Original Article
Slug is associated with poor survival in squamous cell carcinoma of the lung

Heta Merikallio1,4, Taina Turpeenniemi-Hujanen T2, Paavo Pääkkö3, Riitta Mäkitaro1, Riitta Kaarteenaho1,4, Siri Lehtonen1,5, Sirpa Salo6, Tuula Salo6, Terttu Harju1,4, Ylermi Soini7

1Department of Internal Medicine, Pulmonary Division, University of Oulu and Oulu University Hospital, Finland; 2Department of Oncology and Hematology, University Hospital of Oulu, University of Oulu, Finland; 3Department of Pathology, University Hospital of Oulu, Oulu, Finland; 4Medical Research Center Oulu, Oulu University Hospital and University of Oulu, Finland; 5Institute of Biomedicine, Department of Anatomy and Cell Biology, University of Oulu and Department of Surgery, Oulu University Hospital, Finland; 6Department of Dentistry, University of Oulu, Finland; 7Department of Pathology and Forensic Medicine, University of Eastern Finland, Kuopio and Cancer Center of Eastern Finland, Kuopio, Finland

Received July 23, 2014; Accepted August 23, 2014; Epub August 15, 2014; Published September 1, 2014

Abstract: We investigated the expression of slug in a large set of lung squamous and adenocarcinomas to determine common or dissimilar features in its expression in these two most common forms of lung cancer. To investigate slug related tumor spread we studied the expression of vimentin, claudin 1, MMP2 and MMP9 in these tumors and their relation to slug. Addition, cell invasion assays, mRNA analysis and zymographic tests were performed to study epitheliomesenchymal transition (EMT) related changes in slug blocked lung cell lines. According to the results slug expression did not significantly differ between squamous (SCC) and adenocarcinoma (AC) (P = 0.25). In SCC, slug associated with vimentin (P = 0.016). In AC, claudin 1 associated with MMP2 (P = 0.037). In SCC slug expression had a poor prognostic significance (P = 0.006) and it had independent prognostic value (P = 0.037). In AC MMP2 had a worsening impact on survival (P = 0.021) and it had independent prognostic value (P = 0.002). In cell invasion assays, slug knockdown inhibited the invasion and migration of BEAS-2B, SK-LU1 and SK-MES1 cell lines. The mRNA expression of claudin 1 was downregulated in SK-LU1 cell line. Both tumor cell lines expressed MMP2 and in SK-MES1 slug inhibited line MMP2 appeared to decrease. The results show that slug associated EMT is more pronounced in lung SCC than AC. Slug associated with vimentin in SCC and had an independent prognostic value in this tumor type. Forced slug inhibition might be one putative way of treatment of SCC of the lung.

Keywords: Slug, EMT, MMP, claudin 1, vimentin, lung, carcinoma

Introduction

Epitheliomesenchymal transition (EMT) enables tumor cells to invade to neighbouring tissues and metastasize [1]. It is induced by several transcription factors such as snail, slug, twist, zeb1 and zeb2 [2, 3]. In EMT epithelial trait associated genes such as E-cadherin or claudins are downregulated while mesenchymal associated genes such as vimentin or smooth muscle actin are upregulated [2, 3].

Slug/snail2 is a zinc finger transcription factor which is activated at the blastocyst stage of the early mouse embryo taking part in segregation of cellular layers at an early stage of development thus having a role in EMT already during early embryogenesis [4]. Blockade of slug expression delays wound healing due to the failure of basal keratinocyte activation and disturbed loosening of cellular adhesion [5]. In EMT, slug expression is activated by the Notch and transforming growth factor β induced signalling pathways leading to a loosening of cellular cohesion, increased proliferation, repression of E-cadherin expression and increased resistance to apoptosis [6-10]. It activates the E-cadherin switch in tumors and confers resistance to chemotherapeutic drugs such as cisplatin [11, 12].

Claudin 1 belongs to a family of 27 claudins which are located at the tight junctions of epithelial cells [13, 14]. They determine the perme-
Slug in lung carcinoma

ability of the epithelial cell layer, form a fence separating different membrane compartments from each other and contribute to cellular polarisation [13]. Claudin 1 is downregulated by snail and slug in epithelial cells [15]. Some reports suggest that it may, on the other hand, induce EMT. In liver hepatocytes and tumor cells, claudin 1 induced EMT by upregulating slug and snail and in this way it represses E-cadherin and induced vimentin expression [16].

