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Epithelial mesenchymal transition (EMT) and non-small cell lung cancer (NSCLC): A mutual association with airway disease.

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Abstract

Background: NSCLC is a leading cause of morbidity and mortality worldwide. It includes adeno- and squamous cell-carcinoma. In the background, COPD and smoking play a vital role in development of NSCLC. Local progression and metastasis of NSCLC has been associated with various mechanism, but in particular by a process called epithelial mesenchymal transition (EMT), which is implicated in COPD pathogenesis.

Objective: In this study we have investigated whether expression of EGFR (activation marker) and S100A4, vimentin and N-cadherin (as EMT) is different both in central and leading edge of NSCLC and to what extent related to EMT activity of both small and large airways, stage and differentiation of NSCLC.

Methods: We have investigated EMT biomarkers (S100A4, Vimentin, and N-cadherin), an epithelial activation marker (EGFR) and a vascularity marker (Type-IV collagen) in surgically resected tissue from patients with NSCLC (adeno- and squamous cell carcinoma), and compared them with expression in the corresponding non-tumorous airways.

Results: EGFR, S100A4, vimentin, N-cadherin expression was higher in tumour cells located at the peripheral leading edge of NSCLC when compared with centrally located tumour cells of same subjects ($P<0.01$). Type-IV collagen expressing blood vessels were also more at the leading edge in comparison to central parts of NSCLC. EGFR and S100A4 expression was related to differentiation status ($P<0.05$) and TNM stage ($P<0.05$) of NSCLC. Moreover, EMT markers in the leading edge were significantly related to airway EMT activity, while peripheral edge vascularity of squamous cell-carcinoma only was significantly related to large airway Rbm vascularity ($P<0.05$).

Conclusion: EGFR and EMT-related protein expression was markedly high the peripheral leading edge of NSCLCs and related to tumor characteristics associated with poor prognosis. The relationships between EMT-related tumor bio-marker expression and those in the airway epithelium and Rbm, provides a background for utility of airway changes in clinical settings.

Keywords: EMT, S100A4, vimentin, N-cadherin, Adeno carcinoma, Squamous cell carcinoma, TNM

Running Header: EMT in NSCLC progression.

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide [1]. Among NSCLC variants, adenocarcinoma is the most common histological subtype (40% of cases), while squamous cell carcinoma is the second most common type (approx. 25-30%). Surgical resection is the treatment of choice for early-stage tumors though tumor recurrence and metastasis are the common even after resection [2, 3]. Since tumor metastasis is the main obstacle for long-term survival after surgical resection, identification of prognostic molecular markers early on, related to tumor aggressiveness, would be clinically useful.

For tumor metastatic disease, it is widely accepted that invasion of extracellular matrix, vascular dissemination, and homing of cancer cells are major steps [4]. However, tissue invasion, is highly dependent on the structure of the primary organ and seems mechanically difficult due to lack of anatomic lung structures that can serve as routes for invasion. Therefore, in NSCLC, invasion needs newly formed desmoplastic stroma, containing active fibroblasts and a dense network of collagen and elastin, embedded in a ground substance, composed of proteoglycans and glycoproteins [5]. Sakurai and colleagues tried to grade this infiltration process in NSCLC histopathologically by the degree of invasion and showed that grade was relate to prognosis [6]. Travis and colleagues further showed that tumor breaking through epithelial basement membrane is also of major prognostic significance [7].

Invasion into the surrounding tumorigenic desmoplastic stroma is enhanced by epithelial-mesenchymal transition (EMT) activity in cancer cells. EMT is a cellular process of morphologic and functional trans-differentiation from an epithelial to mesenchymal phenotype which is also implicated in the conversion of early-stage tumors into invasive malignancies [8-11].

During EMT, cells lose or redistribute epithelial proteins and acquire mesenchymal proteins, resulting in loss of epithelial polarity and acquisition of a highly motile fibroblastic phenotype. These cells are able to digest through the basement membrane [12-15] and also activate a gene program characteristics of tumor cells [16, 9]. EMT has been shown to be active in a number of epithelial cancers, e.g. pancreatic cancer, gastric, and colorectal carcinomas [17-20] and indeed epithelial cancers constitute the vast bulk of human malignancies. Thus, EMT is an important event in the progression, invasion and metastasis of carcinomas and related in general to a particularly dismal prognosis [13, 15, 21].

Less is known about EMT in NSCLC biology. In this study, we have investigated the heterogeneity of expression of classic EMT-related proteins including S100A4, vimentin and N-cadherin, as well as epithelial activity marker EGFR and a vascularity marker, Type-IV collagen for endothelial basement membrane [22]. We have divided NSCLCs virtually between central and peripheral components, assessed their individual grade of differentiation and also obtained their final recorded histopathological TMN staging. We tested the hypothesis that expression pattern of EMT bio-markers would be greater at the peripheral area 'leading edge' of NSCLC tumors compared with central areas, and correlate with tumour differentiation and staging in both (adeno and squamous cell carcinoma). Finally, we have assessed how EMT expression and vascularity in the tumors may relate to such changes in the large and small airway walls from corresponding individuals in the lung specimens.

