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Immunohistochemical Markers Associated with Brain Metastases in Patients with Non-Small Cell Lung Carcinoma

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Abstract

BACKGROUND—There are no reliable markers able to identify patients with non-small cell lung cancer (NSCLC) likely to metastasize to the brain. We investigated associations between immunohistochemical markers and development of brain metastases in patients with NSCLC.

METHODS—We performed a hospital-based, case-control study of patients with newly diagnosed NSCLC between 1989 and 2003 that developed brain metastases who had available pathology material from both primary NSCLC and brain metastases. The control patients had NSCLC and no evidence of brain metastases. We examined NSCLC for expression of Ki-67, caspase-3, VEGF-A, VEGF-C, E-cadherin and EGFR in 54 surgical pathology specimens using immunohistochemistry and evaluated associations with development of brain metastases.

RESULTS—Brain metastases developed after a median time of 12.5 months (range 1.7-89.4 months) from the diagnosis of NSCLC. A significantly increased risk of developing brain metastases was associated with patients who had high Ki-67 (adjusted odds ratio 12.2, 95% CI, 2.4 to 70.4, $P<0.001$), low caspase-3 (adjusted odds ratio 43.0, 95% CI, 5.3 to >100, $P<0.001$), high VEGF-C (adjusted odds ratio 14.6, 95% CI, 2.0 to >100, $P<0.001$), and low E-cadherin (adjusted odds ratio 3.6, 95% CI, 0.9 to 16.4, $P=0.05$), in the primary NSCLC. No significant risk was observed with VEGF-A and EGFR. A high Ki-67 was also associated with a shorter overall survival ($P=0.04$).

CONCLUSIONS—Patients with NSCLC and high Ki-67, low caspase-3, high VEGF-C, and low E-cadherin in their tumors may benefit from close surveillance since they may have an increased risk of developing brain metastases.

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INTRODUCTION

Non-small-cell lung carcinoma (NSCLC) is the leading cause of death from cancer in both men and women.1 Approximately 210,000 cases of NSCLC are diagnosed annually in the United States.1 Lung cancer is the malignancy that most commonly gives rise to brain metastases which are a devastating complication and a major cause of morbidity and mortality.2 Ten percent of patients have brain metastases at the time of diagnosis, and about 40% of all patients with lung cancer will develop brain metastases during the course of the disease.3 Patients with locally advanced NSCLC treated with chemotherapy, chest radiotherapy with or without surgery have a high rate of developing brain metastases.2, 4-6 These patients also have a 15-30% risk of failing first in the brain.4, 7 Brain metastases from NSCLC received increasing attention because combined-modality therapy has lead to improvements in intrathoracic local control and prolonged overall survival.7-9

It is important to identify patients with NSCLC who are at greater risk of developing brain metastases since they may exist in the absence of neurological symptoms.10 Furthermore, recent reports show that prophylactic cranial irradiation may be an effective modality for preventing brain metastases in patients with NSCLC treated with adjuvant chemoradiation.5 Despite advances in diagnosis and therapeutic modalities, and clinical practice guidelines, it remains unclear whether patients with early stage NSCLC should be screened for brain metastases or not.11-13

Studies have shown that the metastatic cascade is rather complex and involves reciprocal interactions between tumor cells and host tissues, including alterations in tumor cell proliferation, adhesion, proteolysis, invasion, and angiogenesis.8 For the morphological assessment of the fraction of proliferating cells, Ki-67 staining with MIB1 is the most commonly used monoclonal antibody, covering late G1, S, G2 and M phases of the cell cycle.14 TUNEL in situ hybridization or caspase-3 immunohistochemistry are suitable for evaluation of apoptosis as these methods cover the mitochondrial as well as the death-receptor pathways.15 Studies that examined the combination of proliferative and pro-apoptotic factors demonstrated that patients with tumors with a high proliferative activity and no expression of pro-apoptotic factors had the shortest survival times and vice versa.16 VEGF family of proteins modulates angiogenesis which is essential for tumor growth and metastasis.17-19 E-cadherin is a calcium-regulated adhesion molecule expressed in most normal epithelial tissues. Loss of cell surface E-cadherin is considered a defining characteristic of epithelial-mesenchymal transition (EMT), a cellular event that occurs during normal embryo development and might also be associated with tumor cell invasion and metastasis.20, 21 EGFR is overexpressed in 40 to 80% of NSCLC and many other epithelial cancers and is frequently correlated with adverse prognosis.22, 23

Molecular factors investigated in our study have been shown previously to play a role in tumor invasion and metastasis formation, but the association of any of those markers with the development of brain metastasis from NSCLC has not been established.

