Heta Merikallio

CLAUDINS AND EPITHELIOMESENCHYMAL TRANSITION IN LUNG CARCINOMAS AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE
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Academic Dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 8 of Oulu University Hospital, on 1 November 2013, at 12 noon

UNIVERSITY OF OULU, OULU 2013
Lung cancers and chronic obstructive pulmonary disease (COPD) are the most common smoking-related lung diseases and both have high mortality rate. Tight junctions (TJ) are apical junctions between epithelial cells that regulate the permeability of epithelium and form the tight junction along with occludin. Dysfunction of the TJ and dysregulation of TJ proteins leads to a loss of cell-cell adhesion and a loss of cohesion as well as epitheliomesenchymal transition. These increase invasion of lung carcinomas and possibly predispose to the exacerbations in COPD. Therefore, the aim of this thesis was to study expression and regulation of claudins in different lung carcinomas and COPD.

Carcinomas expressed claudins 1, 2, 3, 4, 5 and 7 in different variations. Claudin 5 expression was weak in all carcinoma types. Strong claudin 1, 4 and 7 expression was associated with better survival in squamous cell carcinoma and adenocarcinoma. Claudin 3 expression was associated with COPD in large airways. Claudins 3 and 4 was found to be stronger in small airways of smokers and COPD patients than in non-smokers.

Transcription factor snail had prognostic value in lung carcinomas. Negative snail expression was associated with longer life expectancy in lung carcinoma patients. Negative snail expression was associated with up-regulated claudin 5 and 7 expression, while strong expression was associated with low claudin 1 and 3 expression. Transcription factors slug and twist were inversely associated with claudins 3 and 4 in small and large airways. Slug expression was higher in non-smokers than in COPD patients and smokers.

Transcription factor knockdown increased claudin expression in normal bronchial cell line. Except for claudin 2 and 7, which were decreased. Adenocarcinoma-like cell line was not affected by snail knockdown and in squamous cell carcinoma-like cell line claudin 3, 4 and 7 expression was increased. Transcription factor snail knockdown inhibited invasion of cell lines. Twist knockdown increased transepithelial resistance in normal bronchial cell line indicating higher barrier function in cell layer.

**Keywords:** chronic obstructive pulmonary disease, claudin, epitheliomesenchymal transition, lung carcinoma, tight junction, transcription factor
Tiivistelmä


Asiasanat: epiteliomesenkymaalinen muuntuminen, keuhkoahtaumatauti, keuhkokarsinooma, klaudiini, tiivisliitos, transkriptiotekijä
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Oulu, September 2013

Heta Merikallio
# Abbreviations

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<th>Description</th>
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<tr>
<td>AC</td>
<td>atypical carcinoid</td>
</tr>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>BAC</td>
<td>bronchioloalveolar carcinoma</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>human non-malignant bronchial epithelial cell line</td>
</tr>
<tr>
<td>BEGM</td>
<td>bronchial epithelial growth medium</td>
</tr>
<tr>
<td>BPDE</td>
<td>benzo(a)pyrene diol epoxide</td>
</tr>
<tr>
<td>BRAF</td>
<td>proto-oncogene B-RAf</td>
</tr>
<tr>
<td>CDK4</td>
<td>cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CDK 6</td>
<td>cyclin-dependent kinase 6</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CK 5/6</td>
<td>cytokeratin 5/6</td>
</tr>
<tr>
<td>CK7</td>
<td>cytokeratin 7</td>
</tr>
<tr>
<td>CLD</td>
<td>claudin</td>
</tr>
<tr>
<td>C-MET</td>
<td>proto-oncogene</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CSE</td>
<td>cigarette smoke extract</td>
</tr>
<tr>
<td>DCO</td>
<td>diffusing capacity of carbon monoxide</td>
</tr>
<tr>
<td>DCO/VA</td>
<td>diffusing capacity of carbon monoxide/ alveolar volume</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EML-4</td>
<td>echinoderm microtubule associate protein like-4</td>
</tr>
<tr>
<td>EMT</td>
<td>epitheliomesenchymal transition</td>
</tr>
<tr>
<td>FEV-1</td>
<td>forced expiratory volume in one second</td>
</tr>
<tr>
<td>FHIT</td>
<td>fragile histidine triad protein</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>Gag/Pol</td>
<td>viral core protein</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GOLD</td>
<td>the global initiative for chronic obstructive lung disease</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>HDM2</td>
<td>oncogene</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPF</td>
<td>high power fields</td>
</tr>
<tr>
<td>KRAS</td>
<td>v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LCC</td>
<td>large cell carcinoma</td>
</tr>
</tbody>
</table>
LOH  loss of heterozygosity
LNEC  large cell neuroendocrine carcinoma
MAD  MAX interacting protein
MAX  MYC associated factor X
MMP  matrix metalloprotease
MXI1  MAX-interacting protein-1
MYC  proto-oncogene protein
NCAM-PSA  poly sialylated neural cell adhesion molecule
NSCLC  non-small cell lung carcinoma
NET  neuroendocrine tumor
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PhGP-cells  Phoenix-GP packaging cells
PIK3CA  phosphatidylinositol-4,5-bisphosphate 3-kinase
pRB  retino blastoma tumor suppressor protein
pVSV-G  viral core protein
PTEN  phosphatase and tensin homolog protein
p40  tumor suppressor protein 40
p53/TP53  tumor suppressor protein 53
p60  tumor suppressor protein 60
REV  viral core protein
RNA  ribonucleic acid
RT-PCR  reverse transcription polymerase chain reaction
SCC  small cell carcinoma
SCLC  squamous cell lung carcinoma
SDS  sodium dodecyl sulfate
shRNA  short hairpin RNA
SK-LU-1  lung adenocarcinoma like cell line
SK-MES-1  squamous cell lung carcinoma like cell line
snail  transcription factor snai I
slug  transcription factor snai II
SOX2  transcription factor SOX-2
TC  typical carcinoid
TER  transepithelial electrical resistance
TGF-β  transforming growth factor β
TJ  tight junction
TSG  tumor suppressor gene
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<th>Definition</th>
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<td>TTF-1</td>
<td>thyroid transcription factor 1</td>
</tr>
<tr>
<td>twist</td>
<td>transcription factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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List of original publications

