The Expression of Three Genes in Primary Non–Small Cell Lung Cancer Is Associated with Metastatic Spread to the Brain

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Abstract

Purpose: Brain metastases affect 25% of patients with non–small cell lung cancer (NSCLC). We hypothesized that the expression of genes in primary NSCLC tumors could predict brain metastasis and be used for identification of high-risk patients, who may benefit from prophylactic therapy.

Experimental Design: The expression of 12 genes was measured by real-time quantitative reverse transcriptase PCR in 142 frozen NSCLC tissue samples. Univariate and multivariate Cox regression analysis was used to analyze the correlation between gene expression and the occurrence of brain metastasis. Immunohistochemistry on independent samples was used to verify the findings.

Results: A score based on the expression levels of three genes, CDH2 (N-cadherin), KIFC1, and FALZ, was highly predictive of brain metastasis in early and advanced lung cancer. The probability of remaining brain metastasis–free at 2 years after diagnosis was 90.0 ± 9.5% for patients with stage I/stage IIt tumors and low score compared with 62.7 ± 12% for patients with high score (P < 0.01). In patients with more advanced lung cancer, the brain metastasis–free survival at 24 months was 89% for patients with low score compared with only 37% in patients with high score (P < 0.02). These results were confirmed by immunohistochemical detection of N-cadherin in independent cohort of primary NSCLC.

Conclusions: The expression levels of three genes in primary NSCLC tumors may be used to identify patients at high risk for brain metastasis who may benefit from prophylactic therapy to the central nervous system.

Brain metastases are most commonly associated with both small cell lung cancer (SCLC) and non-SCLC (NSCLC; 50-60%), followed by breast cancer (15-20%), melanoma (5-10%), and colon cancer (4-6%; refs. 1, 2). Prophylactic central nervous system (CNS)–directed therapy is a standard therapy in childhood leukemia (3) and has recently proved to be beneficial to patients with SCLC (4). The high incidence of brain metastases in NSCLC has led to the suggestion of offering prophylactic CNS irradiation (PCI) to these patients as well (5). Identification of patients at high risk for brain metastasis may enable better selection of those likely to benefit from prophylactic therapy to the CNS.

Lung cancer is the leading cause of cancer death worldwide (6). Between 75% and 85% of patients with primary lung malignancy have NSCLC (4, 7–15). Whereas the brain is one of the major sites of relapse in NSCLC, it is currently unclear which patient will develop this complication. Recent studies using microarray technology have shown a correlation between gene expression patterns in NSCLC and patient survival (11, 12, 14, 16, 17). None of these studies, however, has specifically addressed brain metastases. Studies addressing that question (18–20) suggested that brain metastasis was related to an

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The study has been performed as partial fulfillment of the requirement for Ph.D. degree of Helena Grinberg, Sackler School of Medicine, Tel Aviv University.

This research is dedicated to the memory of the father of S. Izraeli who expired from brain metastasis of NSCLC.

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Translational Relevance

Brain metastasis is a common complication of non–small cell lung cancer associated with severe morbidity and mortality. This complication may be prevented or delayed by prophylactic therapy. We have identified a set of genes whose expression in primary non–small cell lung cancer tumors is associated with higher risk of brain metastasis. The expression of these three genes could be used for identification of high-risk patients, who may benefit from prophylactic therapy.

Materials and Methods

Gene expression real-time quantitative reverse transcriptase PCR study

Patients and samples. RNA was extracted from 230 consecutive frozen samples of lung tumor tissues obtained from patients who underwent surgery and was stored at the Pulmonary Institute of Sheba Medical Center. Each sample was obtained following written informed consent, and the study was approved by the institutional review committee (IRB approval/05/3892). One hundred ninety of these samples yielded at least 5 μg high-quality RNA. Review of the pathologic records confirmed the diagnosis of NSCLC in 142 patients. Clinical data were obtained from patients’ files, including radiologic and pathologic records (Table 1). Staging was determined according to the tumor-node metastasis system. The diagnosis of brain metastasis was defined as “time to brain metastasis” from diagnosis of lung cancer until the date of brain imaging records that were performed because of clinical symptoms. The time from diagnosis of lung cancer until the date of brain imaging demonstrating brain metastasis was defined as “time to brain metastasis.” Follow-up period was defined as the time from surgery to death or last visit in hospital.

