

Clinicopathological Implications of EpCAM Expression in Adenocarcinoma of the Lung

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Abstract. *Background: The frequency of epithelial cell adhesion molecule (EpCAM) expression was investigated in non-small cell lung cancer (NSCLC) cells and human tissues, and its clinicopathological significance in adenocarcinoma of the lung was evaluated. Materials and Methods: EpCAM expression was analysed by reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry in human NSCLC cells. EpCAM protein expression was evaluated in 234 adenocarcinoma tissues using immunohistochemistry. Results: A high expression level of EpCAM was observed in human NSCLC cells by flow cytometry and RT-PCR. EpCAM overexpression was detected in 120/234 (51.3%) surgically resected adenocarcinoma tissues. EpCAM overexpression occurred significantly more frequently in adenocarcinoma than in bronchioloalveolar carcinoma ($p=0.02$). The overall survival did not differ significantly between EpCAM-overexpressing and EpCAM-negative patients ($p=0.40$). Conclusion: These findings suggest EpCAM plays a role in the carcinogenesis of adenocarcinoma of the lung and might provide a promising molecule for targeted therapy in NSCLC.*

Lung cancer is the most common cause of cancer-related death worldwide and almost 80% of lung cancers can be classified as non-small cell lung cancer (NSCLC) (1). Despite several improvements in the treatment for NSCLC,

the prognosis and outcome of patients with NSCLC remains unfavourable and the 5-year overall survival (OS) rate is 14-17% (2). Most post-surgical relapses are represented by distant metastases and the risk of a local recurrence is less than 10%. Although several recent randomized studies of adjuvant chemotherapy in completely resected NSCLC showed partially conflicting results, they also indicated a benefit of adjuvant chemotherapy of about 5% at 5 years (3-6). Recent advances in molecular technologies have facilitated the search for genes that are overexpressed in cancers, which might serve as novel targets for cancer treatment.

Epithelial cell adhesion molecule (EpCAM) is a type I transmembrane glycoprotein of about 40 kDa that is expressed on the basolateral surface of most normal epithelial tissues such as the colon, gastric, prostate, and lung epithelia (7). EpCAM is involved in intercellular adhesion and interacts with E-cadherin to induce cell adhesion (8). EpCAM is overexpressed in a variety of epithelial tumours such as breast cancer, gastric cancer, oesophageal cancer and prostate cancer (9-12). Munz *et al.* reported recently that EpCAM overexpression is linked directly to stimulation of the cell cycle and proliferation by upregulating c-myc and cyclin A/E (13). In breast cancer cells, inhibition of EpCAM expression by small inhibitory RNA diminishes cell proliferation, migration and invasiveness (14). However, the relationship between EpCAM overexpression and the invasiveness or metastatic ability of cancer cells and the underlying molecular mechanism remain unclear.

Although a high expression level of EpCAM has been reported in NSCLC, the clinical implication has not been determined fully (9, 15). This study was conducted to investigate the frequency of EpCAM expression in NSCLC cells and human tissues, and to evaluate the clinicopathological significance of EpCAM expression in adenocarcinoma of the lung.

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Materials and Methods

Cell lines. The human NSCLC cell lines A549, PC14PE6, NCI-H661m and NCI-H520, and the human kidney epithelial cell line, HEK-293, were obtained from the American Type Culture Collection (ATCC, Manassa, VA, USA); HEK-293 cells were used as the control and were propagated in minimum essential medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 units/mL of penicillin (base), 100 mg/mL of streptomycin (base) and 25 ng/mL of amphotericin B. H661 cells were propagated in Dulbecco's modified Eagle's medium and A549, PC14PE6 and H520 cells were propagated in RPMI1640.

Flow cytometry. Flow cytometric analysis of cancer cells was performed on a FACS-vantage (Becton-Dickinson, Mountain View, CA, USA). Five hundred thousand NSCLC cells were cultured and prepared in a single cell suspension. One millilitre of cell suspension was stained with fluorescein isothiocyanate (FITC)-labelled anti-EpCAM antibody (Abcam, Cambridge, UK). Ten thousand cells were analysed, and the data were collected and analysed using Cell Quest software.

Patients and tissue specimens. Paraffin-embedded sections were obtained from surgical specimens of 234 patients with adenocarcinoma who underwent curative surgery at Samsung Medical Center between January 2000 and December 2002. The tumour stage was classified according to the TNM classification.