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1 Introduction

Smoking harms nearly every organ in a smoker’s body and causes many diseases. In Finland, about 5000 individuals per year are diagnosed with a smoking-induced disease (Patja 2012). Lungs are in the forefront for cigarette smoke induced damage. There are over 4000 compounds in cigarette smoke, over 60 of them carcinogenic. Cigarette smoke induces damage in airways and in lung tissue. The best-known smoking-related diseases are lung cancer and Chronic Obstructive Pulmonary Disease (COPD). Smoking-related lung diseases also include emphysema, obstructive bronchiolitis, respiratory bronchiolitis-interstitial lung disease, desquamative interstitial pneumonia, pulmonary Langerhans’ cell histiocytosis and acute eosinophilic pneumonia (Galvin & Franks 2009). Emphysema and bronchiolitis are features of COPD, but they can be seen in lung tissue before measurable changes in spirometry fulfilling the criteria of COPD.

Over 90% of lung cancers are smoking-induced cancers and it is also the most common cause of cancer death in the world (WHO 2002). Smoking increases the risk for all types of lung cancer, and the earlier smoking begins the greater the risk, and the risk also increases with long-term smoking. Passive smoking increases also the risk for lung cancer. Lung cancer has poor five-year prognosis: overall survival rate is about 16% of all lung carcinoma patients. Treatment possibilities for lung cancer are limited: early stage tumors can be treated with surgical resection and adjuvant chemotherapy. Advanced tumors are incurable (Gadgeel et al. 2012). Small cell lung carcinomas are usually found in advanced stage with metastases, and survival is very short. Lungs are also a very common site for metastases of other tumors such as colorectal cancer (Sugarbaker 2013).

Smoking increases the risk (90%) for COPD as well as lung cancer. It is suggested that about half of all smokers have some stage of COPD and about one third of all COPD patients have symptomatic disease. It has been estimated that there are 400 000 patients with chronic bronchitis in Finland and about half of them have symptomatic COPD (Finland's ASH 2013). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has estimated that in 2020 COPD will be the third leading cause of death in the world. In Finland about 750 to 1000 patients die of COPD every year.

Chronic inflammation in lungs may induce parenchymal tissue destruction resulting in emphysema, and disruption of normal repair and defense mechanisms results in small airway fibrosis. These mechanisms lead to air trapping and
progressive airflow limitation in COPD. Exacerbations are characteristic for COPD. They are acute events, such as viral or bacterial inflammations, that worsen the patient’s respiratory symptoms and are associated with significant mortality (Celli & Barnes 2007).

The aim of this thesis was to study expression and regulation of the tight junction proteins claudins in smoking-induced lung diseases. The expression of claudins was studied in normal lung tissue, different lung carcinoma tissues and in airways of COPD patients. Expression was compared to lung function, smoking history, survival and lung tumor status and metastases. Transcription factors connected with epitheliomesenchymal transition were compared with the expression of claudins in different lung tissues. The effect of transcription factors on claudin expression was studied in cell culture experiments in vitro.
2 Review of the literature

2.1 Smoking-related lung diseases

Long-term smoking induces several lung diseases such as lung cancer and Chronic Obstructive Pulmonary Disease (COPD). Lung cancer is the most common and deadliest cancer in the world (Brandao et al. 2012, Flieder & Hammar 2008). About 90% of all lung cancer cases are caused by smoking (WHO 2002). COPD is the most common smoking-related disease and the fourth leading cause of death in the world (WHO 2011). Over 50% of long-term smokers with more than 40 pack-years suffer from this preventable public health challenge (Kotaniemi et al. 2003). The risk of lung cancer among cigarette smokers depends on duration of smoking, the amount of smoked cigarettes, the type of cigarettes and inhaling patterns. Genetic risk factors such as alpha-1-antitrypsin deficiency and COPD itself are also risk factors for lung carcinoma. Smokers with COPD have 4- to 6-fold higher lung carcinoma risk compared to smokers with normal lung function (Young & Hopkins 2011). Toxic components in cigarette smoke cause acute and chronic effects in lung tissue. Along with direct cell damage, cigarette smoke causes inflammation, oxidative stress and protease-antiprotease imbalance. These lead to injury in lung tissue and in long-term to damage in smoking different parts of the lung.

2.1.1 Lung carcinomas

Lung carcinomas are divided into two main categories: non-neuroendocrine lung tumors and neuroendocrine lung tumors (NET). About 80% of lung cancers are non-neuroendocrine lung tumors and about 20% NETs (Brandao et al. 2012). The non-neuroendocrine lung tumor group includes squamous cell carcinomas, adenocarcinomas and large cell carcinomas (Table 1). Squamous cell carcinoma is the most common lung carcinoma, but adenocarcinoma has recently exceeded its frequency in some countries. About 44% and 25% of all lung carcinomas in men and women, respectively, are squamous cell carcinoma. Adenocarcinoma is the most common carcinoma in women (42%) and, accounts for 28% of carcinoma in men. Large cell carcinoma comprises about 10% of cases in both sexes. The crude division of lung carcinomas into these two groups is bound to be renewed due to molecular biological discoveries of signal cascades found in the tumors,
especially adenocarcinomas. This gives new opportunities for adenocarcinoma treatment.

