ORIGINAL PAPER

Expression analysis of EML4 in normal lung tissue and non-small cell lung cancer (NSCLC) in the absence and presence of chemotherapeutics

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This paper is dedicated to Prof. Klaus Kayser, on the occasion of his 70th birthday.

Abstract

Despite considerable progress in the development of individualized targeted therapies of tumor diseases, identification of additional reliable target molecules is still mandatory. One of the most recent targets is microtubule-associated human EML4 generating a fusion-type oncogene with ALK demonstrating marked transforming activity in lung cancer. Since EML4 is a poorly characterized protein with regard to expression, function and regulation in human tissue, specimens of human tumor and tumor-free tissues obtained from patients with NSCLC were analyzed to determine the cellular localization. All tissue samples have been previously fixed with the novel HOPE-technique and paraffin embedded. Determination of both gene expression and protein levels of EML4 were performed using RT–PCR, *in situ* hybridization as well as immunohistochemistry, respectively. In human NSCLC tissue samples, possible regulation of EML4 transcription upon chemotherapy with combinations of most established cytotoxic drugs for NSCLC treatment was also studied employing the recently established *ex vivo* tissue culture model STST. In normal lung, both marked mRNA and protein levels of EML4 were localized in alveolar macrophages. In contrast, lung tumor tissues always showed consistent transcriptional expression *in situ* and by RT–PCR. Stimulation of NSCLC tissues with chemotherapeutics revealed heterogeneous effects on EML4 mRNA levels. Based on its expression patterns in both tumor-free lung and NSCLC tissues, human EML4 is likely to be closely associated with processes involved in local inflammation of the lung as well as with tumor behavior. Thus, our results suggest that EML4 may have the potential as a therapeutic target molecule in NSCLC chemotherapy.

Keywords: EML4, NSCLC, chemotherapeutics, HOPE, STST.

Introduction

Despite increasing knowledge regarding onset and progression of tumor diseases, up to now an overall effective therapy regimen for lung cancer is not yet available. Detailed knowledge of tumor biology and the underlying mechanisms is essential for the identification of therapeutically relevant target molecules to develop novel successful strategies for anticancer therapies. Most recently, a fusion-type oncogene generated through chromosome translocation of echinoderm microtubule-associated protein like protein 4 (EML4) and anaplastic lymphoma kinase (ALK), has been discovered with marked transforming activity that is closely associated with lung cancer [1]. To date, EML4–ALK fusion oncogene represents one of the newest molecular targets in a small subset of non-small cell lung cancer (NSCLC), especially of adenocarcinoma histological type [2].

Preclinical studies by Soda M *et al.* using different human cell lines as well as mouse 3T3 fibroblasts transiently transfected with human EML4–ALK and nude mice demonstrated that this fusion protein possesses potent oncogenic potential both *in vitro* and *in vivo* [1]. Genomic analyses in specimens obtained from NSCLC patients subsequently identified several isoforms (variants 1 to 5) of the fusion gene based on diverse break and fusion point regions within human EML4 locus that are always fused to exon 20 of ALK [3]. Yet, within all variants, the presence of the basic coiled coil domain of EML4 is essential for the constitutive homodimerization of EML4–ALK in cells, by which tyrosine kinase activity of ALK is induced. Furthermore, application of ALK inhibiting compounds could effectively suppress cell growth in EML4–ALK expressing BA/F3 cells and further demonstrated serious adverse effects in different NSCLC cell lines

in vitro. As a consequence, research has been focussed on ALK as a potential therapeutic candidate molecule in NSCLC expressing this fusion-type oncogene [4].

In contrast, human EML4 is a poorly characterized protein that belongs to the highly conserved family of echinoderm microtubule-associated protein like proteins (EMAP). To date five human homologues of the echinoderm EMAP, named EML1-5, are known. Human EML4 has been described for the first time by Heidebrecht HJ *et al*. [5], who found an over expression of EML4/ROPP120 (restrictly over expressed proliferation-associated protein 120 kDa) during mitosis. More recent *in vitro* studies using transiently transfected Cos7 and HeLa cells, identified both mouse and human EML4 [6, 7] as microtubule stabilizing molecules that also colocalize with the mitotic spindle of dividing cells. Suppression of EML4 transcription by RNA interference in these cells dramatically reduced proliferation after 72 hours post-transfection with simultaneously increasing numbers of apoptotic cells. Thus, these results indicated an essential role of EML4 for normal cell proliferation and cell survival.

