Protein Expression Differences Between Lung Adenocarcinoma and Squamous Cell Carcinoma with Brain Metastasis

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Abstract. *Aim: We investigated tissue biomarkers in non-small cell lung cancer (NSCLC) to find indicators of brain metastasis and peritumoral brain edema. Patients and Methods: Fifty-two cases were studied out of which 26 had corresponding brain metastatic tissue. Clinicopathological characteristics of tumors were correlated with biomarkers of cell adhesion, cell growth, cell cycle and apoptosis regulation that were previously immunohistochemically studied but never analyzed separately according to histological subgroups, gender and smoking history. Results: Increased collagen XVII in adenocarcinoma (ADC) and increased caspase-9, CD44v6, and decreased cellular apoptosis susceptibility protein (CAS) and Ki-67 in squamous cell carcinoma (SCC) correlated significantly with brain metastasis. Increased β-catenin, E-cadherin and decreased caspase-9 expression in primary SCC, and decreased CD44v6 expression in brain metastatic SCC tissues showed a significant correlation with the extent of peritumoral brain edema. Positive correlation between smoking and biomarker expression could be observed in metastatic ADCs with p16 and caspase-8, while-negative correlation was found in SCC without brain metastasis with caspase-3, and in SCC with brain metastasis with p27. Conclusion: Our results highlight the importance of separate analysis of biomarker expression in histological subtypes of NSCLC.*

Abbreviations: NSCLC: Non-small cell lung cancer, ADC: adenocarcinoma, SCC: squamous cell carcinoma, CAS: cellular apoptosis susceptibility protein.

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Lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for more than 80% of lung cancer and it has two main subtypes, namely adenocarcinoma (ADC) and squamous cell carcinoma (SCC). During the last years it became clearly evident that these two subtypes represent distinct entities regarding not only their histological phenotypes, immunohistochemical and molecular biological profiles, but also their therapeutic approaches (1). About 80% of NSCLC cases are inoperable at the time of diagnosis, with a high frequency of hematogenous dissemination. Lung cancer is the most common primary tumor to metastasize to the brain and brain metastasis is one of the most important factors influencing the quality of life in lung cancer patients (2). Peritumoral brain edema plays a major role in determining symptoms and prognosis, therefore, a better understanding of mechanisms related to its evolution is highly required (3).

Great efforts have been made to predict the sites of metastases from lung cancer. D'Amico *et al*. have used molecular biologic markers and found that patients with isolated brain relapse had significantly higher expression of p53 and urokinase plasminogen activator (4). Li *et al*. have genotyped 33 single-nucleotide polymorphisms from 13 genes in the transforming growth factor-beta (TGF-β) signaling pathway and evaluated their associations with brain metastasis risk by using DNA from blood samples from 161 patients with NSCLC. They found the GG genotype of *SMAD6*: rs12913975 and TT genotype of *INHBC*: rs4760259 to be associated with risk of brain metastasis (5). Recently, associations between single-nucleotide polymorphisms in the PI3K-PTEN-AKT-mTOR pathway and increased risk of brain metastasis in patients with NSCLC were demonstrated by Li and coworkers (6). Singhal *et al*. reviewed molecular markers

Table I. *Patients' characteristics*.

Characteristics	Lung cancer with brain metastasis	Lung cancer without brain metastasis (Control group)
Number of patients	26 (20 metachronous/6 synchronous)	26
Males/females	17/9	19/7
Mean age/(range)	56.8 (36-75) years	56.4 (42-72) years
Histology		
Squamous cell carcinoma	14	15
Adenocarcinoma	9	11
Large cell carcinoma	2	0
Adenosquamous carcinoma	1	0
Stage (at time of primary tumor diagnosis)	IA,1; IB, 7; IIA, 1; IIB, 6; IIIA, 3; IIIB, 2; IV,6	IA, 5; IB, 13; IIA, 2; IIB, 4; IIIA, 2
Extent of peritumoral brain edema (available in 20 cases)	no edema: 4 <10 mm, 10 ≥10 mm, 6	
Smoking history (available in 50 cases)	non-smoker, 1 ex-smoker, 12 current smoker, 11	non-smoker, 1 ex-smoker, 5 current smoker, 20

or proteins that may have prognostic significance in early stage NSCLC. They focused on biomarkers primarily involved in one of three major pathways: cell-cycle regulation, apoptosis and angiogenesis. Although no single marker has yet been shown to be adequately indicative in predicting patients' outcome, the markers with the strongest evidence as independent predictors of patient's outcome included cyclin E, cyclin B1, p21, p27, p16, survivin, collagen XVIII and vascular endothelial cell growth factor (7).