Upregulation of the matrix metalloproteinases (MMPs), such as MMP2 or MMP9 is one feature of EMT [1, 17]. MMP2 and MMP9 are important in cancer cell invasion since these enzymes degrade basement membrane components. In keratin 8/18 depleted cells NK-kB signalling induces expression of MMP2 and MMP9 which claudin 1 silencing reduces [18]. On the other hand, claudin 1 has been shown to promote cleavage of laminin by MMP2 [19]. These results show how these components of the cellular EMT system are complexly intertwined.

Our aim in this study was to investigate slug in two most common histological forms of non small cell lung carcinoma: adenocarcinoma and squamous cell carcinoma. The aim was to study whether expression of slug would be different in these types of tumors and how it would be related to vimentin and claudin 1 expression, the former representing a mesenchymal and the latter an epithelial trait. Additionally, expression of MMP2 and MMP9 were studied both in the histological as well as in cell culture material.

Materials and methods

Study objects

The tissue material consisted of 128 squamous cell carcinomas and 118 adenocarcinomas which originated from the files of the Central University Hospital of Oulu, Finland. The material had been fixed in 4% buffered formalin and embedded in paraffin. The 1.3 mm array blocks were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD) and chosen from two representative areas of the tumor. The diagnosis of the cases was based on the present WHO classification [20]. In adenocarcinomas, tumors were also graded according the growth pattern into lepidic, asinapillary, mucinous and solid [21]. Relevant clinical data were collected from the patient records at the hospital and from the Finnish Cancer Register.

For the cell culture experiments human non-malignant bronchial cells BEAS-2B and human lung adenocarcinoma cell line SK-LU-1 and squamous cell carcinoma cell line SK-MES-1 were obtained from American Type Culture Collection (Rockville, MD) and cultured as recommended by the manufacturer.

Immunohistochemistry for tissue specimen

Four-micrometer sections were mounted on SuperFrost Plus slides (Menzel-Gläser, Germany) and deparaffinized in xylene, rehydrated in graded alcohols, and rinsed in 0.01M phosphate-buffered saline (PBS). Microwave-stimulated antigen retrieval was performed in 10 mM citrate buffer, at pH 6.0. Endogenous peroxidase was blocked with aqueous 0.3% H2O2 for 15 min.

A primary polyclonal rabbit anti-human antibody to slug (RB 1398) was purchased from Abgent (San Diego, CA, USA), polyclonal rabbit anti-claudin 1 (clone JAY.8) was purchased from Zymed laboratories Inc (South San Francisco, CA) and anti-mouse vimentin antibody (Clone 3B4) from Dako (Dakopatts, Copenhagen). An avidin-biotin-peroxidase method was applied using Dako Envision Kit (Dakopatts, Copenhagen) or Histostain™ Kit (Zymed Laboratories Inc). Primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and 0.02 M glycine. PBS was used in all washing steps, the sections were incubated overnight with the antibodies to slug (dilution 1:100), vimentin (1:100) and claudins 1 (dilution 1:100). Diaminobenzidine (Sigma-Aldrich, Steinheim, Germany) was used as the chromogen and the sections were lightly counterstained with Mayer’s hematoxylin. As negative control, the primary antibody was replaced by PBS and rabbit or mouse non-immune serum. Sections of corresponding normal tissues and non-lesional areas in the test sections served as positive controls.

For slug the samples were evaluated for the absence (-) or presence (+) of nuclear immunoreactivity; For vimentin the samples were evaluated for the absence (-) or presence (+) of cytoplasmic immunoreactivity; For claudin localisation of reaction product to cell membrane
Table 1. Comparison of slug, vimentin, claudin 1, MMP2 and MMP9 expression in squamous cell carcinoma (SCC) and Adenocarcinoma (AC)

<table>
<thead>
<tr>
<th></th>
<th>SCC</th>
<th>AC</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slug-</td>
<td>99 (78%)</td>
<td>96 (84%)</td>
<td></td>
</tr>
<tr>
<td>Slug+</td>
<td>28 (22%)</td>
<td>18 (16%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Vimentin-</td>
<td>75 (60%)</td>
<td>69 (62%)</td>
<td></td>
</tr>
<tr>
<td>Vimentin+</td>
<td>50 (40%)</td>
<td>42 (38%)</td>
<td>0.78</td>
</tr>
<tr>
<td>Claudin1-</td>
<td>17 (14%)</td>
<td>12 (11%)</td>
<td></td>
</tr>
<tr>
<td>Claudin1+</td>
<td>107 (86%)</td>
<td>101 (89%)</td>
<td>0.55</td>
</tr>
<tr>
<td>MMP2-</td>
<td>35 (28%)</td>
<td>49 (42%)</td>
<td></td>
</tr>
<tr>
<td>MMP2+</td>
<td>92 (72%)</td>
<td>67 (58%)</td>
<td>0.021</td>
</tr>
<tr>
<td>MMP9-</td>
<td>21 (17%)</td>
<td>18 (15%)</td>
<td></td>
</tr>
<tr>
<td>MMP9+</td>
<td>104 (83%)</td>
<td>99 (85%)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Number of cases is not similar with every antibody due to loss of tumor area during sample processing.