Materials and methods

Study Design

Studies involved 25 lung cancer patient who came to curative-intent operative resection. All participants had a recent diagnosis of primary NSCLC, with an approximate equal mix of squamous cell and adenocarcinoma (well to poorly differentiation), and all were current or recent (within 1 month) smokers. TNM staging was done on post-operative histology, on the basis of tumour size, lymph node involvement and metastasis (lympho-vascular and pleural). (Table 1) Similarly Nine patients demonstrated stage 1 or 2 COPD [23] on spirometry ($FEV_{25-75} < 70\%$), and nine patients had small airway disease only with scalloping of the expiratory limb of the flow-volume curve ($FEF_{25-75} < 68\%$ predicted), thus only 7 (28%) had spirometry technically within normal limits. By selection criteria, none had a history of other chronic respiratory disorder.

Study samples

Surgical resections containing cancer tissue and non-affected large and small airways (<2mm diameter) were fixed in formalin within minutes of surgery, and later blocks were separately embedded into paraffin and cut for 3- μ m thickness section for our analyses.

Immunostaining

At room temperature, optimal sections were stained with the following antibodies: polyclonal anti-S100A4 (Dako cat no. A5114, 1:2000 for 90 minutes), anti-vimentin monoclonal antibody (Dako, cat no. M7020, 1:1000 for 60 minutes), N-cadherin (Abcam cat no. Ab98952, at 1:200 for 60 mins) and collagen IV monoclonal antibody (Dako, cat no. M0785, at 1:100 for 90 minutes) and monoclonal anti- EGFR (Dako cat no. M3563, at 1:1000 dilution for 90 minutes). In each run a section stained with immunoglobulin (Ig) G1-negative control (X0931 clone DAKG01; Dako Cytomation) was included to ensure absence of false positive staining. Bound antibodies were elaborated by using horseradish peroxidase (HRP) conjugated DAKO Envision plus reagent (cat no. K4001, anti-mouse or K4003 anti-rabbit) and diaminobenzidine (DAB) for brown colour resolution (cat. no. K3468; Dako Cytomation). We have extensively used these methods [24-26].

Tissue sections analysis and quantitation

Slides were independently randomly coded, mingled and then assessed blind by a single trained pathologist operator (MM), with quality control provided by a senior academic pathologist (KM). Computer-assisted image analysis was performed by using microscopy at 40x magnification (Leica DM 2500, Microsystems, Germany), a Spot insight 12 digital camera (Spot imaging, USA) and Image Pro V5.1 software (Media Cybernetics, USA). We randomly choose five non-overlapping good fields for both adeno and squamous cell carcinoma from each lung resection, for each of the biomarkers of interest. Peripheral leading edge of tumour was chosen by observing increasing clumping of tumour cells and associated blood vessels in both adeno and squamous cell carcinoma.

Evaluation of cell staining was performed in accordance with the IRS (immunoreactive score) proposed by Remmele and Stegner [27] with slight modification: $IRS = SI \text{ (staining intensity)} \times PP \text{ (percentage of positive cells)}$. SI was determined as 0, negative; 1, weak; 2, moderate; and 3, strong. and PP was ranged from 0-100% in

order to get a wider range for analysis. TNM staging was done on the basis of their final recorded histopathological evaluation. TNM stage was scored according to TNM factor (T x N x M); T (Tumor size), N (Lymph node involvement) and M (Metastasis - Pleural/lympho-vascular/haematogenous). The majority of patients were in stage T1 or T2 plus N1. Even so, because of some patients were at T3 or N2 or M1 with pleural or lymphatic involvement, we were able to have quite a wide range of scoring for correlations.

Statistical analysis

Since data were normally distributed, results for each marker are presented as mean and 95% CI. Comparisons between different markers in central and peripheral area “leading edge” of tumour, used paired t-test. Same for comparing tumour with large and small airway EMT biomarker expression. Associations between variables were assessed using Pearson’s rank test. Statistical analyses were performed using SPSS (statistics version 20.0, IBM Co, USA) for Windows 10.0 and a *p*-value of ≤ 0.05 was considered statistically significant.

Results

EMT biomarker expression in NSCLC (Fig 1, 2, 3 & 4)

For both tumour types, and for all bio-markers, (EGFR for epithelial activation; S100A4, vimentin and N-cadherin, for EMT) and also for collagen-IV for vessels, expression of protein was more marked in the leading edge peripheral area than centrally, $P < 0.01$ to $P < 0.001$ for adeno-carcinoma and $P < 0.05$ for squamous cell carcinoma. Representative images are shown from squamous cell carcinoma, but result were much the same for adeno carcinoma.