Small cell carcinomas (SCC) belong to NETs of the lung including the carcinoid tumors and large cell neuroendocrine carcinoma (Table 2.) (Swarts et al. 2012). These tumors show neuroendocrine features and express neuroendocrine markers such as chromogranin and synaptophysin in their cytoplasm. SCC has a dismal prognosis with about 5% 5-year survival. On the other hand, carcinoid tumors, also called grade I neuroendocrine tumors, show an excellent prognosis but may, however, metastasize in a very small proportion of cases (Tsuta et al. 2011).
Table 1. Non-neuroendocrine lung carcinomas.

<table>
<thead>
<tr>
<th>Carcinoma type</th>
<th>Subtype</th>
<th>Mutations</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell</td>
<td></td>
<td>PIK3CA (10%), BRAF (5%)</td>
<td>TTF-1 negative, CK 5/6, p63, p40, hardly any mutations found (0%-12% in all squamous cell carcinomas)</td>
</tr>
<tr>
<td>Papillary</td>
<td></td>
<td></td>
<td>Small sized, papillary growth into bronchial lumen, well differentiated</td>
</tr>
<tr>
<td>Clear Cell</td>
<td></td>
<td></td>
<td>Clear cell histology, intercellular bridges, or keratinization, poorly differentiated, necrosis</td>
</tr>
<tr>
<td>Small Cell</td>
<td></td>
<td></td>
<td>Poorly differentiated, high nuclear/cytoplasmic ratio, p63 and high molecular weight keratin (34βE12) positive</td>
</tr>
<tr>
<td>Basaloid</td>
<td>SOX2 (20%)</td>
<td></td>
<td>Poorly differentiated, forms, solid lobules or trabecular masses, focal intercellular bridges and keratinization, high mitotic rate, necrosis</td>
</tr>
<tr>
<td>Adeno</td>
<td></td>
<td></td>
<td>CK7 positive, TTF-1 positive</td>
</tr>
<tr>
<td>Adenocarcinoma In situ</td>
<td>EGFR (10-30%), KRAS (10-30%)</td>
<td>≤3 cm sized tumors, lacking stromal, vascular or pleural invasions, lepidic growth, originated from alveolar cells</td>
<td></td>
</tr>
<tr>
<td>Minimally invasive</td>
<td>EGFR (10-30%), KRAS (10-30%)</td>
<td>≤3 cm sized tumors, small ≤ 0,5 cm invasion infiltrating myofibroplastic stroma, tumor grows in lepidic pattern and invasion in different pattern, no tumor necrosis</td>
<td></td>
</tr>
<tr>
<td>Lepidic predominant</td>
<td>EGFR(10-50%), KRAS (10%), BRAF(5%)</td>
<td>≤3 cm sized tumors, small ≤ 0,5 cm invasion infiltrating myofibroplastic stroma, tumor grows in lepidic pattern and invasion in different pattern, no tumor necrosis</td>
<td></td>
</tr>
<tr>
<td>Acinar predominant</td>
<td>KRAS (20%), EGFR (&lt;10%), p53 (40%)</td>
<td>Consist mainly of glands with central luminal space, neoplastic cells and glandular spaces may contain mucin</td>
<td></td>
</tr>
<tr>
<td>Papillary predominant</td>
<td>EGFR (10-30%), KRAS (3%), p53 (30%), BRAF (5%)</td>
<td>Glandular cell growth along central fibrovascular cores</td>
<td></td>
</tr>
<tr>
<td>Micropapillary predominant</td>
<td>KRAS (33%), EGFR (20%), BRAF (20%)</td>
<td>Small tumors, connected to alveolar walls, tumor cell growth in papillary tufts, lack of fibrovascular cores, vascular and stromal invasion</td>
<td></td>
</tr>
<tr>
<td>Carcinoma type</td>
<td>Subtype</td>
<td>Mutations</td>
<td>Identification</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------</td>
<td>------------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Solid predominant</td>
<td>KRAS (10-30%), EGFR 10-30%), p53 form sheets, TTF-1 positive (70%)</td>
<td>Lack of recognizable patterns of adenocarcinoma, mucin production, polygonal tumor cells</td>
<td></td>
</tr>
<tr>
<td>Invasive mucinous</td>
<td>KRAS (80-100%)</td>
<td>Formerly mucinous-BAC, invasive, form satellite tumors or pneumatic consolidations, TTF-1 (0-33% positive)</td>
<td></td>
</tr>
<tr>
<td>Colloid</td>
<td></td>
<td>Extracellular mucin in abundant pools, clusters of mucin-secreting cells, consist of goblet cells or other mucin secreting cells</td>
<td></td>
</tr>
<tr>
<td>Fetal</td>
<td>β-catenin</td>
<td>Glandular elements with tubules, composed of glycogen-rich nonciliated cells, subnuclear vacuoles</td>
<td></td>
</tr>
<tr>
<td>Enteric</td>
<td></td>
<td>Morphologic and immunohistochemical features of colorectal adenocarcinoma, lepidic growth, consist of papillary and glandular structures, luminal necrosis, TTF-1 (50%)</td>
<td></td>
</tr>
<tr>
<td>Large cell</td>
<td></td>
<td>Large peripheral tumors, pleural and chest wall invasions, poorly differentiated, CK7 (80%), TTF-1 (50%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Neuroendocrine lung carcinomas.