We have already investigated the immunophenotypic profile of NSCLC with and without brain metastasis using biomarkers of cell adhesion, cell growth, cell cycle and apoptosis regulation; the tissue microarray approach (TMA) has also been employed (8). We could demonstrate by cluster analysis that the brain metastatic potential of NSCLC may be linked to the elevated levels of cyclin D1, cyclin D3, p16, syndecan-1, p53, caspase-3, caspase-9, CD44v6 and collagen XVII, as well as to the down-regulation of β-catenin and cellular apoptosis susceptibility protein (CAS). In that study, however, different subtypes of NSCLC were analyzed collectively and, therefore, the results yielded could not be interpreted according to the present clinical requirements.

The aim of our present work was to separately re-evaluate the lung ADC and SCC cases both in brain metastatic and control groups in order to explore whether both histological subtypes contribute equally to the observed results. Using the histopathological results of our previous study, we investigated, for the first time, the correlation between clinicopathological characteristics, such as gender and smoking history, and the expression of different tissue biomarkers (8). We also searched for biomarkers that could have predictive value for peritumoral brain edema.

Materials and Methods

Case series. Altogether 78 tumor samples were studies. We have investigated 52 primary NSCLC tissue samples including 29 SCC, 20 ADC, 2 large cell carcinomas and 1 adenosquamous carcinoma. In 26 out of 52 patients corresponding brain metastases were also studied. All tumors were surgically-resected specimens, formalin-fixed and paraffin-embedded tissues. Out of 26 lung cancer patients with brain metastasis 20 had radiomorphologic (by CT or MRI) data on thickness of peritumoral edema that scored 0-2 (0, no edema; 1, edema <10 mm; 2, edema ≥10 mm). In the other 26 patients no brain metastasis was detected during long term follow-up (median, 88 months). Smoking history was available in 50 cases. The clinical and histopathological data for all cases are summarized in Table I. The tumors were classified histologically according to the criteria of the World Health Organization. Permission for using the archived tissue blocks was obtained from the Regional Ethical Committee (TUKEB N° 106/2005).

Immunohistochemistry. In this work we have analyzed 26 previously immunohistochemically studied markers that targeted molecules of cellular differentiation (CK-7, pan-CK, HBME-1, TTF-1, chromogranin-A), cell adhesion (collagen XVII, CD44v6, E-cadherin, β-catenin, syndecan-1), cell growth (EGFR, nm23), cell cycle regulation (Ki67, cyclin-D1, -D3, p16, $p27^{kip1}$), DNA replication/repair (topoisomerase IIα) and apoptosis regulation (bax, bcl-2, CAS, caspase-3, -8, -9, fas-310, p53). For TMA construction and for specification of antibodies and immunostaining conditions used see Figure 1 of Reference 4. Semiquantitative estimations were performed by two independent pathologists. We here present only correlations studied and analyses conducted that have never been performed or published.

Statistical analysis. Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) for the Mann-Whitney *U*-test and Spearman non-parametric correlation, while the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for logistic regression analysis. The Mann-Whitney *U*-test was used to compare immunohistochemical reactions of protein markers in the different groups. The Spearman non-parametric correlation was used to investigate co-expressions of candidate proteins and associations between these protein markers and gender or smoking habits. Logistic regression method was used to predict appearance of metastasis and edema by protein expressions as well.

Results

Two large cell carcinoma samples and one squamous cell carcinoma sample showed markedly different protein expression profiles either from ADCs or from SCCs, therefore, they were excluded from statistical analyses.