In our study we evaluated patients with NSCLC with and without brain metastasis in a unique and unparalleled series that has tumor material from both the primary lung tumor and metachronous brain metastasis obtained from a single institution. These patients are typically only treated for lung cancer, and brain biopsy is rarely recommended or obtained. This gave us the opportunity to interrogate for expression of Ki-67, caspase-3, VEGF-A, VEGF-C, E-cadherin and EGFR in primary lung tumors using immunohistochemistry and investigate the risk of developing brain metastasis and death. We compared patients that developed brain metastases with control patients with NSCLC that had no evidence of brain metastases that were followed for a median period of 2.5 years.

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MATERIALS AND METHODS

Patient Characteristics

Subjects were identified as patients with newly diagnosed NSCLC between 1989 and 2003 that developed brain metastases and had available pathology material from both primary NSCLC and brain metastases (Table 1). The group was identified through a combined search of the registry databases from the Departments of Pathology and Neuropathology maintained by the Department of Pathology and included patients with both confirmed primary NSCLC and metastatic NSCLC to brain who had available pathology material. The cases were reviewed by two pathologists and histologically classified using the WHO criteria for tumors of the lung.24 In each case, the pathologic and immunophenotypic features of brain metastases were compared with the corresponding NSCLC and the diagnosis of NSCLC metastatic to brain was confirmed.

The inclusion criteria for the study were a diagnosis NSCLC (either lung adenocarcinoma, squamous cell carcinoma or NSCLC not otherwise specified), initial treatment by surgery alone, with or without postoperative adjuvant treatment; no other previous or synchronous malignant tumors; histologically confirmed NSCLC metastatic to brain; and no deaths in the perioperative period less than 30 days after surgery, resulting in 21 evaluable cases.

The control group consisted of patients that were seen for follow-up of their NSCLC treated in the same period between 1989 and 2003, in the same clinic from which the case patients were enrolled that had either no metastases or developed metastases to distant sites other than brain (Table 1). Only patients with negative neurologic examination and/or negative brain imaging studies were included in the control group, resulting in 33 evaluable cases.

The clinicopathologic features of the two subgroups were similar, except that patients in the control group were slightly older than the cases at the diagnosis of primary NSCLC (P=0.05, Table 1). The study protocol was approved by the institutional review board of the Brigham and Women's Hospital.

Immunohistochemistry

Immunoperoxidase studies were performed on paraffin embedded sections from 54 NSCLC specimens following deparaffinization and heat induced epitope retrieval with 0.01 M citrate, pH 6.0 or 0.001 M EDTA, pH 8.0 buffers and quenching of endogenous peroxidase with 3% aqueous hydrogen peroxide. Tris buffer, pH 7.5, supplemented with 3% porcine serum, and Tris buffer alone were used for soaking and rinsing tissues, respectively, during processing. Details of the methodology and sources of the antibodies used in this study are summarized in Table 2.

The immunostained slides were examined by light microscopy. Ki-67 and caspase-3 were recorded as labeling indices (Fig. 1). The labeling index (LI) is defined as the number of Ki-67 or active caspase-3-positive tumor cells per 1000 tumor cells. We selected and evaluated five representative high-power fields (1 mm^2, Olympus BX41 microscope, 400x magnification) that have the greatest number of tumor cells staining for Ki-67 or caspase-3 (hot spots). For each patient we recorded Ki-67 LI, caspase-3 LI and the ratio of Ki-67 LI to caspase-3 LI as a marker of tumor survival (Fig. 1). VEGF-A, VEGF-C, and membranous E-cadherin were recorded as percentages of positive tumor cells in the entire tumor using five-percent increments on a representative slide. EGFR was recorded according to published criteria25 as: 0 if tumor cells had complete absence of staining or any staining intensity in less than 10% of tumor cells; 1+ if tumor cells had faint or barely perceptible membrane staining in more than 10% of tumor cells or weak heterogeneous staining in more than 50% of positive cells; 2+ if tumor cells had moderate membrane staining in more than

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10% of cells or moderate heterogeneous staining in more than 50% of positive cells; 3+ if tumor cells had strong membrane staining in more than 10% of cells or strong heterogeneous staining in more than 50% of positive cells. Tumors with 1+, 2+, and 3+ expression were interpreted as positive for EGFR expression, and tumors with no expression (0 score) were interpreted as negative for EGFR expression.25

Statistical analysis

The primary end point of this study was the occurrence of brain metastasis. Brain metastasis (present v absent) was considered a binary response variable.