<table>
<thead>
<tr>
<th>Carcinoma type</th>
<th>Subtype</th>
<th>Mutations</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small cell</td>
<td></td>
<td>p53, MYC, RAS, pRB,</td>
<td>Mainly centrally located, metastasize aggressively, necrosis, small cell size, TTF-1 (90%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCAM-PSA</td>
<td></td>
</tr>
<tr>
<td>Large cell neuroendocrine</td>
<td></td>
<td></td>
<td>Large cell carcinoma with neuroendocrine morphology, TTF-1 (50%)</td>
</tr>
<tr>
<td>Carcinoid tumor</td>
<td>Typical</td>
<td>FHIT, p53 absent</td>
<td>Small tumors, no necrosis, less than 2 mitoses/2mm², rarely forms metastases, TTF-1 (20%)</td>
</tr>
<tr>
<td></td>
<td>Atypical</td>
<td>FHIT, p53 absent</td>
<td>Displays 2-10 mitoses/2mm², necrosis, forms metastases, TTF-1 (&gt;50%)</td>
</tr>
</tbody>
</table>

Carcinomas with no neuroendocrine features

Carcinomas formerly known as non-small cell lung carcinomas (NSCLC) are now called non-neuroendocrine lung carcinomas. About 30% of non-neuroendocrine lung carcinomas are squamous cell lung carcinomas (SCLC). SCLC is a smoking-related lung cancer and is histologically divided into four different types: papillary, clear cell, small cell and basaloid (Beasley et al. 2005). This carcinoma type arises from metaplastic basal cells and usually begins to develop in the main, lobar, segmental or subsegmental bronchi. One third of SCLCs arise from small peripheral airways (Hammar 2008). SCLCs are detected early because of the early clinical symptoms and tumors do not usually become large. SCLCs have a tendency to cavitate. Lesions are different depending on stromal desmoplasia, keratin production and necrosis. Central tumors often infiltrate into peribronchial tissue, lung, hilar and mediastinal lymph nodes and mediastinal structures. Well-differentiated tumors tend to spread locally in the chest and involve mediastinal structures. Poorly differentiated tumors metastasize to distant sites. SCLCs are histologically diagnosed by the keratinization or intracellular bridges (Travis et al 2004). Immunohistochemically they express high molecular weight cytokeratins (CK5/6) and do not usually express thyroid transcription factor 1 (TFF-1) (Perez-Moreno et al. 2012).

Adenocarcinoma is the most common cancer type in non-smokers and women (Brambilla et al. 2001). Adenocarcinomas are associated with parenchymal scarring. On the other hand, adenocarcinoma has become more common among smokers. Adenocarcinoma is a malignant epithelial tumor with glandular differentiation or mucin production. This carcinoma type usually arises from the peripheral lung tissue and is probably developed from Clara cells or type
II pneumocytes. Central carcinomas develop from bronchial epithelium or bronchial gland epithelium. Adenocarcinomas are currently divided by the predominant growth pattern into lepidiform, papillary, acinar, solid and micropapillary predominant types (see Table 1). The former diagnosis of bronchioloalveolar carcinoma corresponds to the lepidiform type. The precursor lesions of adenocarcinoma are atypical adenomatous hyperplasia and adenocarcinoma in situ, describing a lesion with a lepidiform growth and with no destructive invasion present (Travis et al. 2011). There are also some unusual types of adenocarcinoma, such as fetal type or intestinal type, which are listed as separate entities. By immunohistochemistry, adenocarcinomas often show strong expression of TTF-1 (Brambilla et al. 2001).

Less than 10% of non-neuroendocrine lung carcinomas are large cell carcinoma (LCC). Large cell carcinoma is an undifferentiated malignant epithelial carcinoma which originates from epithelial cells of the lung but does not show any signs of squamous or glandular differentiation (Travis et al. 2004). LCCs are usually large peripheral tumors and often necrotic and hemorrhagic. Pleural and chest invasion are common for LCCs (Hammar 2008).

Carcinomas with neuroendocrine features

About a fourth of primary lung cancers are neuroendocrine tumors (NET). Small cell lung carcinoma (SCLC) is the most common NET (20%). Large cell neuroendocrine carcinomas (LCNEC), typical carcinoids (TC) and atypical carcinoids (AT) are also NETs. Carcinoids are more often found in younger patients with no predilection for sex or smoking history. SCLC is found in older patients with smoking history. Patients are also often males (Rekhtman 2010).

Small cell lung carcinoma is the most aggressive lung carcinoma types and is strongly tied to cigarette smoking. It normally arises in the central region of the lungs and the carcinoma cells are small and round, many times also elongated with high nuclear-cytoplasmic ratio and neuroendocrine differentiation. Mitoses, necroses and apoptotic bodies are abundant. SCCs tend to surround the major bronchi and extensively spread within the lung in a lymphangitic pattern (Hammar 2008). Immunohistochemically they express neuroendocrine markers such as synaptophysin and chromogranin, cytokeratin often with a dot-like pattern, and they also express TTF-1.
Carcinoid tumors are mainly central tumors, about 25% them peripheral. These tumors have a coarse granular chromatin, they lack prominent nucleoli and cells are overall uniform. Vascularity is also typical for the tumors. Carcinoid tumors are divided into atypical carcinoids (AC) and typical carcinoids (TC) (Rekhtman 2010). TC is a low-grade neuroendocrine tumor that has relatively well-demarcated nodule. Peripheral tumors may have multiple satellite nodules. Tumors show < 2 mitosis per 10 high power fields (HPF) and without necrosis. Tumors may show a large spectrum of growth patterns, usually trabecular or mosaic. The nucleus is round-shaped with finely granular chromatin (Morandi et al. 2006). AC is an intermediate-grade tumor which form metastases. AC is defined as a neuroendocrine tumor with mitotic rate of 2 to 10 per HPF or with necrosis. AC shows more variability in cell size and shape than TC and has less neuroendocrine granules than TC. Large cell neuroendocrine carcinomas have large tumor cells and show a mitotic frequency of over 10 mitosis per HPF. Necrosis has a punctate appearance in the center of the tumors (Rekhtman 2010).