Regression analysis for predicting brain metastasis showed that increased collagen XVII (p=0.0110) in ADC, increased caspase-9 (p=0.0080), CD44v6 (p=0.0310), and decreased CAS (p=0.029) and Ki67 (p=0.0319) in SCC correlated significantly with brain metastasis (Table II) (Figure 1).

During the metastatic progression in the ADC subgroup, significant marker loss of β-catenin (p=0.0370) was observed, while the E-cadherin and bax marker loss were nearly significant (p=0.054 and p=0.059, respectively). When examining marker gain in the ADC subgroup, significant changes were found in p16, topoIIα, syndecan-1, cyclin D1 and CAS expression, while a positive trend was observed regarding cyclin D3 and Ki67 expression (p=0.066, and p=0.083, respectively) (Table II).

The correlation between smoking rate and biomarker expression could be observed in certain cases (Table III). A significant positive correlation was found in the metastatic ADC subgroup with p16 (p=0.024) and caspase-8 (p=0.045) expression. In SCC a negative correlation was found in the non-metastasizing subgroup with caspase-3 (p=0.039) expression. In primary SCC with brain metastasis there was a negative correlation between smoking and p27 expression (p=0.038). It is, however, of importance that apart from one case all patients were ex- or present smokers.

The correlation between female gender and biomarker expression was also examined. In ADC, decreased cyclin-D1 showed a significant correlation with female gender, but only in the non-metastasizing primary tumor group (p=0.016), while in SCC we found no correlation between biomarker expression and gender (Table III).

When we studied markers' characteristic for wider peritumoral brain edema, we found no significant correlations either in primary or in metastatic ADC. In primary SCC, however, increased β-catenin (p=0.0259) and E-cadherin (p=0.0259), as well as decreased caspase-9 (p=0.0373) expression showed a significant correlation. In brain metastatic SCC tissues decreased CD44v6 expression showed highly significant correlation with the extent of peritumoral brain edema (p=0.0021) (Table III).

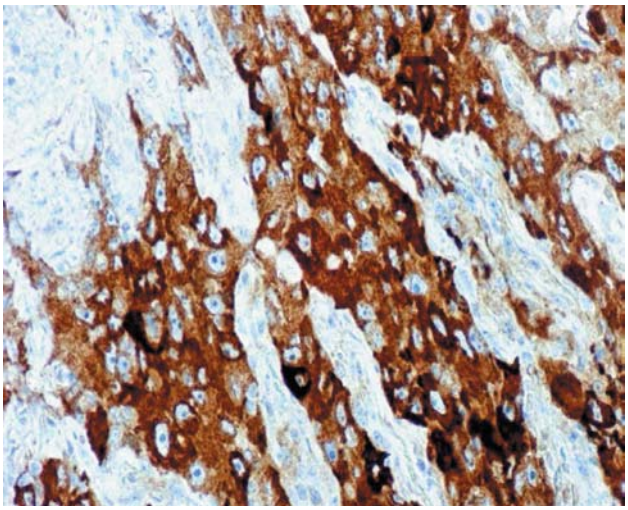

Figure 1. *Collagen XVII expression in lung SCC (hematoxylin eosin staining ×200). Collagen XVII expression is diffusely-expressed in the cytoplasm of cancer cells, but is undetectable in adjacent stromal cells.*

Discussion

Early brain metastasis or cerebral relapse remains an important cause of morbidity and mortality in NSCLC, with the mechanisms of brain metastatic process remaining poorly understood. In breast cancer for example, certain cell-surface markers, such as nm23 and CD44 are indicators and/or predictors of distant metastasis (9). Similarly, breast cancer cells that are Her2-positive are more likely to metastasis to the brain (10).