The distribution of patient characteristics was compared between study and control groups using the Wilcoxon rank-sum test and Fisher's exact test for continuous and discrete variables, respectively. The distribution of protein expression was compared between study and control groups also using the Wilcoxon rank-sum test. In addition, the test was stratified according to <60, 61-70 and >70 years of age in order to adjust for the age difference between the two groups. The association between development of brain metastases and each molecular marker was based on the age-adjusted Mantel-Haenszel estimate of the odds ratio.

Overall survival was calculated from the time of NSCLC diagnosis to the time of death from any cause or to the time of the last follow-up, at which point the survival time was censored. Overall survival curves were estimated using the Kaplan-Meier method, and the log-rank test was used to evaluate the survival difference between patient groups. The statistical analysis was computed using SAS 9.1 (SAS institute, Cary, NC), while the plots were produced using S-Plus 6.2 for Windows (Insightful Corp, Seattle, WA). All significance tests were based on a two-sided hypothesis.

RESULTS

Patient and Tumor Characteristics

The patient characteristics are summarized in Table 1. In patients with newly diagnosed NSCLC included in our study, 21 (39%) developed brain metastases after a median time of 12.5 months (range 1.7-89.4 months), and 33 (61%) had no evidence of brain metastases by negative neurologic examination and/or negative brain imaging. The follow-up period of the control group (median 3.5 years; range 0.8-6.6 years) was at least as long as the study period of the patient group (median 2.6 years; range 0.7-8.4 years, P=0.54).

Expression of Molecular Markers and the Risk of Developing Brain Metastases

Table 3 shows the distribution of case patients and control subjects according to the expression of molecular markers and cutoff values used in the study. The primary analysis in Table 3 is focused on the results for differential expression that is meaningful from a pathology perspective. For each marker, we reported the cutoff point associated with the highest odds ratio, in terms of quantifying the risk of developing brain metastases. Nevertheless, the strong association between biomarker expression and development of brain metastases was maintained generally over a broad range of different cutoff values of the semiquantitative score. In general, we explored the range between the 25^{th} and 75^{th} percentiles of marker expression in the combined study and control groups to ensure reasonable patient numbers in the comparison subgroups for reasons of statistical power and clinical utility.

The study patient group had 15 tumors (71%) with a high Ki-67, whereas the control group had 7 tumors (21%, Table 3). A high caspase-3 LI was present in seven (33%) tumors from

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the study group whereas almost all the tumors (97%) in the control group had a high caspase-3 (21%, Table 3).

An increased risk of developing brain metastases was associated with a Ki-67 LI of 30 or higher (adjusted odds ratio 12.2, 95% CI, 2.4 to 70.4, $P<0.001$). Caspase-3 LI was also significantly associated with development of brain metastases ($P<0.001$), but the correlation was negative. Therefore, lower staining percentages of caspase-3 were observed in NSCLC from patients who developed brain metastases (adjusted odds ratio 43.0, 95% CI, 5.3 to >100, $P<0.001$). The ratio of Ki-67 LI to caspase-3 LI was also significantly associated with development of brain metastases (odds ratio 68.0, 95% CI, 7.7 to >100, $P<0.001$). Staining percentages of VEGF-C and E-cadherin were also significantly associated with development of brain metastases (P values of 0.001, and 0.05, respectively, Table 3, Fig. 2). No significant risk of developing brain metastases was observed with VEGF-A and EGFR expression (Table 3, Fig. 2).

Outcome of Patients with NSCLC

We found no significant differences in overall survival between patients in the study group and those in the control group ($P=0.46$, Fig. 3).

As in previous studies,[2, 4] brain metastases developed after a median time of 12.5 months (range 53 days to 89.4 months) from the primary diagnosis of NSCLC. When the study and control groups were analyzed together, a high Ki-67 LI was significantly associated with poor prognosis ($P=0.04$). Patients who had a Ki-67 LI of 30 or higher had a shorter overall survival than those with a Ki-67 LI of less than 30 (median overall survival times 26 and 47 months, respectively, Fig. 3). The other molecular markers did not show any association with overall survival.