Immunofluorescent staining of EpCAM. For immunofluorescent labelling of EpCAM in the explants, fixed explants were rinsed in phosphate-buffered saline (PBS) supplemented with bovine serum albumin (BSA), rinsed twice in PBS-BSA supplemented with 0.05% Tween 20 and then rinsed once in PBS-BSA. The explants were incubated with 3% BSA in PBS to reduce non-specific labelling. The excess BSA was removed and the explants were incubated overnight at 4°C with FITC-conjugated antibody to EpCAM (Abcam). The explants were rinsed with PBS, mounted, and examined using a fluorescence microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 2 µg of total RNA using an Oligo(dT) primer (Invitrogen) and Omniscript Reverse Transcription kit (Qiagen, Valencia, CA, USA). β-Actin was used as the endogenous expression standard. Each PCR program involved a 3 min initial denaturation step at 94°C, followed by 25 cycles at 94°C for 30s, 55°C for 30s and 72°C for 1min on a gradient thermal cycler (Biometer, Göttingen, Germany). The primer sequences were: EpCAM forward, 5'GAA GGC TGA GAT AAA GGA GAT GGG3' and EpCAM reverse, 5'TTA ACG ATG GAG TCC AAG TTC TGG3'.

Immunohistochemistry for EpCAM expression in lung adenocarcinoma tissues. Formalin-fixed paraffin-embedded tissue blocks were sectioned to 4 µm thickness. The tissue sections were deparaffinized in xylene and then rehydrated in serially graded alcohol. Endogenous peroxidase activity was eliminated by preincubation in 3% hydrogen peroxide in 10% methanol for 15 min followed by three washes in PBS. For antigen retrieval, the sections were heated in microwave oven for 10 min in 10 mM sodium citrate buffer (pH 6.0). After washing in

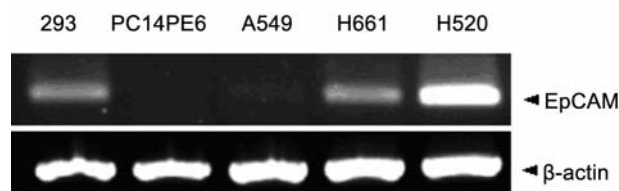


Figure 1. mRNA expression levels of EpCAM and β-actin as a control were assessed in the indicated carcinoma cell lines by standard RT-PCR.

Tris-buffered saline, each slide was preincubated in 100 µL of 5% normal blocking solution (goat serum) for 10 min to reduce non-specific binding. The slides were incubated at room temperature with mouse monoclonal antibody to epidermal growth factor receptor (EGFR) (NCL-EGFR-384; Novocastra, Newcastle, UK) at 1:100 in a humid chamber for 1 h. The primary antibody was visualized with an avidin-biotin complex system (Dako, Glostrup, Denmark).

The immunohistochemical evaluation was performed independently by two pathologists with no prior knowledge of the clinical data. EpCAM expression was evaluated by calculating the total immunostaining score as the product of the proportion score and the intensity score. The proportion score described the estimated fraction of positively stained tumour cells (0, none; 1 <10%; 2, 10-50%; 3, 50-80%; and 4, >80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; and 3, strong). The total score ranged from 0 to 12. The overexpression of EpCAM was defined as a total score >4 as described previously (16).

Statistical analysis. The clinical and pathological variables were compared between groups using the Pearson χ^2 -square test for categorical variables. Survival curves were calculated using the Kaplan-Meier method and compared with other prognostic variables using the log-rank test. Stepwise Cox regression analysis was performed to identify prognostic factors for survival. In all tests, $p < 0.05$ was considered significant.

Results

EpCAM expression in non-small cell lung cancer cells. Four different NSCLC cell lines were chosen to study the expression level of EpCAM. The expression of EpCAM mRNA was detected in two of these NSCLC cell lines, NCI-H520 and NCI-H661. However, EpCAM mRNA was not detected in another NSCLC cell line, PC14PE6, and was detected only modestly in the A549 cell line (Figure 1). EpCAM protein expression at the cell surface was analysed by flow cytometry using fluorescently labelled EpCAM antibody. Consistent with the pattern of EpCAM transcript level, EpCAM protein was strongly detected in H520 and H661 cell lines, and weakly detected in A549 cells. PC14PE6 and HEK293 kidney epithelial cells did not express a detectable level of EpCAM protein at the cell surface (Figure 2). In cell culture slides examined immuno-cytochemically with fluorescently labelled EpCAM antibody,