There is no information for the role and regulation of wild-type EML4 expression, neither in normal human lung nor in lung tumor tissues, except for one recent study that analyzed the overall frequency for all known fusion transcripts in specimens of human NSCLC patients. Here, amplification of wild-type EML4 could also be detected in several cases, strongly suggesting a possible role of this molecule in malignant transformation [2]. Based on the potential importance of EML4 in human lung disease, the cellular expression of EML4 was analyzed in a total of 25 clinical tissue specimens of normal human lung as well as in 40 samples of NSCLC (22 adenocarciomas, 15 squamous cell carcinomas, three others) using both *in situ* hybridization (ISH) and immunohistochemistry (IHC). Additionally, two selected tissue samples (one adenocarcinoma, one squamous cell carcinoma) and the human lung tumor cell line A549 (adenocarcinoma-like) were also subjected to immunofluorescence staining method (F–IHC). All tested human lung (cancer) tissue samples were fixed by the novel hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) technique and embedded in paraffin, as described elsewhere [8, 9]. In addition, in a different set of 18 lung tumor tissues (11 adenocarcinomas, five squamous cell carcinomas, two others) the effects of most common chemotherapeutics, such as carboplatin, gemcitabine, vinorelbine and paclitaxel in different combinations, upon transcription of EML4 were analyzed by RT–PCR. For this purpose, a currently established human *ex vivo* cultivation model designated STST (Short-Term-Stimulation of Tissues) was employed [10–13].

Material and Methods

Tissue specimens and human cell line

Tumor samples were obtained from patients by lobe- or pneumonectomy because of lung cancer. A total number of 40 tumor samples were tested including 22 adenocarcinomas, 15 squamous cell carcinoma, two carcinoids and one basaloid carcinoma, all of them with a differentiation grade of 2 or 3, except for one adenocarcinoma and one squamous cell carcinoma with grades 1. For comparison reasons, a total of 25 lung tissue samples were taken far enough (>3 cm) from the tumor tissues to serve as normal human lung. In addition, the human NSCLC cell line A549 (adenocarcinoma histological type) was also analyzed by fluorescence immunohistochemistry.

In all cases, the different tissue samples and cytospins of A549 cells were HOPE-fixed and paraffin embedded as previously described [9]. Accordingly, subsequent analyses were always performed after deparaffinization of the tissue samples.

Chemotherapeutical treatment of NSCLC specimens

A selected set of 19 specimens of NSCLC tissues (11 adenocarcinomas, six squamous cell carcinomas, one carcinoid and one basaloid carcinoma) were stimulated with several, most common chemotherapeutic agents in the novel *ex vivo* cultivation model designated STST [11]. Briefly, vital tumor samples were cultured in 2 mL RPMI 1640 at 37^0C and 5% CO_2 for 16 hours in the absence or presence of gemcitabine (0.31 µg/mL) in combination with either carboplatin (8.25 µg/mL), paclitaxel (2.41 µg/mL) or vinorelbine (0.76 µg/mL), respectively, as well as in the presence of vinorelbine combined with paclitaxel. The indicated concentrations were calculated based on human clinical dose regimens.

In situ hybridization (ISH)

Another 18 tumor tissue specimens of NSCLC (10 adenocarcinomas, seven squamous cell carcinomas and one pleomorphic carcinoma) and the corresponding normal lung areas were analyzed by *in situ* hybridization, as previously described [12]. Using PCR products of EML4 from below (described in RT–PCR), double stranded, digoxigenin (DIG) labeled DNA-probes (DIG-High-Prime, Boehringer Mannheim, Germany) were synthesized and used at a final concentration of 2 ng/µL [14].

Immunohistochemistry (IHC)

A different set of 18 NSCLC specimens (11 adenocarcinomas, seven squamous cell carcinomas) and their corresponding normal lung tissues (n=13) as well as one exemplary sample of both adeno- and squamous cell carcinoma treated with a combination of gemcitabine and carboplatin, were stained by IHC as reported earlier [13, 15]. The primary antibody EML4 (mouse anti-human EML4, monoclonal, clone 3C10, Abnova, Taipei City, Taiwan) was applied in a final dilution of 1:200 for 50 minutes at ambient temperature. Visualization was performed by incubating the detection system (ZytomedPlus HRP polymer kit, Zytomed Systems, Berlin, Germany) for 30 minutes at room temperature and using AEC (aminoethylcarbazole) as chromogen. Negative controls were always included omitting the primary antibody.