and the characteristics of the stain were recorded and the staining was quantified as follows: 0 = negative, 1 = less than 25% of cells positive, 2 = 25-50% of cells positive, 3 = 50-75% of cells positive, 4 = over 75% of cells positive. In each case two separate array samples from each case were studied. The samples were studied by two independent observers (YS and RK). For the final analysis, the cases with less than 25% of cells were considered negative and the data was divided in two groups; negative (<25%) and positive (>25%).

Immunohistochemical staining for MMP2 and MMP9 was conducted as follows: Four-micrometer sections were cut on slides coated with poly-L-lysine (Sigma Chemicals, St. Louis, Mo) and incubated overnight at 37°C. The slides were deparaffinized and hydrated and treated with 0.5% pepsin (2000 FIP-U/g, Merck, Darmstadt, Germany) for 10 minutes at 37°C. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 10 minutes and nonspecific binding was blocked with 10% goat serum for 15 minutes. Mouse monoclonal antibody (24 μg/ml, for MMP2 (CA-4001, Diapor, Oulu, FI) and mouse monoclonal antibody to MMP9 (10 μg/ml) (GE-231 Diapor, Oulu, FI) were used as primary antibodies. The slides were incubated with primary antibody at room temperature overnight after which the Histostain bulk kit was used (Zymed Laboratory Inc., San Fronsisco, Ca, USA). The sections were counterstained with haematoxylin and mounted with Immuno-mount (Shanon Inc., Pittsburgh, PA, USA). For negative controls, the primary antibody was replaced by mouse non-immune IgG or PBS. The evaluation of the results was as follows: <5% = negative (-), 5-25% = weakly positive (+), 25-50% = intermediately positive (++), >50% = strongly positive (+++). The results were evaluated by two investigators (TTH, YS). In each case two separate array samples from each case were studied. For the final analysis, the cases with less than 25% of cells were considered negative and the data was divided in two groups; negative (<25%) and positive (>25%).

Invasion assays

Cell invasion was studied by the gel matrix assay (Culturex BME cell invasion assay, Trevigen, Gaithersburg, MD) and myoma organotypic model.

In the myoma organotypic assay 400,000 cells were cultured on the myoma tissue for ten days and fixed with formalin for the histochemistry and immunohistochemistry [22]. The 4 µm thick slides were stained with haematoxylin and eosin and with anti-human cytokeratin (Dako-Cytomation, Glostrup, Denmark) to evaluate the distance for the maximal invasion depth (the distance from the lower surface of the non-invasive cell layer to the deepest invaded cell).

Recombinant retroviruses and shRNA blocking in cell lines

Lentiviruses were produced in Phoenix-GP packaging cells (PhGP-cells). PhGP-cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For the cell were given low glucose medium just before transfection. For the transfection was prepared a mix of Optimem (Invitrogen), lipofectamine 2000, wanted retroviral vector Slug 865 (Sigma-Aldrich) and core protein plasmids (REV, pVSV-G and Gag/Pol). Mix was added to the cells. 24 hours post transfection medium was removed and changed to fresh medium for virus-collection. Viruses were collected three times. BEAS-2B, SK-LU-1 and SK-MES-1 cells were infected with fresh virus-stocks. Polybrene (Sigma-Aldrich) was added in each infection to enhance the efficiency of the infection. After the infec-
Slug in lung carcinoma

After electrophoresis, SDS was removed by 2.5% Triton X-100 to renature the gelatinases. Gels were incubated in 50 mM Tris-HCl buffer (pH 7.8, 150 mM NaCl, 5 mM CaCl₂, 1 M ZnCl₂) overnight at 37°C. A set of corresponding gels were incubated overnight at 37°C with 10 mM EDTA in 50 mM Tris-HCl to inhibit the metalloproteinase activities. The gelatin degradation was visualized under long-wave ultraviolet illumination followed by 0.5% Coomassie Blue R-250 staining of the gels. The gels were photographed and cleavage rates of gelatin were estimated by determining the rates of disappearance of gelatin by densitometric scanning of the photographed gels.

