the accepted limitations of IHC in detecting mutational events (18), overexpression of this magnitude is notable, even without mutation analysis. It indicates that MMP-9 production is a common feature of brain metastasis. In each case, if the expression was positive in the brain metastasis, it was also positive in the primary tumor (mean percentage of immunoreactive cells, >90%). This suggests a role for MMP-9 not only in degradation of the ECM of the brain but earlier in tumor propagation by influencing disassociation from the primary site. Especially interesting was the finding that the inhibitor of MMP-9, TIMP-1, was negative in the majority of primary tumors and brain metastases studied. This implies a disruption of the normal balance of degradation of the ECM by MMPs and TIMPs in the metastatic process in the brain and in those tumors that eventually spread to the brain. Others have shown that gliomas secrete metalloproteinases in vivo (19). This suggests that both primary brain tumors and metastatic tumors use MMPs to invade the brain parenchyma. Whether the microenvironment of the brain is especially susceptible to MMPs or requires them for invasion is unknown. The next step will be to analyze MMP-9 and TIMP-1 mutations in these brain metastases, as well as to analyze the expression of other metalloproteinases in brain metastases.

On the basis of previous studies of advanced stage cancers (5), we expected and found that a highly significant proportion of brain metastases (91%) overexpress p53, as do the tumors from which they metastasize (100%). In comparison to surrounding lung and brain parenchyma and to positive controls, p53 expression was much higher in tumor tissue. All primary tumors that eventually metastasized exhibited p53 overexpression, and it is likely that deregulation of the G_{1}-S cell cycle checkpoint is necessary for metastasis, although this is probably not specific to brain metastases, given that p53 is frequently mutated in metastatic tumors (5). Wild-type p53 expression has been shown to be associated with increased sensitivity to irradiation in brain tumor cell lines (20). Of note, the patients with intermediate p53 expression showed a variable response to irradiation (one had CNS recurrence in a previously irradiated site, and two patients died of intercurrent infections).

We predicted a variable level of bcl-2 expression based on the primary site. Especially interesting was the finding that the inferior of MMP-9, TIMP-1, was negative in the majority of primary tumors and brain metastases studied. This implies a disruption of the normal balance of degradation of the ECM by MMPs and TIMPs in the metastatic process in the brain and in those tumors that eventually spread to the brain. Others have shown that gliomas secrete metalloproteinases in vivo (19). This suggests that both primary brain tumors and metastatic tumors use MMPs to invade the brain parenchyma. Whether the microenvironment of the brain is especially susceptible to MMPs or requires them for invasion is unknown. The next step will be to analyze MMP-9 and TIMP-1 mutations in these brain metastases, as well as to analyze the expression of other metalloproteinases in brain metastases.
previous observations that a fraction of nonlymphoid malignancies overexpress bcl-2 (21, 22, 23). In some series, positive bcl-2 expression correlates with an improved survival in early stage lung cancer (6, 20); however, in one series of 427 lung cancers, bcl-2 was not associated with a statistically significant difference in survival (24). In our series, bcl-2 expression was variable in both primary tumors and brain metastases and agrees with the study above (24); it was not correlated with the patient’s response to therapy or survival from metastatic diagnosis to death. The lowest survival occurred in patients with intermediate expression, but this association may be misleading because of the small sample size. Another explanation may be that other apoptotic pathway influences may play a role in the development of brain metastases. For example, the balance of positive and negative apoptotic factors in the bcl-2 family is well described–Bax counteracts Bcl-2, executing a death command, whereas Bad provides a connection to upstream regulators of cell death in a proapoptotic fashion (25). Because of the limited amount of tissue available, Mcl-1, Bax, and Bad IHC could not be performed, and therefore, the functional state of apoptosis within brain metastases is not completely clear from our study. As well, we did not find an inverse relationship between p53 and bcl-2 expression (data not shown), as observed by other authors (26). Clearly, the complexities of the apoptotic pathway need to be further assessed in brain metastases by studying many members of the bcl-2 family of proteins.

We hypothesized that EC expression would be markedly reduced in brain metastases, as has been observed in metastasis to other sites; however, we found quite the opposite phenomenon. EC expression was not lost in the majority of brain metastases, nor did its expression differ between primary and brain metastasis pairs in 71% of patients, whereas expression was greater in the brain metastasis in 29% of patients. This suggests that loss of adherence is not central to brain metastasis development. Adhesion may be lost early in cancer development, as has been shown by others (7, 8), but may be necessary for successful growth in the brain once a metastatic focus arises and may be a specific requirement for brain metastasis. Of note, we did not observe a correlation between the intensity of membranous or cytoplasmic staining and degree of differentiation or prognosis (data not shown) reported previously by Mattijsen et al. (8).

Characterizing brain metastases will permit optimal utilization of new therapies including metalloproteinase and angiogenesis inhibitors. From a molecular standpoint, understanding how brain metastases differ from other metastases allows us to clarify the mechanism of brain metastases specifically and to potentially target these mechanisms for novel drug design.

In summary, we have found that MMP-9 and p53 were overexpressed in all tumors that eventually metastasized to the brain and in the metastases themselves. TIMP-1 was negative, whereas EC expression was not lost and bcl-2 expression was variable. Metastases to the brain appear to differ from metastases to other organs in their high expression of EC (rather than loss of expression) and their marked overexpression of MMP-9 in the face of negative TIMP-1 expression. Brain metastases, like metastases at other sites, exhibit p53 nuclear accumulation, confirming the global role of p53 in tumor progression and regulation. This study suggests that the functional balance of MMP-9 and TIMP-1 is shifted toward ECM degradation in brain and that deregulation of cell cycle control by p53 also exists in brain metastases. EC expression was not lost, indicating that the need for adherence may reappear at late stages of metastasis. Future studies need to further define the role of MMPs, TIMPs, and EC in brain metastasis and to compare tumors that do and do not metastasize to the brain.

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REFERENCES


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