Immunofluorescence staining of both one exemplary sample of adenocarcinoma and of squamous cell carcinoma as well as of cytospins of A549 cell line was also

performed as described above except for using a secondary antibody labeled with green fluorescence (goat anti-mouse fluorescence isothiocyanate FITC, Alexa Fluor 488, Invitrogen, Darmstadt, Germany) for visualization, requiring an additional incubation period of 40 minutes in the dark. One drop of mounting medium containing DAPI (4',6-diamino-2-phenylindole, Vector Laboratories, Burlingame, USA) was applied for DNA staining and mounting.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

RT–PCR was performed on 18 different NSCLC tissue specimens (11 adeno-, six squamous cell carcinomas, one carcinoid and one basaloid carcinoma), which were treated with either two different toxic drugs in combination or with RPMI as controls. Total RNA was extracted from all samples according to the manufacturer's recommendations (RNeasy Mini Kit, Qiagen, Hilden, Germany). RT–PCR was performed as previously described in detail [14] using EML4 specific primers (forward: CAA GTC ATA CCA GTG CTG TC; reverse: GGT GAT GCT CGA ATT TGT GG) spanning an amplicon of 156 bp. Specific primers targeting the mRNA of the housekeeping gene glyceraldehyde 3- phosphate dehydrogenase (GAPDH) (forward: AGA ACG GGA AGC TTG TCA TC; reverse: TGC TGA TGA TCT TGA GGC TG) spanning an amplicon of 257 bp were always run in parallel as control. RT–PCR products of EML4 were normalized to those of GAPDH for direct comparisons between the different treatment conditions. The EML4 products were sequenced using an ABI 377 sequencer and cycle sequencing.

Statistical analysis

Statistical comparisons of the normalized values between treated NSCLC samples (gemcitabine and carboplatin) and the corresponding untreated control tissues (medium) following short-term cultivation were performed using nonparametric Mann–Whitney-test for matched-paired samples (InStat 3.06, GraphPad Software Inc. San Diego, CA, USA). A two-tailed p-value ≤ 0.05 was considered significant.

Results

Cellular EML4 expression in human tissues

Normal human lung

Representative results of cellular localization in normal human lung tissues are shown in Figure 1, A–D.