Regression Analyses:

Adeno-carcinoma: (Fig 5 & 6)

For EGFR and S100A4, there were strong relationships, between peripheral tumor expressions (IRS assessment) and both TNM stage ($P < 0.05$) and differentiation ($P < 0.05$).

For S100A4 and vimentin, peripheral tumor cell expression was positively correlated to corresponding small airway epithelial S100A4 ($P < 0.05$) and vimentin ($P < 0.02$) expression, but not to large airway expression. There were no relationships found between tumor vascularity and that in either the large or small airways.

Squamous cell-carcinoma: (Fig 5, 6 & 7)

Again peripheral leading edge area expression of EMT biomarker (S100A4 and Vimentin) and EGFR (IRS assessment) positively correlated with more advanced stages ($P < 0.01$) and poor differentiation ($P < 0.05$) of tumors.

There were significant relationships found between tumor EMT bio-marker expression (by IRS assessment) and corresponding large and small airway wall EMT marker expressions. There was also a strong positive association between tumor peripheral leading edge, vessel density and large airway epithelial reticular basement membrane (Rbm) vascularity ($P < 0.05$).

Discussion

Non-small cell lung cancer (NSCLC) is the most predominant type of lung cancer and the leading cause of cancer deaths worldwide [28]. However, NSCLC has been relatively little studied for the potential importance of EMT in determining its biology and outcomes. We have now shown that there is obvious heterogeneity in central and peripheral leading edge of NSCLC (adeno and squamous cell-carcinoma), not only in terms of EMT biomarker expression (S100A4, Vimentin and N-cadherin) and EGFR expression but in vessel density as well. In addition, there was a strong correlation between EMT biomarker expression in the leading edge and with advanced stage and poor differentiation of NSCLC. Here was also a positive correlation between leading edge EMT expression in both tumor types and airway epithelial EMT activity, and for squamous cell-carcinoma only between peripheral tumor and large airway Rbm vascularity.

The current literature has included a focus on gene deletions and insertions in exon 19 and point mutations in exons 18 and 21 in the epidermal growth factor receptor (EGFR) [29, 30], expression of onco-fetal protein IMP3 [31] and specific gene promoter methylation [32] as prognostic markers in NSCLC. However, there has been little work solely on metastasis-related markers including EMT related proteins. Some studies have highlighted that centrally located tumor cells stained more positively for epithelial markers, but was absent at the invasive front of the tumor in lung cancer [33-35], but did not look at the inverse, i.e. more mesenchymal tumor cell expression at the leading edge. Both lower epithelial marker and higher mesenchymal marker expression suggest active EMT.

As EMT activity is considered to be one of the causes of morphological tumor heterogeneity in general [36] and now we have observed in NSCLC that S100A4 and vimentin and N-cadherin expression was relatively low in the central region of tumors and so was blood vessel density, in comparison with leading edge peripheral parts. In the central area of NSCLC, the tumor cells usually form irregular-shaped nests associated with marked desmoplastic stroma production [37]. In comparison, in the peripheral areas of primary NSCLC, the tumor cell infiltrate fills and destroys the alveolar spaces, but is associated with a weak desmoplastic reaction [38, 39]. This latter area is also where we observed increased S100A4 and vimentin expression and an associated increase in tumor blood vessels. Our observations are possibly in contrast to the study by Ugadawa and colleagues who observed expression of molecular markers (EGFR, S100A4, CD44 and E-cadherin) at both peripheral and central area of squamous cell-carcinoma [40]

The current study clearly showed that the expression level of N-cadherin was significantly higher at the tumor periphery than in the central area of NSCLC of both sorts studied. In digestive tract cancers [41] and oral squamous cell carcinoma [42], a decrease in E-cadherin expression was found at the invasive front of the tumor but they did not look at the “flip-side” expression of N-cadherin; a switch from E-cadherin to N-cadherin is a strong bio-marker of EMT [43].

EGFR is an example of a receptor tyrosine kinase (RTKs), a family of transmembrane proteins that serve as receptors for many growth factors, which then activate signalling pathways leading to cell proliferation and anti-apoptotic activity. Heterogeneous expression of RTKs in cancer cells is well recorded [44] and in NSCLC heterogeneous EGFR expression within the same tumor was reported [45, 46]. Compatible with this in our study, EGFR expression was significantly higher in the peripheral area than in the central area of the primary NSCLCs. However, Ugadawa et al observed EGFR expression to be greater in the central tumor area than peripherally [47],

though this study dealt only with squamous cell-carcinoma. A retrospective analysis of the FLEX study suggested that chemotherapy with an anti- EGFR antibody, improved the overall survival of patients with tumors showing high EGFR expression, but not in those showing low EGFR expression [48]. It might be that early assessment of lung cancer for leading-edge EGFR expression could be especially useful for deciding on anti-EGFR therapy.