Molecular biology of lung carcinomas

Lung carcinomas develop through accumulation of multiple molecular abnormalities. The carcinogenic process includes abnormalities in self-sufficiency of growth signals due to mutations in proto-oncogenes, insensitivity in anti-growth signals as a result of mutations of the tumor suppression genes, evading apoptosis by up-regulating anti-apoptotic proteins or down-regulating pro-apoptotic molecules, replicative potential becoming limitless due to the activation of telomerase, sustained angiogenesis and tissue invasion capability (Hanahan & Weinberg 2011). These alterations include genetic abnormalities of tumor suppressor gene (TSG) inactivation and over-activity of cell growth-promoting oncogenes. Smoking along with abnormalities in DNA repair and a genetic predisposition induces lung cancer development (Fong et al. 2003).

Cell growth is stimulated by oncogenes. These genes are strictly controlled and regulated because alterations in oncogenes lead to uncontrolled proliferation of the cells. As an example, epidermal growth factor receptor (EGFR) signaling is important for normal cell proliferation, but deregulation leads to carcinoma pathogenesis, metastasis and inhibition of apoptosis. EGFR dysregulation is associated with poor survival and resistance to chemotherapeutic agents in NSCLCs. In advanced NSCLC EGFR is overexpressed (Fong et al. 2003). Detection of EGFR-activating mutations is especially important in
adenocarcinoma where carcinoma cell growth may be retarded by specific kinase inhibitors (Okayama et al. 2012, Travis 2011). In fact, about 20% of lung adenocarcinomas harbor activating EGFR mutations while other types of lung carcinomas hardly ever do (Paez et al. 2004). EGFR mutations are more frequently found in non-smokers, women and in oriental people (Planchard 2013, Travis 2011). Activating mutations are commonly found between exons 18–21 (Planchard 2013). HER-2/neu/EGFR2 is a transmembrane glycoprotein like EGFR and they have high homology. This protein is overexpressed in adenocarcinomas and is associated with drug-resistant phenotype and higher prevalence of metastases, but the overexpression is low and found only in about 4% of cases (Hirsch et al. 2002).

The RAS proto-oncogene family includes HRAS, KRAS and NRAS. These genes have an important role commanding the activation effectors that mediate cell proliferation, apoptosis vesicle movement and T-cell activation. The RAS protein is activated when guanine nucleotide exchange factors (GEFs) bring activation signals near the inactive Ras protein. GEF promote GTPase in RAS protein to convert GDP to GTP which activates the RAS protein (Diaz et al. 2004). Due to a point mutation in the RAS gene RAS protein conformation may be altered leading to GTPase activity that is locked to active state with persistent stimulation of cell proliferation. RAS mutations are found in 15–20% of NSCLC and in up to 50% in adenocarcinomas. EGFR and Ras mutations commonly exclude each other, and Ras mutations are more common in adenocarcinomas originating from bronchial epithelium such as mucinous bronchioloalveolar carcinoma (Kakegawa et al. 2011). In SCLC these mutations are rare. KRAS mutation has been found to correlate with smoking (Fong et al. 2003). Benzo[b]pyrene diethyloxide (BPDE), nitrosamines and other DNA adduct-forming agents cause the majority of the mutations in the RAS gene. These agents are found in tobacco smoke.

Recently also anaplastic lymphoma kinase (ALK) mutations have been given attention because of potential therapeutic use of kinase inhibitors also in these cases. In such cases ALK- protein fuses with EML4 protein (Echinoderm Microtubule associated protein Like-4), thus creating a fusion protein favouring proliferation, cell migration and survival (Soda et al. 2007). The frequency of ALK translocations varies between 1–6.7% of adenocarcinomas. It is present in younger patients, in males but on the other hand also in patients of more advanced clinical stage (Inamura et al. 2009, Kwak et al. 2010).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
<th>Large cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>20%</td>
<td>4.4%</td>
<td>4%</td>
</tr>
<tr>
<td>RAS</td>
<td>7%</td>
<td>1.5%</td>
<td>4%</td>
</tr>
<tr>
<td>ALK</td>
<td>1-6.7%</td>
<td>4%</td>
<td>8.3%</td>
</tr>
<tr>
<td>PTEN</td>
<td>7.0%</td>
<td>10%</td>
<td>27.3%</td>
</tr>
<tr>
<td>BRAF</td>
<td>1-3%</td>
<td>3%</td>
<td>0%</td>
</tr>
<tr>
<td>C-MET</td>
<td>≤5%</td>
<td>≤5%</td>
<td>0%</td>
</tr>
</tbody>
</table>

The MYC proto-oncogene encodes transcription factors that are involved in cell growth control and apoptosis and also effect chromatin structure. MYC is localized in the nucleus and it regulates transcription factors through partner proteins such as MAX, MAD or MX11. Activated MYC heterodimer induces alterations in chromatin structure which modulates the gene transcription. MYC gene amplification or overexpression is found in 80–90% of SCLC and only 10% of NSCLC. Mutation in this gene is also a late event in SCLC lung carcinoma pathogenesis (Danesi & Paolo 2003).