Selection of genes for RNA quantification. Based on our initial gene expression studies (8, 22) and published studies, we chose 12 genes hypothesized to be associated with increased brain or general metastasis (Supplementary Data and Supplementary Table S1). These genes represent three general functional categories: (a) cell proliferation and mitosis [KIFC1 (kinetin family member C1), KIF2C, KIF14 (23–27), CCNB2 (cyclin B2), S100A1 (secretogranin I; ref. 7)], TNPO1 (transportin 1), LMNB1 (lamin B1; refs. 30, 32, 33)], (b) neuronal genes [CDH2 (N-cadherin; refs. 34–40), SGNE1 (secretogranin I; ref. 7), FALZ (fetal Alzheimer antigen; ref. 30)], and (c) genes coding extracellular matrix proteins [ADAM8 (a disintegrin and metalloprotease 8; ref. 41) and SPP1 (osteopontin; refs. 27, 42–48)].

Table 1. Clinical characteristics of patients and tumors

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RQ-PCR study</th>
<th>CDH2 immunohistochemistry study</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cases</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis of primary tumor, y</td>
<td>64.95 ± 1.29</td>
<td>61.06 ± 1.22</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>93 (65.5)</td>
<td>76 (71.0)</td>
</tr>
<tr>
<td>Female</td>
<td>49 (34.5)</td>
<td>31 (29.0)</td>
</tr>
<tr>
<td>Cancer cell type, n (%)</td>
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<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>66 (46.5)</td>
<td>43 (40.2)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>61 (43)</td>
<td>50 (46.7)</td>
</tr>
<tr>
<td>Other</td>
<td>15 (10.5)</td>
<td>14 (13.1)</td>
</tr>
<tr>
<td>Stage at diagnosis, n (%)</td>
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<td></td>
</tr>
<tr>
<td>I or II</td>
<td>97 (68)</td>
<td>76 (71)</td>
</tr>
<tr>
<td>III or IV</td>
<td>45 (32)</td>
<td>31 (29)</td>
</tr>
<tr>
<td>Follow-up period, mo</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>Median</td>
<td>1-81</td>
<td>1-72</td>
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</table>

*Either large cell or atypical adenocarcinoma and squamous NSCLC; SCLCs were excluded.

Follow-up period was defined as the time from surgery to death or last visit in hospital.

Immunohistochemical study

Tumor specimens. Tumor sections were taken from 107 formalin-fixed, paraffin-embedded NSCLC tumor specimens with known clinical outcomes (25 with known brain metastases and 82 without known brain metastases). Forty-four samples were from tumors already analyzed by RQ-PCR, and 63 were additional independent cases (Table 1). Immunostaining was performed on 4-mm-thick sections. Antigen was detected with a labeled avidin-biotin method (Zymed Laboratories). Monoclonal mouse anti-human antibody (DakoCyto) for CDH2 diluted at 1:20 was used. A malignant mesothelioma

RNA processing and real-time PCR. Total RNA was isolated using Trizol (Invitrogen). RNA isolated from a NSCLC cell line (H1299) served as a calibrating control. Quantitative reverse transcriptase PCR (RQ-PCR) was then performed on cDNA synthesized from the RNA using Apgene Reverse-IT First Strand Synthesis kit (Apgene). RQ-PCR assays were performed with the ABI Prism 7900 sequence detection system using the SDS 2.2 software application. Taqman Gene Expression Assays were developed using two specific oligonucleotide primers and a unique Taqman MBG probe for the fluorescently marked target sequence (Supplementary Table S2). Experiments were performed on 96-well plates containing duplicates for 10 tumor samples, a sample of H1299, and a negative control (no cDNA). In each plate, two target genes and two endogenous control genes, ActB and HPRT1, were tested (Supplementary Fig. S1). To control for possible variations among PCR runs in different plates, the expression of all the analyzed and endogenous control genes was compared with their expression in the H1299 cell line included in every plate. Because the normalization to the endogenous control genes ACTB and HPRT1 led to similar results and conclusions, we presented the data normalized to the mean of both of these genes. The relative gene expression for all analyzed genes was calculated using the “Relative Quantification Study” program (SDS 2.2 software applications; ref. 49).
tumor sample with high-CDH2 expression served as control. All of the immunostained sections were examined independently by two pathologists (M.P. and E.O.) blinded to clinical outcomes. Immunostain scoring was determined by estimation of the percentage of immunoreactive tumor cells in each section reviewed. Only those tumor cells showing both cytoplasmatic and strong membranous staining were considered positive. Cases showing up to 2% immunoreactive tumor cells were assigned a negative score, whereas cases with ≥2% immunoreactive tumor cells were assigned a positive score.