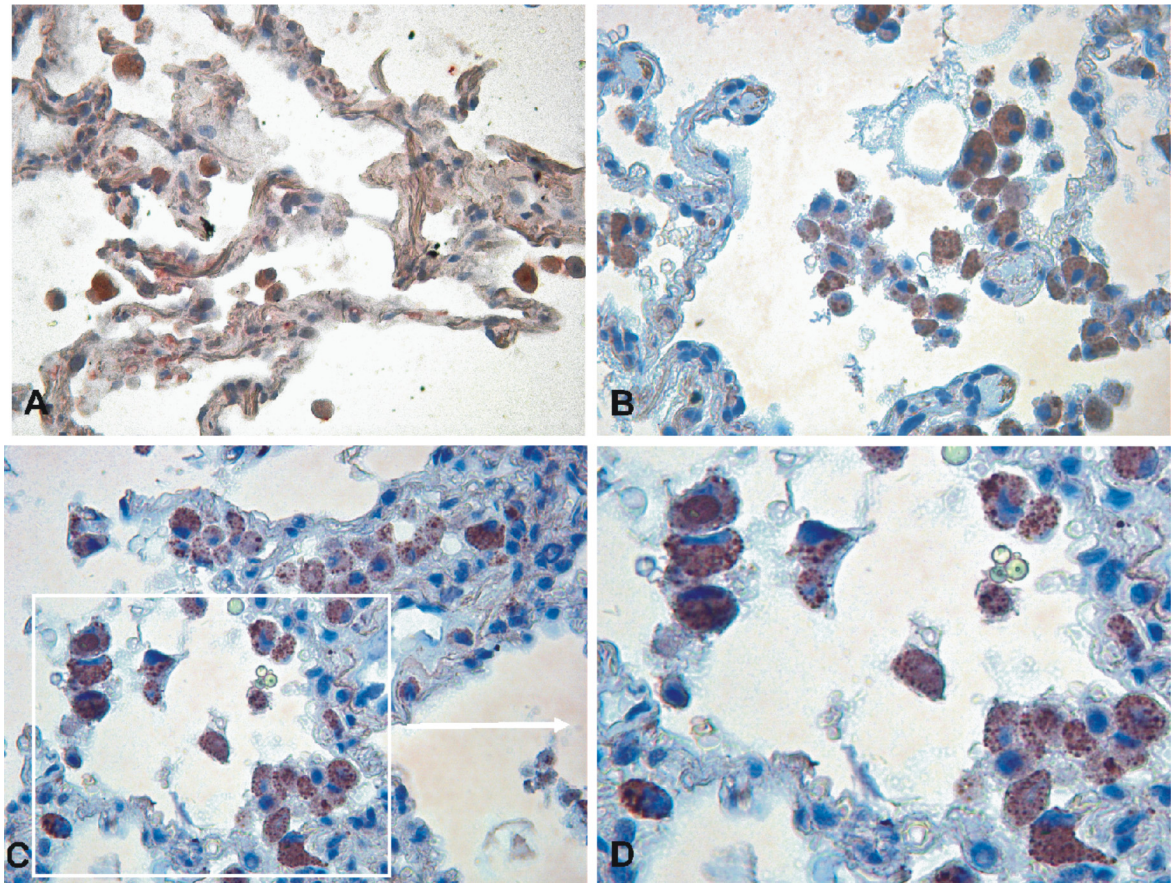

Figure 1 – ***Detection of EML4 in alveolar macrophages of human non-malignant lung tissues by in situ hybridization (ISH) (A) or immunohistochemistry (IHC) (C–D), whereby an area of (C) is shown enlarged as indicated in (D). (Magnification 400× and 800×, respectively).***

Expression of EML4 mRNA was detected *in situ* in all tested specimens of normal human lung (n=18). In the majority of the cases, transcripts appeared in the alveolar macrophages (AM) (Figure 1A). Some occasional signals were also observed in cells reflecting the morphology of alveolar epithelial type II cells

(AECII). In addition, immunohistochemical EML4 signals were detected in 8/13 (62%) analyzed lung tissue samples with localization in the AM only, revealing both perinuclear and strong granular staining patterns in the cytoplasm (Figure 1, B–D).

NSCLC specimens

Likewise, the results of the cellular distribution of EML4 in tumor cells of adenocarcinoma type are depicted in Figure 2, A–D that are representative for the different histological types of NSCLC.