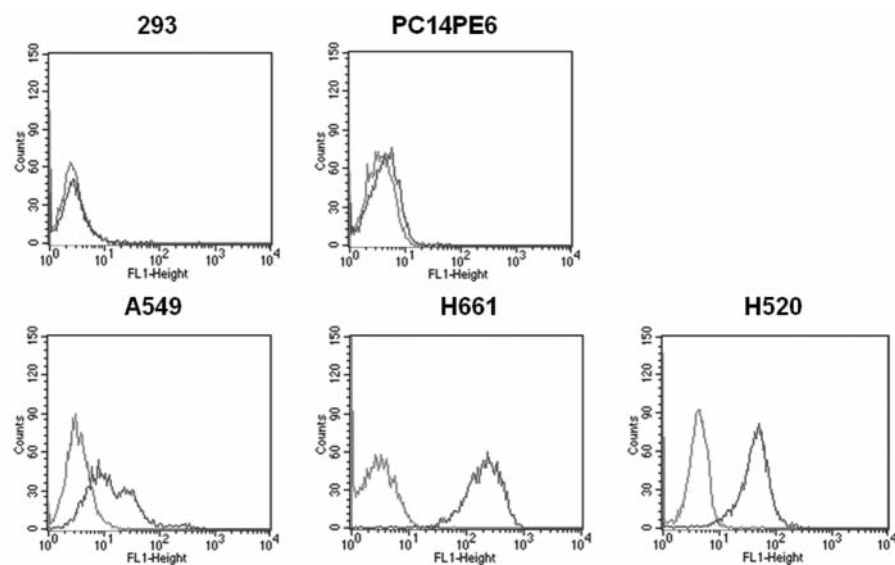


Figure 2. Cell surface expression of EpCAM was analysed by flow cytometry of the indicated cell lines.

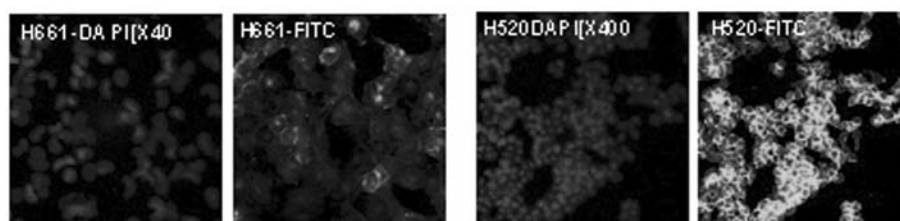


Figure 3. Expression of EpCAM protein in NCI-H520 and NCI-H661 cell lines was analysed by immunofluorescence staining.