**Statistical analysis**

The expression data for most of the genes (CDH2, SIL, TNPO1, SPP1, KIFC1, KIF2C, KIF14, LMNB1, CCNB2, and SGNE1) were normalized using log10 transforms. The FALZ and ADAM8 expression data were normalized using a square root transformation (Supplementary Table S3; Supplementary Fig. S2; Supplementary Data). After transformation, results for each gene were centered and scaled to an average of 0 and an SD of 1. Univariate Cox regression analyses were run for each gene and summary expression variable, with time to diagnosis of brain metastasis as the dependent variable. Wald χ² statistics were used to summarize statistical significance. Genes and expression variables achieving P values of <0.1 (Wald statistic, >2.71) were then entered as potential predictors in a stepwise, multivariate Cox regression analysis, which retained those predictors that had P values of ≤0.05. The resulting regression equation was used to define a predictive score.

**Results**

**Development of a model predicting the risk for brain metastasis based on gene expression levels in primary tumors.** We hypothesized that increased expression of certain genes in primary NSCLC could identify patients at high risk for the development of brain metastasis. We first performed univariate COX regression analysis of the normalized RQ-PCR values (see Materials and Methods) and ranked the genes according to their effect on brain metastasis risk (Table 2). Figure 1 schematically illustrates the relative effect of each gene on the risk for brain metastasis based on its Wald significance score. Genes in the center had no significant effect, whereas genes on the right had a positive effect, and genes on the left had a negative effect. Three genes had a significant (Wald score, >2.71; P < 0.1) positive-predictive value (CDH2, KIFC1, and SPP1) with the SIL gene just below 2.71. Surprisingly, one gene, FALZ, had a borderline significant negative-predictive value (P = 0.08).

Multivariate COX regression (Table 2) analysis revealed a statistically significant positive-predictive effect for CDH2 and KIFC1 (P = 0.009, P = 0.021) and a statistically significant negative effect for FALZ (P = 0.011). Once the effect of those three genes was taken into account, the marginal effect of SPP1 was not statistically significant.

The multivariate COX analysis was then used to define a brain metastasis score given by the following equation:

\[
\text{Score} = 0.382 \times \text{CDH2} - 0.586 \times \text{FALZ} + 0.475 \times \text{KIFC1}
\]

wherein CDH2, FALZ, and KIFC1 refer to the normalized, centered, and scaled results for each gene. The patients were then ranked into three groups based on their score. A score of < -0.5 was ranked 1 (low), a score between -0.5 and 0.5 was ranked 2 (intermediate), and a score above 0.5 was ranked 3 (high). These divisions were chosen to achieve approximately equal numbers of patients in each group.

Brain metastasis developed in 3 of the 45 patients (6.7%) in the low-ranking (a) group, in 11 (20%) of the 54 intermediate ranked (b) patients, and in 17 (41%) of the 41 patients classified to the highest rank (c; Supplementary Table S4). Thus, the higher the score is, the higher risk for brain metastasis.

The clinical significance predicting brain metastasis in patients with an early-stage disease, who may benefit from prophylactic therapy, is of great clinical importance. We, therefore, calculated the score separately for the group of patients with early-stage (I-II) disease and for the group with advanced-stage (III-IV) disease. The results are depicted by Kaplan-Meier curves in Fig. 2. Patients with low-stage NSCLC who had a high score had ~40% incidence of brain metastasis within the first 2 years after diagnosis compared with a 10% risk for patients in the low and intermediate groups (P < 0.02, log-rank test; Fig. 2A). The scores remained significant also in patients with more advanced lung cancer (Fig. 2B). The brain metastasis–free survival at 24 months was 89% for patients with low score compared with only 37% in patients with high score (P < 0.02, log-rank test). Thus, the combined score based on the gene expression level of all three genes in primary NSCLC is a powerful predictor of the risk for brain metastasis.