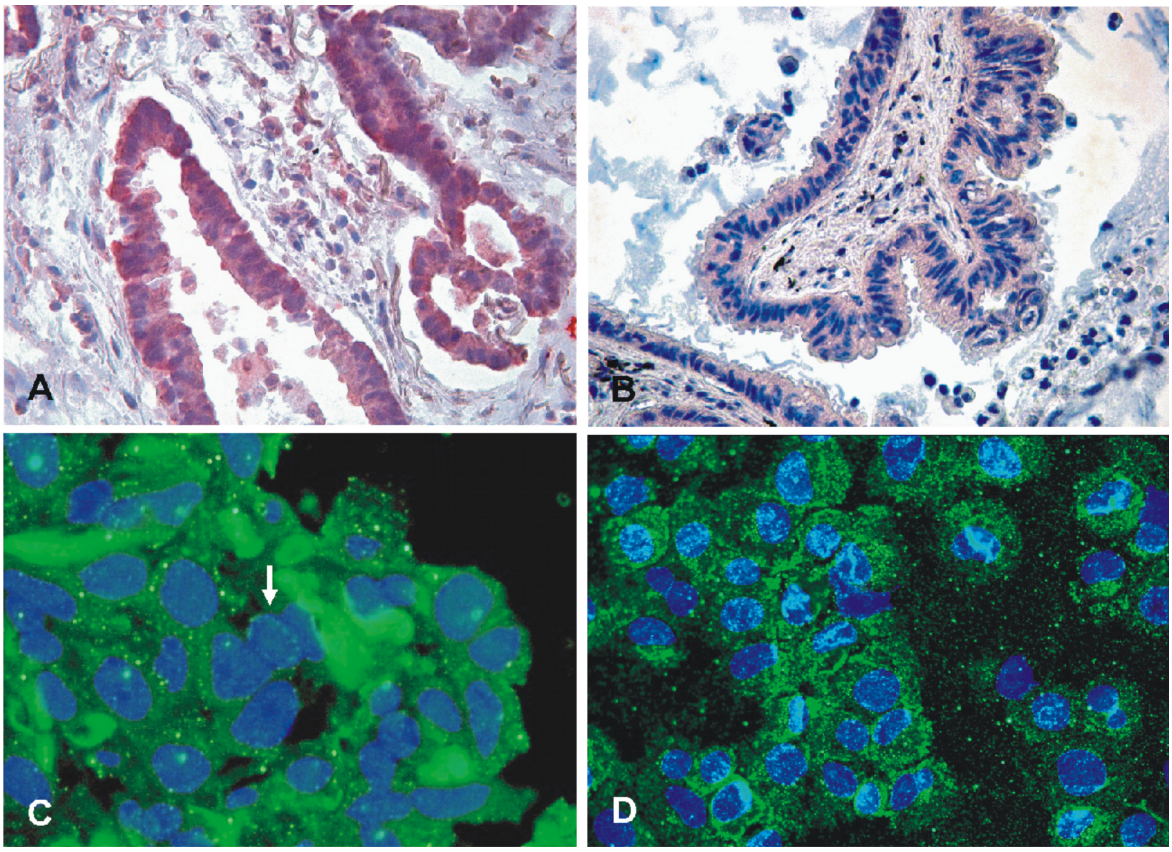

Figure 2 – ***Detection of EML4 in tumor cells of a selected adenocarcinoma, which is representative for all different histological types of NSCLC by ISH (A) or IHC (B). Immunofluorescent signals of EML4 protein are shown in a different adenocarcinoma type of NSCLC (C) as well as in the adenocarcinoma-like A549 cell line (D). Perinuclear alignment of EML4 is indicated by the arrow. (Magnification 400× for A, B, D and 1000× for C).***

The transcripts of EML4 were detected in all but one analyzed NSCLC specimens (n=18), revealing a strong and homogenous expression pattern for EML4 mRNA within the whole lung tumor tissue, irrespective of the histological type (Figure 2A). Furthermore, EML4 protein levels as determined by IHC, were detected in 13/18 (72%) tested NSCLC specimens, with a similar, albeit weaker diffuse staining patterns in the cytoplasm of all tumor cells as compared to the normal lung (Figure 2B). Immunofluorescence staining further revealed distinct perinuclear EML4 signals in two different NSCLC tissue samples (Figure 2C) as well as in A549 cells (Figure 2D).

Combined chemotherapy effects

Alterations of EML4 transcription in human NSCLC

The results of gene expression of EML4 in lung tumor specimens, which were cultivated in the absence or presence of two chemotherapeutic agents gemcitabine and carboplatin in combination as determined by RT–PCR, are summarized for adenocarcinomas (left side) and squamous cell carcinomas (right side) separately in Figure 3A. The values of the specific transcripts were normalized to the corresponding GAPDH mRNA bands for direct comparisons. Both stimulated and control NSCLC tissue samples (n=18) consistently revealed specific EML4 mRNA in all cases. In 4/18 (22%) NSCLC cultures, up-regulation of EML4 expression occurred after simultaneous stimulation with these two toxic agents in both adeno- and squamous cell carcinomas as compared to the corresponding untreated controls. Furthermore, in 5/18 (28%) NSCLC tissue cultures, down-regulation of the EML4 transcripts was detected in stimulated adenocarcinomas and in one carcinoid, when compared to their medium controls. No alterations in EML4 transcription were seen in all other tested tumor tissue samples (50%) following chemotherapeutical treatment.

In addition, one selected tissue sample of both adeno- and squamous cell carcinomas, which have shown clear alterations upon chemotherapy, was further treated with additional combinations of gemcitabine and either vinorelbine or paclitaxel as well as a combination of vinorelbine and paclitaxel, to study the effects of chemotherapeutic agents with particular toxicity on microtubules on EML4 transcription. EML4 gene expressions in response to these treatment conditions are demonstrated for both cases in Figure 3B.