Tumor suppression genes (TSG) control the normal cell growth. These genes inhibit the tumorigenic processes and are involved in the DNA damage response and repair. TSGs are inactivated when one allele in the chromosomal locus is lost and the other is damaged by a gene mutation or epigenetic hypermethylation of the promoter of the gene. In pathogenesis and earliest genetic events of lung carcinomas (96%) there are several allelic loci losses in chromosome arm 3p. The most frequent loss of heterozygosity (LOH) in lung carcinomas is found in the chromosomal arms of 1p, 3p, 4p, 4q, 5q, 8p, 9p, 9q, 10p, 10q, 13q, 15q, 17p, 18q, 19p, Xp and Xq (Fong et al. 2003). One TSG is fragile histidine triad gene (FHIT) the loss of which causes accumulation of diadosine tetraphosphate. Through this, FHIT induces DNA synthesis and cell proliferation. The FHIT gene is frequently found to be abnormal in lung carcinomas (Sozzi et al. 1996). pFHIT expression is associated with cigarette smoking indicating that FHIT might have a role in initiating smoking-related lung carcinogenesis (Panov 2005). TP53 tumor-suppressor gene (p53) is located in chromosome arm 17p. p53 maintains the integrity of the genome after cellular DNA damage induced by UV or γ irradiation, hypoxia and other carcinogens. p53 prevents the cells from moving from phase G1 to phase S or induces apoptosis if the cell has already transferred to S phase. Human double minute 2 (HDM2) protein suppresses the p53 regulation of target
genes and enhances its proteasome-dependent degradation (Oren 2003). p53 is an autoregulated gene that up-regulates HDM2 expression and down-regulates its own expression. Due to this autoregulatory loop p53 expression is not seen in normal cells. Mutations in the TP53 gene enhance the half-life of p53 protein and suppress the protein activity in lung carcinomas. p53 is inactivated in 75% of SCLC and about 50% of NSCLC (Kitamura et al. 2008). Smoking is correlated with the frequency of mutation in the p53 gene in NSCLC. Retino Blastoma tumor-suppression gene (RB gene) controls and regulates the cell cycle progression along with p53. In lung carcinomas there are deletions, nonsense mutations, pathogenic splicing and chromosomal deletions which cause RB gene dysfunction (Kitamura et al. 2008). Abnormal activity of the RB gene induces progression of cell cycle and makes cells insensitive to antigrowth factors that control transition through phase G1. Over 90% of SCLC cases have abnormal or no expression of RB gene (Rom et al. 2000). The p16 gene product binds to CDK4 and CDK6 which inhibits their interaction with cyclin D1. The inhibition of cyclin D1-CDK complex activity prevents RB protein phosphorylation and releases E2F. These actions inhibit the G1/S phase transition in the cell (see Figure 1) (Fong et al. 2003).
Fig. 1. Regulation of cell cycle by Rb protein.

Transforming growth factor-β (TGF-β) has potential function as tumor suppressor and tumor promoter. As suppressor TGF-β inhibits the epithelial cell proliferation by binding in the cell receptors and prevents cell proliferation into G1 phase. As promoter it can increase invasiveness especially in patients with later stages of carcinomas (Elliott & Blobe 2005). TGF-β is down-regulated in NSCLC (Malkoski et al. 2012).

2.1.2 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is mainly a smoking-related disease, which is associated with long-term and progressive obstruction of airways, chronic bronchitis and emphysema. This major public health problem is an important cause of morbidity and mortality worldwide. Patients with COPD suffer from this disease for years and die prematurely, and the global burden of COPD has been estimated to increase during the decades to come because of a continuing exposure to COPD risk factors as well as aging of the population.
COPD is an underdiagnosed disease in which half of the people that have it are ignorant of the disease (Lindberg et al. 2006). Symptoms of COPD are long-term cough, mucus secretion and shortness of breath especially on exercise. When the symptoms are apparent the disease is far advanced and half of lung function measured as forced expiratory volume in one second (FEV1) is lost. A distinct feature and a remarkable prognostic factor of COPD are the exacerbations of the disease that appear with or without infections in respiratory tract, leading to hospitalizations, acute respiratory insufficiency, risk of dying and further progression of the disease (Burge & Wedzicha 2003).

Risk factors for COPD are smoking, occupational exposures, air pollution, aging, gender, infections, socio-economic status and bronchial hyper-responsiveness. Besides known risk factors there are genetic factors which increase the risk for COPD (Mannino & Buist 2007). Smoking is the most important risk factor for COPD. Tobacco smoke causes oxidant stress in the lungs, which has an important role in inflammation and tissue damage. Over 70% of COPD mortality in high-income countries is related to smoking compared to 40% in low- and middle-income nations (Lopez et al. 2006a, Mannino & Buist 2007).

The best-known genetic risk factor is α-1-antitrypsin deficiency, which is found in 1–3% of COPD patients (Stoller & Aboussouan 2005). TGFβ1, tumor necrosis factor α, microsomal epoxide hydrolase 1, α-1-chymotrypsin and glutathione-s-transferases are other candidate genes associated with the risk for COPD (Celedon et al. 2004, Cheng et al. 2004, Keatings et al. 2000, Mannino & Buist 2007, Stoller & Aboussouan 2005). Occupational exposures are usually chronic exposures to mineral or metallic dust, gases, fumes or combinations of these products. In lower income countries the emission regulations in mines and factories are less stringent than in high-income countries. Smoking attached to occupational exposures increases the risk factor to triple. Indoor and outdoor pollutants are a greater risk in low-income countries than in high-income countries. Exposure to biomass fuels such as coal, straw, animal dung, crop residues and wood, which are used for cooking in poorly ventilated households, increases the risk of developing COPD (Mannino & Buist 2007).

Bacterial and viral infections have an important role in the development and progression of COPD. Half of the infections are bacterial and half are viral infections. Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis are the most common bacteria found in exacerbations of COPD (Message & Johnston 2002). In earlier times COPD was more common in men
than in women due to smoking and occupational exposure. Since smoking has become more common among women, COPD is found more frequently in women as well (Mannino & Buist 2007). Lower socioeconomic status increases the risk for COPD. Poor nutrition, poor access to health care, early respiratory infections, high smoking rates and high work exposures are socioeconomic factors that influence the risk for developing COPD (Mannino & Buist 2007).

**Diagnosis and classification of COPD**

Clinical diagnosis of COPD should be suspected when a patient with exposure to COPD risk factors suffers from dyspnea, chronic cough or sputum production. Spirometry is required to make a definite diagnosis in this context. According to the international definition of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) COPD is confirmed when post-bronchodilatation FEV$_1$ is less than 70% of forced vital capacity (FVC), i.e., there is irreversible airflow obstruction. When FEV$_1$ is over 80% of reference value, the airway obstruction is considered to be mild (Stage I). Moderate COPD (Stage II) is diagnosed when FEV$_1$ is 50–80%, severe (Stage III) when FEV$_1$ is 30–50%, and very severe (Stage IV) when FEV$_1$ is under 30% of the predicted value (GOLD 2013). The use of this fixed FEV1/FVC-ratio has raised several concerns: underdiagnosis of COPD in young patients and overestimation of obstruction in elderly population. The risk of misdiagnosis and overtreatment is limited, since spirometry is only one of three components in building COPD diagnosis, together with symptoms and risk factors (Cerveri et al. 2008, Hardie et al. 2002).