**Immunohistochemistry for N-cadherin.** Next, we wished to verify the results of the gene expression analysis in an independent group of patients. As we had no access to RNA from a large independent cohort of frozen NSCLC tissues with clinical information on the occurrence of brain metastasis, we opted for an immunohistochemical study. Of the three genes identified in the multivariate Cox regression model associated with brain metastasis, only N-cadherin expression can reliably be detected by immunohistochemistry on paraffin-embedded tissues with commercially available antibodies. We therefore

<table>
<thead>
<tr>
<th>Genes</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>df</th>
<th>P</th>
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<tr>
<td>KIFC1</td>
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<td>0.193871</td>
<td>1.78</td>
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<td>KIF14</td>
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<td>0.190735</td>
<td>1.49</td>
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<td>CCNB2</td>
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<td>1</td>
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<td>SIL</td>
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<td>2.61</td>
<td>1</td>
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<td>CDH2</td>
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<td>0.162251</td>
<td>4.97</td>
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<td>FALZ</td>
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<td>SGNE1</td>
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<td>0.163295</td>
<td>0.54</td>
<td>1</td>
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<td>SPP1</td>
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<td>Multivariate COX regression</td>
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<tr>
<td>CDH2</td>
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<td>FALZ</td>
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<td>KIFC1</td>
<td>0.475</td>
<td>0.207</td>
<td>5.297</td>
<td>1</td>
<td>0.021</td>
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</table>

Abbreviations: B, regression coefficient; SE, standard error; WALD, Wald significance score; df, degrees of freedom.

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attempted to corroborate our RQ-PCR findings immunohistochemically on 107 samples, 63 of which were independent, i.e., not among the 142 samples evaluated previously by RQ-PCR.

Figure 3 depicts examples of N-cadherin stains. In most of the samples, the staining was focal ranging from 2% to 80% of the cells and varied in different areas of the tumor section. As mentioned previously, only those tumor cells showing both cytoplasmic and strong membranous staining were considered positive. Of the 39 sections scored as positive, 14 were positive in 2% to 25% of the tumor cells, 13 were positive in 25% to 50% of the cells, and 12 were positive in >50% of the tumor cells within the sections.

Sixty percent of the tumor samples from patients who developed brain metastasis were positive for N-cadherin compared with only 29% of the tumor samples from patients who did not develop brain metastasis. The cumulative incidence of brain metastasis in CDH2-positive cases at 24 months after diagnosis was twice as high as that seen in N-cadherin–negative samples (35% versus 17%; \( P = 0.022 \), log-rank test; Fig. 4A). Separate analysis of the 63 independent specimens showed that the brain metastasis–free survival at 24 months was 86% for patients with N-cadherin–negative tumors compared with only 66% in positive cases (Fig. 4B; \( P < 0.03 \), log-rank test). These results for immunohistochemical staining of N-cadherin provide independent verification of the RNA expression study, suggesting its importance as a predictor of brain metastasis in NSCLC.

Discussion

About 25% of patients with NSCLC will develop brain metastases (50). To the best of our knowledge, this work is the first genomic-based study whose specific goal is to identify patients with NSCLC at high risk for this serious complication. The three-gene model we propose, based on a multivariate Cox regression analysis of the expression levels of 12 genes in primary NSCLC tumors, identifies a group of patients with high risk for developing brain metastasis during the first 2 years after surgery. These results were verified by immunohistochemical staining of N-cadherin in independent samples.

Cadherins are transmembrane proteins that mediate cell-to-cell adherence (51, 52). During the process of activation, its extracellular domain is cleaved by ADAM10 (53) and released to the cytosol. This is consistent with the immunohistochemical staining pattern that we and others have observed, namely a membranous staining pattern with or without cytoplasmatic staining. The expression of N-cadherin with suppression of
E-cadherin occurs during epithelial to mesenchymal transition that has been linked to invasion and metastasis of several types of cancers (37–40), although there is one report associating E-cadherin with brain metastasis of adenocarcinomas (54). Although N-cadherin is expressed in many tissues, it is highly expressed in the brain and is critical for many aspects of neuronal development through interactions with neural growth factors (34–36). It is tempting to speculate that N-cadherin may mediate the endurance of brain metastases through interactions with the neuronal parenchyma, as observed in Supplementary Fig. S3.