DISCUSSION

In this study we evaluated patients with NSCLC with and without brain metastasis in a unique series that has tumor material from both the primary lung tumor and matched metachronous brain metastasis obtained from a single institution. These patients are typically only treated for lung cancer, and brain biopsy is rarely recommended or obtained. This gave us the opportunity to confirm that the brain tumors are metastases from lung primary NSCLC and to interrogate for expression of a broad panel of biomarkers reported to be involved in the pathogenesis of brain metastasis.[8, 9, 14-16, 18, 19, 22, 26] Our goal was to determine if any of these markers or a combination of markers could identify the primary NSCLC tumors that are more likely to metastasize to the brain. We have found that patients with high Ki-67, low caspase-3, high VEGF-C, and low E-cadherin in their primary NSCLC tumors have a higher risk of developing brain metastases when compared with a control group of NSCLC diagnosed during the same period of time (Fig. 2). These biomarkers are promising for predicting the development of brain metastases, and additional studies on larger series of patients are needed to validate the findings from our study.

Recent studies on the molecular biology in human cancers have shown that numerous factors affect the behavior of malignant neoplasms.[27] Tumors with a high Ki-67 LI are frequently more aggressive than those with low Ki-67 LI.[28] In addition, factors involved in angiogenesis such as VEGF are associated with tumor growth and metastasis.[17-19] Furthermore, reduced expression of molecules involved in epithelial mesenchymal transformation (EMT) such as E-cadherin, could induce tumor cells with high metastatic potential.[21] Although studies have shown association between expression of biomarkers and metastatic potential of other cancers,[18, 28-30] the value of these molecular markers in predicting brain metastasis in patients with NSCLC has not been explored yet.

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To date, it remains unclear whether patients with early stage NSCLC should be screened for brain metastases according to published clinical guidelines.11¯13 In its latest joint statement, the American Thoracic Society and the European Respiratory Society recommend no preoperative imaging of the brain in patients with NSCLC.11 In fact, it is believed that the routine screening is cost-ineffective due to the low incidence of brain metastasis in asymptomatic patients.31¯34 Some reports argue that early detection of occult brain metastases will avoid increased morbidity either by allowing earlier treatment of the brain or by avoiding futile thoracotomies.10, 35¯38 However, evidence suggests an increased incidence of cerebral metastases after preoperative radiotherapy and chemotherapy in patients with locally advanced NSCLC.39 In addition, studies have shown contrasting results regarding the correlation between tumor histology and the risk of developing brain metastases.4, 12 Therefore, there is a need to identify biologic markers to help identify patients with NSCLC who are at increased risk of developing brain metastases.

Patients with NSCLC who develop brain metastases are most likely to benefit from therapy addressing the brain that would substantially improve the quality of life.7 Analysis of subgroups of patients with NSCLC shows that certain populations are at particularly high risk of brain metastasis.7

Studies have investigated predictors of survival in patients with NSCLC and brain metastases. Similar to our results, Gaspar et al.2 found an increased risk of brain metastases in patients younger than 50 years of age. It is also known that performance status, extent of extracranial disease, control of primary cancer, and age are predictors of overall survival in patients with NSCLC.

Immunostaining with the Ki-67 antibody is a widely accepted method for evaluating proliferative activity in a variety of human tumors. Ki-67 LI correlates well with the predicted growth fraction despite that it has been consistently shown to overestimate the proliferative activity of tumors and is therefore not an exact reflection of tumor growth.40 Sarbia et al.41 found a significant association between Ki-67 LI and the mitotic activity in tumor tissue. Numerous studies have shown that Ki-67 LI correlates with other markers of cell proliferation.42, 43 Our data showed a strong correlation between high Ki-67 LI and development of brain metastases (Fig. 2). Patients with Ki-67 LI of ≥30% had a 12 times increased risk of developing brain metastases (P<0.001, Table 3) compared to those with lower proliferative activity. Furthermore, in our study, a high Ki-67 LI was associated with poor prognosis (Fig. 3).