**Mechanisms involved in the development of COPD**

Several mechanisms are involved in the development of COPD. Protease-antiprotease balance and oxidant-antioxidant balance are the most widely studied mechanisms. Protease-antiprotease balance is disturbed in COPD. Lung tissue is protected against proteases by protease-inhibitors, such as α-1-antitrypsin which is primarily secreted into the bloodstream from the liver (Fregonese & Stolk 2008), but they can be secreted locally from monocytes (Paakko et al. 1996). Normally these protease-inhibitors are mainly present in blood at low levels. Matrix metalloproteases (MMP) are secreted from inflammatory cells, such as macrophages and neutrophils. MMPs degrade matrix proteins such as elastin, collagen, proteoglycans, fibronectin and laminin. This causes damage in lung
tissue and destruction of alveolar walls inducing dilated alveolar spaces and reduced gas-exchanging capacity (Churg \textit{et al.} 2012). Degradation of elastin in airways walls leads to significant reduction of elasticity in small airways and alveoli. This adds to the airflow limitations (Black \textit{et al.} 2008). Tobacco smoke contains oxygen radicals that are involved in oxidant-antioxidant balance. Environmental oxygen radicals activate inflammatory cells that produce more oxygen radicals. These radicals induce cytokine expression, lipid-peroxidation, inactivate antiproteases and inhibit antioxidant defense. These actions also induce tissue damage and alveolar wall disruption. Antioxidants such as glutathione, uric acid, bilirubin and vitamins C and E inactivate the oxygen radicals in normal lung. In COPD patients the antioxidant levels are low (Barnes & Hansel 2004).

Environmental agents such as air pollution or cigarette smoke induce defense mechanisms in airways. Airway epithelial cells are involved in inflammation process by inducing inflammatory transmitters and influencing inflammatory cells through cell-cell adhesion molecules. Exaggerated inflammatory response leads to aberrant airway epithelia and matrix remodeling, where excessive growth factors are released and MMPs are elevated. Aberrant immune-surveillance and secondary autoimmune processes may contribute to maintaining or amplifying pulmonary inflammation. This aberrant response might also involve processes that lead to excessive oxidative stress. Epithelial mesenchymal transition (EMT) is induced by expression of growth factors and MMPs. This leads to matrix remodeling. Epithelial cells express inflammatory cytokines (interleukin-8, interleukin-6 and tumor necrosis factor-α) which promotes the oxidative stress, EMT, remodeling and repair of the epithelium. Excessive cytokine expression might lead to aberrant epithelial and airway remodeling leading to pathological changes (Young \textit{et al}. 2011).

Collagens I and III are found in the fibrotic layer under the bronchial epithelial cells in the airways. Collagens are found to be involved in the remodeling of the airway epithelia. Remodeling is caused by inflammatory processes and oxidation stress by continuous exposure to toxic agents. Small airways are the major source of airflow resistance. In COPD small airway walls are thickened and alveolar walls have been partly destroyed. Expression of pro-collagens I and III is up-regulated in the large airways of smokers and stage I-II COPD patients. Pro-collagen I is not found in the small airways, but pro-collagen III is present in bronchioli. Pro-collagen III expression has been found to decrease the in end-state COPD, which might indicate that remodeling is diminished, cells
are not able to produce collagen or that the protein is degraded. Remodeling also occur also in the large airways not only in the small airways of COPD patients (Harju et al. 2010).

Connection between COPD and lung carcinomas

Patients with COPD have a greater risk of developing lung cancer compared to smokers with corresponding smoking history and normal lung function (Brenner et al. 2011, Papi et al. 2004, Powell et al. 2013). Reduced FEV1 is an important risk factor for COPD and for lung cancer as well as smoking. There are also genetic factors that are found in both pathogeneses. Inflammatory pathways and immune-modulating pathways involved in the intracellular signaling are found in COPD and lung cancer (Young et al. 2011). EMT is also present in both diseases. The conversation of epithelial cells to motile, invasive and migratory mesenchymal cells is characteristic of EMT in lung cancer. Epithelial cells lose their cell-cell adhesion and their polarity is changed. EMT promotes malignant transformation of the respiratory epithelium and invasive spread of lung carcinoma (Soini 2012). The role of EMT in COPD has not been studied much. EMT-promoting factors are present in the lung matrix remodeling in COPD (Milara et al. 2013).

2.2 Tight junctions

Cell junctions consist of multiprotein complexes that provide contact between neighboring cells or between a cell and the extracellular matrix. Junction types between epithelial cells are tight junction, gap junctions, adherens junctions, desmosomes and hemidesmosomes (Mitic et al. 2000). Tight junctions (TJ) form a morphological and functional boundary between the apical and basolateral cell surface. They regulate paracellular permeability and cell polarity sealing the paracellular pathway by forming a paracellular diffusion barrier or gate. Claudins and occludin are the main proteins that form the backbone of the TJs (Balda & Matter 2000). (Figure 1)
TJs form a continuous, circumferential, belt-like structure at the luminal end of the intercellular space. On the cytoplasmic side of the TJ, the TJ plaque is the site of a great number of TJ-associated cytoplasmic proteins such as ZOs (ZO-1, ZO-2 and ZO-3) that are bound to the TJ backbone proteins and to actin microfilaments (Schneeberger & Lynch 2004). The complexity of the TJ network varies greatly among different epithelia, leading to the suggestion that there is a direct relationship between these and the measured transepithelial electrical resistance (TER). High TER indicates low permeable epithelial layer which indicates that TJs are very tight (Schneeberger & Lynch 2004). In airways tight junctions are connected with relatively low TER, ~ 100 $\Omega$cm², and described as being moderately leaky (Coyne et al. 2003).