We also found strong relationships between EMT activity and both small and large airway EMT activity in squamous cell tumors though in adeno-carcinoma there was such a relationship only with EMT expression in small airways. Further, there was a strong association between peripheral squamous cell carcinoma vascularity with large airway Rbm vascularity. This positive association is in agreement to our previous studies in which Type-3 EMT (hype vascular Rbm) is thought to be a pro-malignant features in large airways [26]. This was not in case of adeno-carcinoma.

These similarities with airway regions of likely origin, i.e. squamous cell-carcinoma from large airways and adeno-carcinoma from small airways suggest that those local environments are important for development of specific cancer types and for imprinting on the tumors local EMT-related features which then influence tumor aggressiveness. This suggests that sampling of the airway wall to assess EMT and vascularity in large airway could be useful in prognosticating, especially for squamous cell tumors.

The strengths of the present study included the use of relevant human tissue in well phenotyped individuals, the inclusion of both adeno and squamous cell carcinoma patients and the fairly robust numbers giving sufficient power to detect these fascinating findings. We also focused on staging and differentiation of tumors, in order to be able to relate tumor characteristics to these important clinical factors. There were also some limitations to this study. Firstly, it was cross-sectional at a single time point and lacked the potential strength of a longitudinal study with early tumor assessment that could be related prospectively to out-comes, and although difficult this should now be done in a replication cohort.

Conclusion and Summary

In conclusion, we found that EGFR and EMT-related protein expression was markedly high at the peripheral leading edge of NSCLCs and related to tumor characteristics associated with poor prognosis. These changes could be potentially useful as early prognostic bio-markers. There were also interesting relationships between EMT-related tumor bio-marker expression and those in the corresponding airway epithelium and Rbm, which means that background airway changes may also be of clinical utility in tumor assessment.

List of abbreviations:

CI	Confidence interval
EMT	Epithelial mesenchymal transition
EGFR	Epidermal growth factor receptor
IRS	Immuno reactive score
NSCLC	Non-small cell lung cancer
Rbm	Reticular basement membrane
TNM	Tumor, node, metastasis

Conflict of interest: The authors declare they have no conflict of interest.

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Ethics approval

The Tasmanian health and medical Human Research Ethics Committee approved this study (#EC00337). Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.”

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Figure Legends

Fig-1: Representative photomicrograph of EGFR and S100A4 expression in squamous cell carcinoma.: A & C) Central Portion of tumor, B & D) Peripheral leading edge of tumor. Original magnification, $\times 400$. Scale bar =50 μm .

Fig-2: Photomicrographs of Type-IV collagen expressing blood vessels, both in squamous cell and Adeno carcinoma: A & C) Central Portion of tumor, B & D) Peripheral leading edge of tumor. Original magnification, $\times 400$. Scale bar =50 μm .

Fig-3: Comparison of (A) S100A4 and (B) EGFR expression (IRS score), between centre and peripheral leading edge of squamous cell carcinoma.

Fig-4: Comparison of number of Type-IV collagen expressing vessels, between center and peripheral leading edge of (A) squamous cell carcinoma and (B) Adeno carcinoma

Fig-5: Regression analyses: A) Correlation between IRS score of EGFR at leading edge of Squamous cell carcinoma, and TNM stage (B) Correlation between IRS score of EGFR at leading edge of Adeno carcinoma, and TNM stage C) Correlation between IRS score of EGFR at leading edge of Squamous cell carcinoma, and differentiation (D) Correlation between IRS score of EGFR at leading edge of Adeno carcinoma, and differentiation.

Fig-6: Regression analyses: A) Correlation between S100A4 (IRS score) at leading edge of Squamous cell carcinoma, and number of basal epithelial cells per mm of Rbm, positive for S100A4, in large airway (B) Correlation between S100A4 (IRS score) at leading edge of Adeno carcinoma, and number of basal epithelial cells per mm of Rbm, positive for S100A4, in large airway C) Correlation between S100A4 (IRS score) at leading edge of Squamous cell carcinoma, and small airway's number of basal epithelial cells per mm of Rbm, positive for S100A4 (D) Correlation between S100A4 (IRS score) at leading edge of Adeno carcinoma, and small airway's number of basal epithelial cells per mm of Rbm, positive for S100A4.

Fig-7: Regression analyses for squamous cell carcinoma: Correlation between number of Type-IV collagen positive vessels at leading edge and number of Rbm vessels per mm of Rbm, in large airways.