Statistical analysis

The statistical analyses were performed with SPSS for Windows software (SPSS, Chicago, IL, USA) using analysis of variance (ANOVA), Fisher’s exact test and the Kruskall-Wallis-test when appropriate. Kaplan-Meier survival analy-

Figure 1. A: Slug immunostaining in squamous cell carcinoma. The nuclei stain positively. B: A case of lung adenocarcinoma strongly positive for vimentin. C: In this case of a lung adenocarcinoma, membrane bound positivity for claudin 1 can be seen. D: In this case of a lung adenocarcinoma, cytoplasmic positivity for MMP9 is present.
Slug in lung carcinoma

Ethical considerations
The study was approved by the ethical committee of Northern Ostrobothnia Hospital District. The survival data was obtained from the Finnish Cancer Register after receiving permission from the Ministry of Health and Social Welfare and the National Supervisory Authority for Welfare and Health.

Results

Results of immunohistochemistry
The expression of slug, vimentin, claudin 1, MMP2 and MMP9 in squamous cell and adenocarcinoma is shown in Table 1 (Immunostaining shown in Figure 1). There was a significant difference only with MMP2 (P = 0.021), its expression being stronger in SCC than AC.

In SCC slug had a positive association with vimentin (P = 0.016) but not with claudin 1, MMP2 or MMP9 (P = 0.58, P = 0.57, P = 0.46, respectively). Additionally, MMP2 associated strongly with MMP9 (P < 0.001).

In AC slug had no association with vimentin, claudin 1, MMP2 or MMP9 (P = 0.53, P = 0.46, \( P = 0.51, P = 0.44 \), respectively). Claudin 1 associated with MMP2 (P = 0.037), and MMP2 had a near significant association with MMP9 (P = 0.062).

In SCC, slug was more strongly expressed in grade I-II tumors (P = 0.033). No association was found with tumor size (P = 0.76), presence of nodal metastases (P = 0.68), or distant metastases (P = 0.48). Other markers did not show any significant associations with TNM factors.

In AC, slug associated with grade (P = 0.019), there being more grade III cases in tumors with slug expression. No association was found with tumor size (P = 0.49), presence of nodal metastases (P = 0.59), or distant metastases (P = 0.61). Of other markers, MMP2 associated with the presence of lymph node metastases (P = 0.031).

Cases of predominantly acinar morphology had more often strong claudin 1 expression than other types (P = 0.030). No other significant associations were observed.

Survival analysis in squamous cell carcinoma
Cases with strong slug expression had a worse prognosis (P = 0.006, log rank) (Figure 2). Vimentin (P = 0.46), MMP2 (P = 0.56) or MMP9 (P = 0.40) did not influence patient survival. Low claudin 1 expression associated with poor survival in one statistical tests (P = 0.11, log rank, \( R = 0.040 \) Breslow, \( P = 0.053 \) Tarone-Ware). In Cox regression analysis including the size, nodal and distant metastases, vimentin, MMP2 and MMP9 expression, the age and sex of the patients and grade of tumors, slug expression had an independent prognostic value (P = 0.037) along with tumor size (P = 0.006), grade (P = 0.039) and low claudin 1 expression (P = 0.026).

Survival analysis in adenocarcinoma
In contrast to SCC, strong slug expression did not influence survival in AC (P = 0.66, log rank). Neither had vimentin expression any significant
Slug in lung carcinoma

Invasion assays and claudin 1 expression

Invasion capability of the cell lines was studied by two different assays, gel matrix assay and myoma organotypic invasion model. In the gel matrix assay (Figure 3) BEAS-2B cells were the less invasive cell line as expected. The SK-MES-1 cell line had more invasive properties than SK-LU-1 as could be demonstrated by both assays (Figure 3). After slug knockout all cell lines were less invasive than the normal cells. Curiously, in myoma invasion assay model BEAS-2B cells were as invasive as the SK-MES-1 cells as measured by the invasion distance to the myometrium (Figure 3). This property was,

\[ \text{Invasion assays and claudin 1 expression} \]

\[ \text{Invasion capability of the cell lines was studied by two different assays, gel matrix assay and myoma organotypic invasion model. In the gel matrix assay (Figure 3) BEAS-2B cells were the less invasive cell line as expected. The SK-MES-1 cell line had more invasive properties than SK-LU-1 as could be demonstrated by both assays (Figure 3). After slug knockout all cell lines were less invasive than the normal cells. Curiously, in myoma invasion assay model BEAS-2B cells were as invasive as the SK-MES-1 cells as measured by the invasion distance to the myometrium (Figure 3). This property was,} \]
not show evident bands in zymography. SK-LU-1 did not produce the MMP9 protein. Knockout of slug did not affect MMP2 in zymography. SK-ME-S-1 cells produced MMP2 protein, but not MMP9 enzymatic activities. The slug knockout cells had a decreased amount of MMP2 and produced faint bands most likely representing MMP9 pro and active forms. All the gelatinolytic activities disappeared after EDTA treatment of the gels.