In multivariate Cox regression analysis, KIFC1 came second after CDH2 in the association with brain metastasis. KIFC1 is one of three kinesin family proteins and is one of the five mitotic regulators that we have included in our panel of genes tested. Increased expression of such mitotic spindle checkpoint genes, including aurora B kinase, MAD2, survivin, and others, have been noted to be associated with progression and metastasis of many types of cancers. Accordingly, novel antimitotic and specifically kinesin-related drugs are being developed and introduced into the clinic (22, 24–27, 55–60). Whereas KIF2C and KIF14 have been previously reported to be associated with progression of lung and breast cancers, KIFC1 has never been associated with cancer. We included it in the current study because of its striking overexpression in brain metastasis in our initial gene expression studies. It is unclear whether KIFC1 has a unique role in promoting the dissemination of NSCLC (into the brain) or if it simply represents the kinesin family or mitotic checkpoint proteins. Combining the expression of all the three kinesins or all the five mitotic regulators into our statistical model did not improve the predictive power for brain metastasis compared with inclusion of KIFC1 alone (data not shown). Whereas it is impossible to exclude the possibility that other mitotic regulators may have a similar or even better predictive power, in our cohort, KIFC1 seems to be the strongest predictor of brain metastasis.

Surprisingly, the neuronal transcriptional factor FALZ (also called BPTF for bromodomain PHD finger transcription factor) was found to be a negative predictor of brain metastasis in our cohort. FALZ was first identified by a monoclonal antibody, which recognizes neurofibrillary pathology associated with Alzheimer disease and subplate neurons in the developing human brain (61). Recent studies suggest that it is a chromatin remodeling protein sensing methylated H3K4 chromatin marks (62, 63). Except for one publication, in which its overexpression in primary adenocarcinomas was predictive of metastasis, there is no data linking FALZ to cancer (30). We have included it in our RQ-PCR study because of that publication and because of its neuronal expression. The results observed in the analysis of the whole cohort were, therefore, unexpected.
Our study did not include prospective evaluation of the brain metastasis by imaging studies. The results of this study are, therefore, meaningful in relation to diagnosis of symptomatic brain metastasis. Whereas the specific gene score may also be relevant to the occurrence of metastases of other sites, we focused on the brain because of the prospects of prophylactic therapy.

Prophylactic CNS-directed therapy has revolutionized the outcome of childhood acute lymphoblastic leukemia. Currently, the intensity of the prophylactic therapy in acute lymphoblastic leukemia is adjusted to clinical variables predicting the risk for CNS relapse (64). PCI has long been accepted as a standard treatment for limited disease in SCLC, wherein improved survival is achieved at a small cost of CNS toxicity from the radiation. A prospective study 3 has shown that PCI given to patients with extensive SCLC responding to systemic chemotherapy reduced the 1-year occurrence of brain metastasis from 40% to 13%, thus extending the indication for PCI to include all patients with SCLC who respond to chemotherapy. The incidence of CNS metastases in NSCLC is lower, and there has, therefore, been reluctance to test the hypothesis of PCI with its associated CNS toxicity in these patients (reviewed and discussed in ref. 65). A recently published study showed that PCI reduced the occurrence of brain metastases at 5 years in patients with operable stage IIA lung cancer from 34.7% to 7.8% (5), suggesting the potential benefit of PCI to a selected group of patients with NSCLC.

If these results are confirmed in a prospective study, then our identification of genes whose expression in primary NSCLC predicts the occurrence of brain metastasis may enable selecting patients for clinical trials of prophylactic CNS directed therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank the many physicians, scientists, and laboratory technicians for their assistance in this study, Drs. Vitezslav Kolek, Jiří Kleinh, and Lenka Radová for providing data on the Czech patients, Dr. Naftali Kaminsky for his contribution to the microarray studies that formed the basis of this work, and Dr. Amir Onn for the critical review of the manuscript.

References


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