Our group has already demonstrated that brain metastatic potential of NSCLC may be linked to the elevated levels of CD44v6 and caspase-9, and decreased level of CAS expression. In that work separate analysis regarding histologic subtypes of NSCLC has not been carried out, therefore, this is the first time that we could demonstrate that the previously observed correlation was mainly related to the SCC subgroup. Recently, an interesting work demonstrated effects of different sequences of pulmonary artery and vein ligations during pulmonary lobectomy on CD44v6 mRNA expression and blood micrometastasis of NSCLC (11). In the study of Afify *et al*. CD44v6 expression correlated significantly with lymph node metastases and tumor size in lung adenocarcinoma (12). In squamous cell carcinoma of the esophagus CD44v6 protein expression was found to

Table II. *Significant differences in biomarker expression in ADC and SCC (p<0.05 in all cases; bold when p≤0.01).*

	ADC	SCC
Lung cancer with brain metastasis compared to lung cancer without brain metastasis	+ collagen XVII	+ caspase-9, + CD44v6 – CAS, – Ki67
Marker gain in brain metastasis	p16, topoIIα, CAS, syndecan-1, cyclin D1	caspase-3
Marker loss in brain metastasis	β-catenin	

+: Positive correlation; –: negative correlation. ADC: adenocarcinoma; SCC: squamous cell carcinoma; CAS: cellular apoptosis susceptibility protein.

Table III. *Biomarker expression differences in ADC and SCC according to gender, smoking history and peritumoral brain edema (p<0.05 in all cases).*

	ADC			SCC		
	Primary tumor non-metastatic group	Primary tumor with brain metastasis	Brain metastasis	Primary tumor non-metastatic group	Primary tumor with brain metastasis	Brain metastasis
Smoking		+ p16 + caspase 8				
				– caspase 3	– p27	
Female gender	- cyclin D1					
Peritumoral edema					+ β-catenin + E-cadherin – caspase-9	– CD44v6

+: Positive correlation; –: negative correlation. ADC: adenocarcinoma; SCC: squamous cell carcinoma.

correlate with the infiltration and the metastases (13).

Peritumoral brain edema plays a crucial role in deterioration of the quality of life in patients with metastatic lung cancer; however, the exact pathomechanisms related to its development is poorly understood. Sawada *et al*. evaluated aquaporin-4 protein expression in different brain tumors by immunohistochemistry but only glial tumor cells exhibited a positive reaction for aquaporin-4 (14). Recently, higher transcript and protein levels of aquaporin-4 in well-differentiated lung adenocarcinomas were described and an association with a more favorable prognosis was observed (15). In our work, decreased caspase-9 and increased β-catenin and E-cadherin expression were markers for wider peritumoral brain edema in SCC. In brain metastatic tissues in ADC no such marker was identified, while in SCC low CD44v6 expression showed a significant correlation.

It might be of interest that in the SCC subgroup markers with positive correlation for brain metastasis showed a negative correlation for the extent of peritumoral brain edema, such as CD44v6 and caspase-9 expression. Further studies are required to elucidate the relevance of this observation.

Regarding previously studied biomarkers, we demonstrated for the first time that in ADC increased cyclin-D1, p27 and EGFR expression showed a significant correlation, while in the SCC subgroup increase in topo-II α and decrease in caspase-9 expression proved to correlate significantly with smoking, although the proportion of never smokers in both ADC and SCC subgroups was very low. Nevertheless, the observed correlation between smoking and decreased caspase-9 expression might be in accordance with the findings of Chen *et al*. who demonstrated that cigarette smoking suppressed the activity of caspase-3, which could have led to the proliferation and growth of lung cancer cells in human cell lines (16).

There are hardly any data on gender differences regarding the studied biomarkers. In our present work, only the decreased cyclin D1 levels had significant correlation with female gender in ADC, whereas decreased CD44v6 was nearly significant (p=0.0528). On the contrary, increased p27, caspase-3 and caspase-9 expression in SCCs all showed a positive correlation with a high level of significance. Interestingly, the observed decreased CD44v6 expression in our female ADC patients is the opposite of what Vermeulen *et al*. reported recently in breast cancer patients, where

CD44v6 expression was increased in females when compared to male breast cancer patients (17).

Molecular markers that are of predictive value for brain metastasis might help clinicians to change follow-up and treatment strategies or to diagnose brain metastasis early enough for effective therapy. The present study highlights the importance of epigenetic factors, such as smoking and gender differences that might influence the expression of biomarkers playing a role in the metastatic process. Our results may facilitate further studies aiming to select NSCLC patients with high risk for brain metastasis who might benefit from prophylactic cranial irradiation. A better understanding of the formation of peritumoral edema in lung cancer brain metastasis might facilitate the development of novel therapeutic strategies.

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