Abnormalities in the molecular mechanisms of apoptosis are important in the pathogenesis of malignant processes because they increase the longevity of neoplastic cells, develop resistance to generally harmful stresses, and increase invasiveness resulting in disease progression and metastasis.29 This process is the result of the interaction between several proteins involved in either inhibition or activation of the apoptotic cascade. Several studies have shown that activation of caspase system is involved in gefitinib-induced apoptosis in patients with NSCLC.44, 45 Our results showed that a low caspase-3 LI was associated with a significant increase in the risk of developing brain metastases (Table 3, Fig. 2).

VEGF family of proteins modulates angiogenesis which is essential for tumor growth and metastasis.46 Since its identification around 15 years ago, this family has expanded considerably to include VEGF-A, -B, -C and -D.17, 47, 48 Although VEGF has been linked to angiogenesis in various tumors,49, 50 its role in the metastatic ability of NSCLC has been poorly characterized. VEGF-C production by a tumor is associated with increased incidence of lymphatic metastasis, but the mechanism remains unclear.51 It is believed that tumors that overexpressed VEGF-C were surrounded by lymphatic vessels that have abnormal

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lymphatic flow when compared to controls.52, 53 Our results showed a strong correlation between high expression of VEGF-C and increased risk of developing brain metastases in patients with NSCLC (Table 3). When more than 5% of tumor cells express VEGF-C, the risk of developing brain metastases increases 15 times ($P<0.001$, Table 3). There was no association between expression of VEGF-A and the risk of developing brain metastases (Fig. 2).

E-cadherin is a calcium-regulated adhesion molecule expressed in most normal epithelial tissues that plays an important role in cell-sorting mechanisms by offering adhesion specificities that govern tissue integrity. It has been demonstrated that this molecule may regulate the invasiveness of neoplastic cells.54 Our results showed that loss of E-cadherin is associated with an increased risk of developing brain metastases in patients with NSCLC ($P=0.05$, Table 3).

EGFR is overexpressed in 40 to 80% of NSCLC and many other epithelial cancers.22, 23 Despite the fact that anti-EGFR agents are being used in clinical trials for patients with advanced disseminated disease, the value of EGFR in predicting brain metastases is unknown. In this study we found that EGFR was overexpressed in 90% of patients in the study group and 85% of patients in the control group, but the difference was not statistically significant (Table 3, Fig. 2).

Our study shows that patients with NSCLC and high Ki-67, high VEGF-C, low E-cadherin and low caspase-3 in their tumors have a higher risk of developing brain metastases (Table 3, Fig. 2). Our study suggests that patients with NSCLC and a specific biomarker expression may benefit from an individualized surveillance regimen and preventive therapeutic intervention designed to prevent or delay the development of brain metastases.

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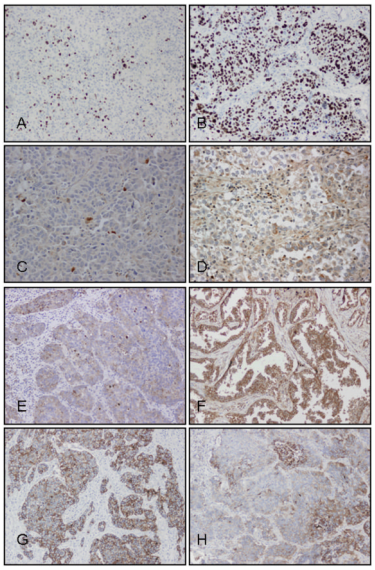


Figure 1.

Immunohistochemistry with antibodies against Ki-67 (**Panels A and B**), caspase-3 (**Panels C and D**), VEGF-C (**Panels E and F**), and E-cadherin (**Panels G and H**), in a patient who did not develop brain metastasis (left column) and a patient who developed brain metastasis (right column).

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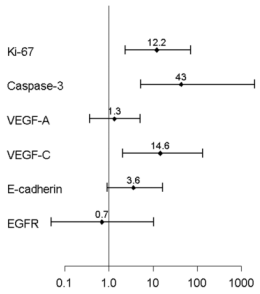


Figure 2.

Adjusted odds ratios (OR) with 95% confidence intervals (CI) of developing brain metastasis according to expression of Ki-67, caspase-3, VEGF-A, VEGF-C, E-Cadherin, and EGFR in the primary NSCLC.