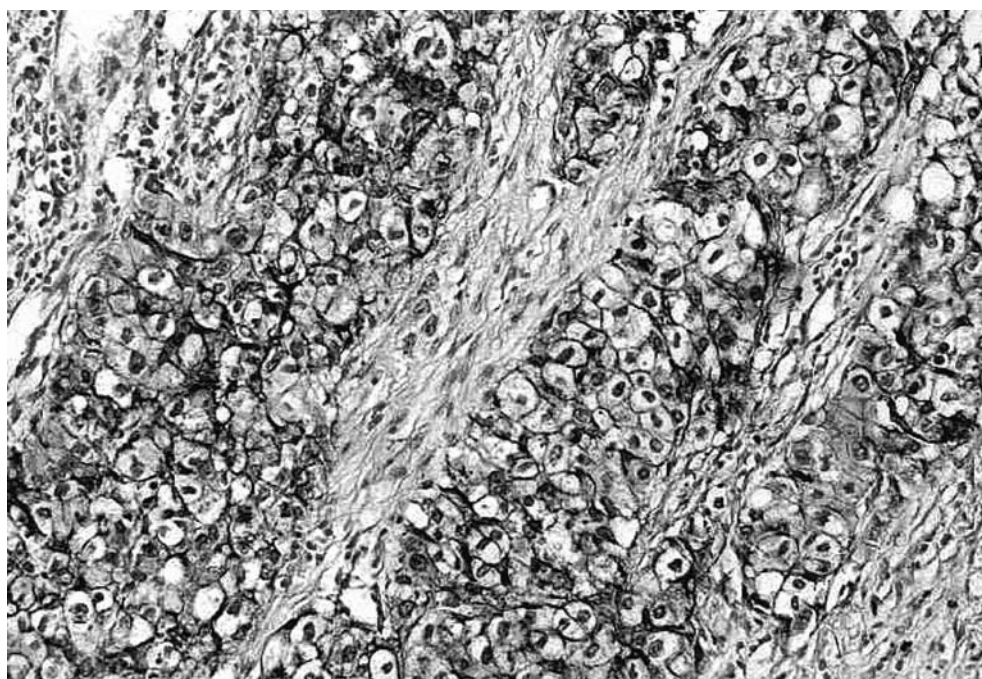


Figure 4. Examples of EpCAM immunohistochemical staining of lung cancer samples. High EpCAM reactivity was detected mainly at the cell surface of the tumour tissue.

Table I. Patients characteristics according to EpCAM expression in adenocarcinoma.

	No. of cases	EpCAM overexpression		p-value
		-	+	
Age (median)	61(13-82)			
<61	115	88	27	0.19
≥61	119	82	37	
Gender				
Male	127	88	39	0.21
Female	107	82	25	
Histology				
Adenocarcinoma	199	139	60	0.02
BAC or BAC combined	35	31	4	
Primary tumor				
pT1	65	44	21	0.29
pT2-T4 (T2/T3/T4)	169 (137/16/16)	126	43	
LN status				
pN0	160	112	48	0.18
pN1-N2 (N1/N2/N3)	74 (34/39/1)	58	16	
Stage				
I (A/B)	51/92	102	41	0.78
II (A/B)	5/30	27	8	
III (A/B/IV)	38/16/2	41	15	

*BAC: bronchioloalveolar carcinoma.

EpCAM immunoreactivity was confined mainly to the cell surface of EpCAM-positive cancer cell lines (Figure 3).

EpCAM expression in surgically resected adenocarcinoma of lung. EpCAM expression was assessed by immunohistochemical staining of the primary tumour specimens obtained from patients (Figure 4). EpCAM expression at any expression level was observed in 51.3% (120/234) of patients. Interestingly, positive focal EpCAM staining was also seen in the normal bronchial epithelium. However, only 27.3% (64/234) of tumours displayed overexpression of EpCAM, which was defined as a total score >4. Clinicopathological parameters such as sex, age, T stage or regional lymph node metastasis were not related to EpCAM expression (Table I). However, EpCAM overexpression was significantly more frequent in adenocarcinoma specimens than in bronchioloalveolar carcinoma (BAC) of the lung specimens ($p=0.02$). For the total study population, the median follow-up was 82.0 months (range, 66.1-104.2 months) and the median OS was 76.0 months (95% CI, 61.5-90.5 months). The OS did not differ significantly between the EpCAM-overexpressing and EpCAM-negative groups (72.1 vs. 78.0 months, $p=0.40$; Figure 5). The disease-free survival did not differ significantly between the two groups ($p=0.63$, data not shown).

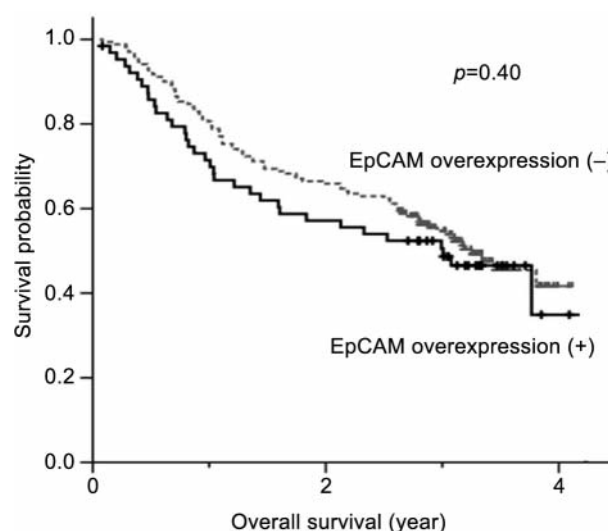


Figure 5. Overall survival according to the EpCAM expression level in patients with lung adenocarcinoma who underwent surgical resection.