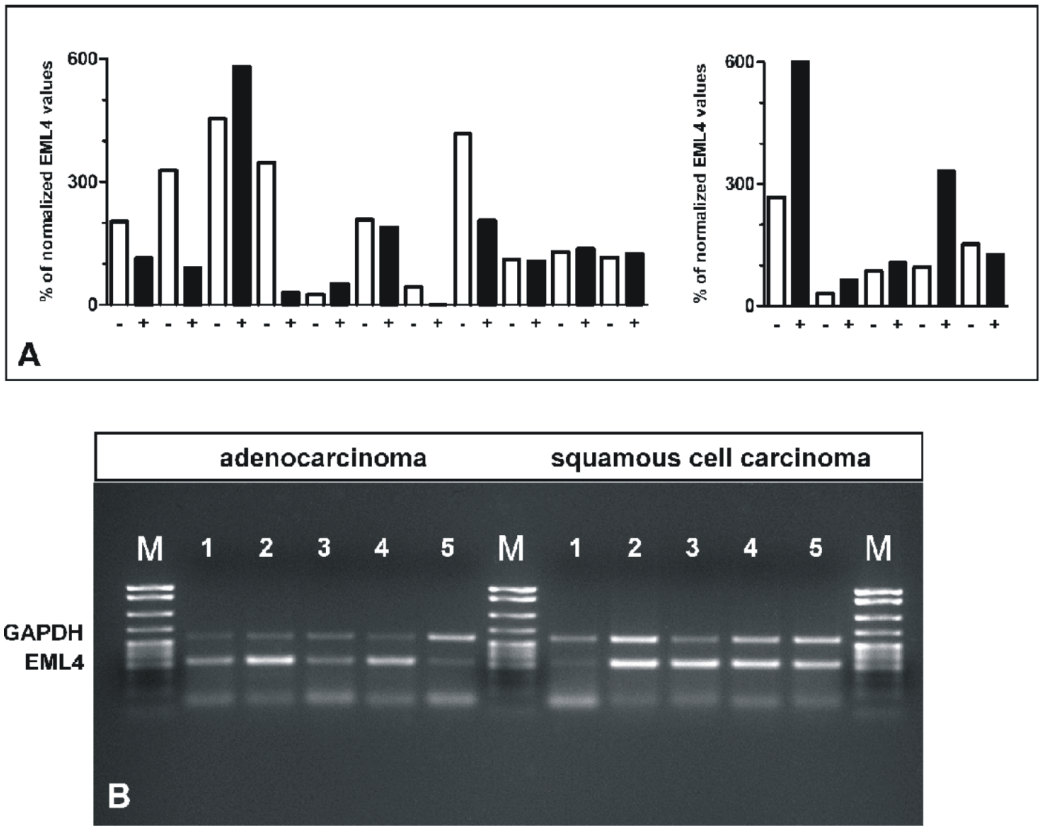

Figure 3 – ***The effects of combined chemotherapy on EML4 transcription in human NSCLC specimens. RT–PCR results of both adenocarcinomas (upper left graph) and squamous cell carcinomas (upper right graph) in the absence (-) or presence (+) of a combination of gemcitabine and carboplatin are shown as values of EML4 mRNA levels normalized to the corresponding GAPDH mRNA bands for all cases separately (A). The results of RT–PCR targeting GAPDH (upper bands) or EML4 transcripts (lower bands) in two selected NSCLC specimens following different combined chemotherapy conditions are shown in (B). One adenocarcinoma (on the left) and one squamous cell carcinoma (on the right) were treated for 16 hours with none (1) or with a combination of gemcitabine and carboplatin (2), gemcitabine and navelbine (3), gemcitabine and paclitaxel (4) or a combination of vinorelbine and paclitaxel (5). The molecular weight marker (M) used with this agarose gel electrophoresis was pBR 322-Msp$^{1/2}$.***

After normalization to the corresponding GAPDH bands, EML4 mRNA levels of the adenocarcinoma previously revealing down-regulated EML4 transcription upon stimulation were consistently attenuated by the additional treatment conditions to around 70% of the corresponding medium controls (set as 100%). EML4 transcription appeared to be almost completely diminished with vinorelbine being one of the two compounds. Likewise, gene expression of EML4 in the squamous cell carcinoma, previously exhibiting up-regulation of EML4 transcription following stimulation, was constantly enhanced up to 4–5 folds above the corresponding unstimulated cultures without considerable disparities between the different treatment conditions.