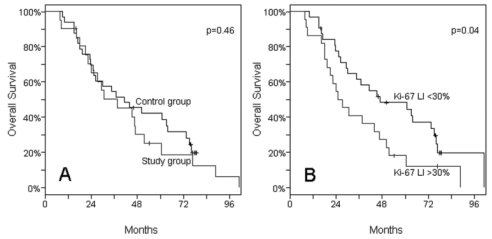


Figure 3.

Kaplan-Meier Estimates of Overall Survival among Patients with NSCLC. **Panel A**. There is no difference in overall survival between the patient and control groups. **Panel B**. Overall survival is significantly shorter in patients with high Ki-67 LI (*P*=0.04).

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TABLE 1

Clinical and Pathologic Characteristics of the Study Group and Control Group*

Characteristic	Study Group (N=21)	Control Group (N=33)	*P* Value
Sex - no. (%)			1.00
Male	10(48)	15(45)	
Female	11(52)	18(55)	
Age - yr			0.05
Mean			
Median (range)	60(42-75)	65(39-84)	
Histology - no. (%)			0.57
Adenocarcinoma	12(57)	22(67)	
Squamous cell carcinoma	9(43)	11(33)	
Primary location - no. (%)			1.00
Right lung	12(57)	18(55)	
Left lung	9(43)	15(45)	
Tumor size - cm			0.63
Median (range)	2.2(0.3-9.5)	2.6(0.6-7.3)	
Tumor stage - no. (%)			0.45
T1	13(62)	18(55)	
T2	5(24)	13(39)	
T3	2(10)	2(6)	
T4	1(5)	0(0)	
Node stage - no. (%)			0.54
N0	14(67)	25(76)	
N1	7(33)	8(24)	
Pathologic pTNM stage - no. (%)†			0.19
I	12(57)	23(70)	
II	7(33)	10(30)	
III	2(10)	0(0)	

*Because of rounding, not all percentages total 100.

†pTNM denotes pathologic tumor-node-metastasis stage.

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Table 2

Characteristics of the Antibodies Used in the Study

Antibody to	Clone	Source	Dilution	Retrieval	Detection
Ki-67	MIB-1	Dako[a]	1:150	Steamer/EDTA buffer	Envision[a]
Caspase-3 (cleaved, ASP-175 rabbit antibody)		Cell Signaling[b]	1:250	Microwave/citrate buffer	ABC[c]
VEGF-A (A20) (rabbit antibody)		Santa Cruz[d]	1:150 (overnight incubation)	Pressure cooker/citrate buffer	Envision+
VEGF-C (N-19) (goat antibody)		Santa Cruz	1:250 (overnight incubation)	Steamer/EDTA buffer	LSAB+[a]
E-Cadherin	36B5	Novocastra[e]	1:50	Pressure cooker/citrate buffer	Envision+
EGFR	EGFR.25	Novocastra	1:50	Pressure cooker/citrate buffer	Envision+

[a] Dako Corp., Carpinteria, CA

[b] Cell Signaling Technologies, Inc., Beverly, MA

[c] Vector Laboratories, Burlingame, CA

[d] Santa Cruz Biotechnology, Inc., Santa Cruz, CA

[e] Vision-Biosystems, Norwell, MA

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Table 3

Risk of Developing Brain Metastases According to the Expression of Molecular Markers in NSCLC

	Study Patients		Control Patients				
Characteristic	No.	(%)	No.	(%)	Adjusted Odds Ratio*	(95% CI)†	*P* Value
Ki-67 LI							
<30	6	(29)	26	(79)	1.0		
≥30	15	(71)	7	(21)	12.2	(2.4 to 70.4)	<0.001
Caspase-3 LI							
≥ 2	7	(33)	32	(97)	1.0		
<2	14	(67)	1	(3)	43.0	(5.3 to >100)	<0.001
VEGF-A (%)							
0-90	8	(38)	16	(48)	1.0		
91-100	13	(62)	17	(52)	1.3	(0.4 to 5.1)	0.77
VEGF-C (%)							
0	2	(10)	18	(55)	1.0		
1-100	19	(90)	15	(45)	14.6	(2.0 to >100)	0.001
E-Cadherin (%)							
91-100	5	(24)	17	(52)	1.0		
0-90	16	(76)	16	(48)	3.6	(0.9 to 16.4)	0.05
EGFR							
Negative	2	(10)	5	(15)	1.0		
Positive	19	(90)	28	(85)	0.7	(<0.1 to 10.4)	1.00

*Odds Ratios were adjusted for age.

†CI denotes confidence interval.