Discussion

In this study, EpCAM was expressed robustly at the transcriptional and protein expression levels in several NSCLC cell lines. Immunohistochemistry showed that EpCAM proteins were localized at the cell surface membrane. In primary tumour specimens, EpCAM expression at any expression level was observed in 51.3% of adenocarcinoma patients. However, the pattern of EpCAM expression was heterogeneous at all expression levels. The overexpression of EpCAM was noted in only 27.3% (64/234) of tumours after definition of the overexpression of EpCAM as a total score >4. The frequency of EpCAM expression observed in this study is consistent with or slightly lower than those of other recent EpCAM studies of various human tumour samples. In the retrospective analysis of thousands of samples from prostate, lung, colon, gastric and renal cell cancer patients using standardized staining conditions in tissue microarray (9), about 85% of adenocarcinomas and 72% of squamous cell carcinomas overexpressed EpCAM in a homogeneous manner. In the subset of lung cancer array of this study, 823 of 1287 cases (63.9%) showed a high level EpCAM expression (9).

Interestingly, a significantly higher frequency of EpCAM overexpression was found in adenocarcinoma compared with BAC. BAC is a subtype of lung adenocarcinoma without evidence of stromal, vascular or pleural invasion and is thought to be a less aggressive *in situ* carcinoma than adenocarcinoma. EpCAM expression might play a role in the carcinogenesis of adenocarcinoma of the lung. However, no significant difference was found in lymph node metastasis or

stage between the EpCAM-overexpressing and EpCAM-negative group. Further studies are needed to determine the exact role of EpCAM expression in terms of the invasiveness or metastatic ability in adenocarcinoma of the lung.

Given the possible prognostic significance of EpCAM overexpression in cancers suggested by several investigators (10, 11, 14, 16), whether survival differed according to EpCAM expression level was also assessed. No significant difference was found in disease-free survival or OS between the EpCAM-overexpressing and EpCAM-negative groups, suggesting that EpCAM overexpression has no prognostic significance. The results are consistent with previous reports showing no definite correlations between EpCAM expression and tumour grade or OS in any of the histological entities (9,15). The prognostic impact of EpCAM overexpression on survival varies widely between studies of various tumours. In human breast cancer, for example, the expression of EpCAM was negatively correlated with survival parameters in node-positive patients (18). A similar negative correlation was observed in patients with gall bladder cancer (19) and oesophageal cancer (10). In contrast, EpCAM upregulation was associated with improved survival in patients with clear cell renal carcinoma (17), moderately differentiated stage II colon cancer (9) and NSCLC at stage pT2 (9). These discordant findings with different cancer types might be attributable to several factors. Possible explanations include the limitations of the semi-quantitative nature of immunohistochemical staining, such as the different antibodies used, interobserver or intraobserver variation, and the variable cut-off values for EpCAM positivity. In many cases, the studies were performed on a relatively small sample size, which may have been insufficient to show significant differences. A standardized method remains to be established and validated in larger series of patients in prospective studies.

It is also possible that the functions and actions of EpCAM in cancer progression may differ in different tissue microenvironments. Although an *in vitro* study showed rapid upregulation of c-myc by EpCAM overexpression, suggesting a direct link between EpCAM and c-myc-mediated cell cycle control (13), the exact mechanism responsible for the contribution of EpCAM to the malignant potential of tumour cells is not understood fully.

Despite the controversy about the clinicopathological significance of EpCAM expression in human malignancies, the various EpCAM studies are consistent in showing the frequent, high level of expression of EpCAM in diverse human cancers. This observation makes EpCAM a prime target for immunotherapies using antibodies to EpCAM to treat several cancers. Monoclonal antibodies (*e.g.*, edrecolomab, adecatumumab), EpCAM-specific immunotoxins (*e.g.*, proxinium), multi-target-specific EpCAM antibodies (*e.g.*, catumaxomab) and various EpCAM cancer vaccines are being investigated in

ongoing clinical trials (18) and have shown partial activity in several tumour types.

The validation of EpCAM as a therapeutic cancer target remains largely unexplored. The precise understanding of the molecular role of EpCAM in tumour progression will be crucial in rationalizing the various clinical attempts to employ EpCAM-specific antibodies and in identifying subsets of patients who would benefit most from EpCAM-targeted cancer therapy. In parallel with a number of EpCAM-directed immunotherapies, the focus of future EpCAM studies will be on elucidating EpCAM signalling networks, determining its oncogenic or metastatic capacities in relation to other membrane proteins and identifying its possible role in contributing to cancer cell “stemness”.

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