Discussion

To our knowledge, the distribution pattern of human wild-type EML4 has been demonstrated for the first time in tissues of both normal human lung and of primary NSCLC in detail. While EML4 mRNA was expressed in 100% of the different lung (cancer) tissue samples, 62% and 72% of lung or NSCLC tissues, respectively, revealed positive staining of EML4 protein. In the present study, cellular localization of EML4 transcripts was closely associated with alveolar macrophages and also, yet infrequently, with alveolar epithelial type II cells in tumor free lung tissues. On the protein level, strong granular staining was both cytoplasmic and occasionally perinuclear. Thus, our data indicated substantial transcriptional activity and protein synthesis mostly in alveolar macrophages as the major cell type of the lung. A comparable staining pattern as in the present study was also demonstrated *in vitro* in human transiently transfected HeLa cells. Using immunofluorescence staining method, strong staining of the cytoplasm was associated with mitotic cells, whereas interphase cells revealed distinct dot like patterns [7]. The only other EML4 related data examining human non-tumor lung tissues are provided by Martelli MP *et al*., who could demonstrate the presence of EML4-ALK fusion gene but not of the corresponding protein in human non-neoplastic lung tissues neither by IHC nor by Western Blotting or by immunoprecipitation [17]. The authors were interested in the appearance of the fusion oncogene in human NSCLC patients, thus using antibodies exclusively specific for ALK in the immunohistochemical detection, which explains the conflicting results with the present study.

Knowledge about the potential function(s) of wild-type EML4 in general is scarce but the two known publications about this particular protein conclusively

demonstrated that EML4 is a microtubule stabilizing protein that is essential for both proliferation and survival of cells [6, 7]. Hence, based on our data related to human tissue, it appears to be likely that EML4 is involved in various immunoreactive processes such as local innate host defense mechanisms in the normal human lung.

EML4 gained clinical importance when it has been identified to form a fusion-like oncogene in conjunction with ALK that is highly associated with NSCLC, representing the major histological type of human lung cancer. Subsequent clinical investigations were focused on possible gene rearrangements of EML4 and ALK loci in patients suffering from lung cancer. Determination of the resulting oncogene was performed in all cases by selective detection targeting the ALK partner by various assays such as *in situ* hybridization reaction or immunohistochemistry. Since ALK becomes activated in conjunction with EML4, this protein was thought to be suitable for new therapeutic strategies by using already available tyrosine kinase inhibitors [1,4]. Up to date, clinical interest in EML4 has been focussed on its possible contribution to activate ALK but not as a potential target molecule itself.

Therefore, the expression of the wild-type protein in primary NSCLC was also investigated in the present study. Here, marked EML4 gene expression was demonstrated in all tested tissue samples within the tumor cells. There was only one specimen of squamous cell carcinoma failing to show neither transcriptional activity nor immunohistochemical staining of EML4, which was due to extensive necrosis. Further-more, clear protein staining was seen within a high percentage of lung tumor tissues irrespective of the histological type. In one other more recent investigation on the fusion transcript in human NSCLC, focal high-level amplification of EML4 itself in several cases among two independent cohorts of NSCLC patients has been reported by using multi-fluorescent *in situ* hybridization as well as RT–PCR [2]. These authors hypothesized that EML4 over expression may contain additional oncogenic abnormalities or could be tumorigenic itself, which would support our results suggesting a possible role for EML4 in tumor behavior related to proliferation and progression.

Furthermore, in response to different combinations of widely used cytotoxic drugs, the observed effects comprised both up regulation and suppression of EML4 transcripts in adeno- and squamous cell carcinomas as well as no regulation in half of the tested tumors. These alterations remained statistically insignificant due to the small number of tested specimens. The heterogeneity of our experimental data corresponds well with the overall characteristics of lung tumors themselves and also reveals close correlation with the clinical situation *in vivo*. Accordingly, in NSCLC patients, a strong association between EML4-ALK with resistance has been found revealing any response to EGFR tyrosine kinase inhibitors erlotinib and gefitinib [18]. This was consistent with preclinical studies using a EML4-ALK-containing NSCLC cell line, which was resistant to erlitinib [19]. We therefore hypothesize that the effects of chemotherapy onto EML4 transcripts might be valuable hints towards an understanding of the broad chemotherapy resistance found in NSCLC.

Conclusions

Our study is the first to determine that human wild-type EML4 plays an important role both in non-malignant processes involved in local inflammatory responses of the human lung and in certain aspects of tumor behavior. Due to its known role in organizing and stabilizing the microtubules of cells, our results suggest that EML4 itself may have the potential as a therapeutic target molecule in NSCLC chemotherapy.

Acknowledgements

The authors thank Dr. Elvira Richter and Dr. Doris Hillemann of the National Reference Center for *Mycobacteria* for sequencing the PCR products and Tanja Zietz, Jasmin Tiebach, Maria Lammers and Jessica Hofmeister for their excellent technical assistance.

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Received: August 24th, 2010

Accepted: October 25th, 2010