Discussion

In this study we investigated the expression of slug in a set of lung SCC and ACs by immunohistochemistry and compared it with the clinical data of the patients, expression of claudin 1, vimentin MMP2 and MMP9. In a non-neoplastic (BEAS-2B) and two neoplastic (SK-LU1 and SK-MES1) cell lines invasion was determined both in slug expressing and slug knockdown cell lines. RT-PCR was performed for claudin 1 mRNA and cell zymography for MMP2 and MMP9 for blocked and non-blocked cell lines.

We did not find any significant difference between SCC and AC in their nuclear expression of slug. The prognosis of patients with SCC was poor and it was an independent prognostic factor. Additionally, slug expression was associated with vimentin suggesting an upregulation of mesenchymal trait in SCC and in this way promoting EMT related transformation in these tumors.

Figure 4. Zymogramms of the cell. MMPs were studied from the 48 hour exposed medium in (A) BEAS-2B cells, (B) BEAS-2B slug knockout cells, (C) EDTA-treated BEAS-2B slug knockout cells, (D) SK-LU-1 cells, (E) SK-LU-1 slug knockout cells, (F) EDTA-treated SK-LU-1 slug knockout cells (G) SK-MES-1 cells, (H) SK-MES-1 slug knockout cells and (l) EDTA-treated SK-MES-1 slug knockout cells.

however, diminished by slug knockout in these cells.

Knockdown of slug mRNA increased the expression of claudin 1 mRNA. SK-LU-1 cells expressed significantly less claudin 1 mRNA ($P = 0.038$) than the slug knock-out cell line. SM-MES1 had a similar tendency but the results were not statistically significant. Curiously, in normal BEAS-2B cells claudin 1 mRNA was more highly expressed than in slug-knockout line.

Cellzymographic experiments

Zymographic analysis for MMP2 and MMP9 was performed in slug non knock out and slug knock out cell lines (Figure 4). BEAS-2B cells did...
Slug in lung carcinoma

In oral squamous cell carcinoma vimentin was one of the top 20 upregulated genes induced by slug which is in line with our finding of an association between vimentin and slug [26]. Slug knockdown also most efficiently retarded the migration of the SK-MES1 cell line which is reported to be differentiated towards squamous epithelium [27]. In an earlier report elevated slug mRNA was found to be correlated with a worse outcome of the patients in ACs [28]. Our results are not in line with these findings but our results were based on immunohistochemistry. Moreover, ACs have been found to represent several different types of tumors, at least as far as growth pattern and driver mutations are concerned which could lead to erroneous results when a mixture of different growth patterns is studied [21]. To examine the effect of growth pattern we tested the differences in claudin 1, vimentin, slug, MMP2 and MMP9 expression and different growth patterns in the array material and found only increased claudin 1 protein expression in tumor samples with acinar growth pattern compared to others.

Matrix metalloproteinases have an important role in EMT [17] and also in our study this was evident but the tendencies were more prominent in ACs where MMP2 was an independent prognostic factor and it also associated with a positive lymph node status. Even though slug has been reported to increase the expression and activation of MMP2 in AC cell line [28] we did not observe any significant association between slug and MMP2 in our cell line material. Slug knockdown, however, decreased MMP2 in the SK-MES1 cell line.

In summary, our results show that slug influences lung cancer growth especially in SCC. It is an independent prognostic factor in these tumors and is associated with vimentin promoting EMT. In ACs slug was not related to survival. In AC claudin 1 was associated with MMP2 suggesting that claudin 1 might have EMT promoting functions as suggested in a recent report [16]. Claudins have, after all, been shown to be able to activate MMP2 through MT-MMP2 [29].

Acknowledgements

The financial support of The Finnish Anti-Tuberculosis Foundation, The Northern Savo and Joensuu Hospital District and Jalmari and Rauha Ahokas Foundation are appreciated.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ylermi Soini, Department of Pathology and Forensic Medicine, Institute of Clinical Medicine, Pathology and Forensic Medicine, School of Medicine, University of Eastern Finland, Cancer Center of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland. E-mail: ylermi.soini@uef.fi

References

Slug in lung carcinoma