In human carcinomas TJ dysfunctions and abnormal structures are associated with tumorigenesis by loss of cell-cell adhesion, loss of cohesion and invasiveness (Oliveira & Morgado-Diaz 2007, Oliveira & Morgado-Diaz 2007, Olivera et al. 2007). They also lead to disturbances of barrier function and cause unbalanced ion and molecule change and nutritional balance. It is particularly clear that TJs are targets of pathogens because of their localization as the most apical junction between epithelial cells. Bacteria, viruses and allergens that enter the human body affect the barrier function of TJs by changing the function of TJ proteins and actin organization. Barrier function abnormalities are involved in a number of pathological conditions (Sawada et al. 2003, Soini 2012).
2.2.1 Claudins

Claudins are transmembrane proteins which are essential for the formation and function of TJs. Claudins have four hydrophobic transmembrane (TM) domains and two extracellular loops (ECLs), an intracellular tail and N-and C-terminal cytoplasmic domains (Furuse et al. 1998, Morin 2005) (Figure 2).

![Fig. 3. Structure of claudin.](image)

There are 27 members in the claudin family known so far and their molecular weight is 20–27 kDa (Mineta et al. 2011, Oliveira & Morgado-Diaz 2007). TJs and Claudins are present in epithelial, endothelial and mesothelial cells (Soini 2005, Soini et al. 2006). Most tissues derived from these cells express several Claudins and they have specific distribution patterns (Morin 2005). Some Claudins have sealing functions in TJs and some increase paracellular permeability. In general, Claudins 2, 7, 10, 15 and 16 increase paracellular permeability by forming pores in tight junctions while Claudins 4, 5, 8, 11, 14 and 18 have a permeability decreasing function (Krause et al. 2008). Airway bronchi and bronchiole express Claudins 1, 3, 4, 5, and 7. Claudins 1, 3, 4 and 7 have been localized in a circumferential pattern along the cell membrane with different polarity. Claudin 2 have cytoplasmic and granular positive expression in normal
bronchial cells (Moldvay et al. 2007). Claudins 1 and 3 have been shown to tighten the epithelium while claudin 5 makes the epithelium leakier (Coyne et al. 2003).

Claudins are expressed in the normal epithelial cells in different variations. Claudins 1, 3, 4, 5, and 7 are expressed in the normal lung. Claudin 1 is expressed only in bronchial epithelium whereas claudins 3, 4 and 7 are also positive in alveolar epithelium as well as bronchial epithelium (Kaarteenaho et al. 2010). Claudins play a role in several human diseases. In the kidney mutations of claudins 16 and 19 lead to hypomagnesemia due to disturbances in tubular cells of the nephron (Hou & Goodenough 2010). Knockdown of claudin 1 is lethal and affected mice die of dehydration (Kirschner et al. 2013). Mutations of some claudins also lead to deafness and sensorineural deficiencies (Table 3). Claudins acts as barriers in TJs and several pathogens may disrupt these barriers and gain entry to the subepithelial space. The bacteria Clostridium perfringens excrete a toxin which attaches to claudins 3 and 4 inhibiting their function and causing opening in the TJs. Hepatitis C virus uses claudin 1 as an entry to get into the liver cells (Farquhar et al. 2012).
Table 4. Some characteristics of different claudins present in humans.

<table>
<thead>
<tr>
<th>Claudin</th>
<th>Chromosomal site</th>
<th>Molecular weight (kDa)</th>
<th>Disease association</th>
<th>Other aspects</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLD1</td>
<td>3q28-29</td>
<td>22.7</td>
<td>Ichthyosis, sclerosing, cholangitis</td>
<td>Receptor cofactor for hepatitis C virus, regulates right-left patterning in embryogenesis, knockdown causes water loss through the skin</td>
</tr>
<tr>
<td>CLD2</td>
<td>Xq22.3-q23</td>
<td>24.5</td>
<td></td>
<td>Knockdown hinders bile canalicular development</td>
</tr>
<tr>
<td>CLD3</td>
<td>7q11.23</td>
<td>23.3</td>
<td>Clostridium perfringens toxin receptor</td>
<td></td>
</tr>
<tr>
<td>CLD4</td>
<td>7q11.23</td>
<td>22.0</td>
<td>Same as above</td>
<td></td>
</tr>
<tr>
<td>CLD5</td>
<td>22q11.21</td>
<td>23.1</td>
<td>Present in endothelium, important in heart development in Xenopus, knockdown causes disturbances in blood-brain-barrier function</td>
<td></td>
</tr>
<tr>
<td>CLD6</td>
<td>16p13.3</td>
<td>23.3</td>
<td></td>
<td>Entry cofactor receptor for hepatitis C virus</td>
</tr>
<tr>
<td>CLD7</td>
<td>17</td>
<td>22.4</td>
<td></td>
<td>Knockdown decreases chloride permeability</td>
</tr>
<tr>
<td>CLD9</td>
<td>16p13.3</td>
<td>22.8</td>
<td></td>
<td>Entry cofactor for hepatitis C virus</td>
</tr>
<tr>
<td>CLD11</td>
<td>3q26.2-q26.3</td>
<td>21.9</td>
<td>Deafness, Sertoli, cell dysfunction</td>
<td>Knockdown causes nerve conduction disturbances, male sterility, deafness</td>
</tr>
<tr>
<td>CLD12</td>
<td>7q21</td>
<td>27.1</td>
<td></td>
<td>D vitamin induced Ca absorption from enterocytes</td>
</tr>
<tr>
<td>CLD14</td>
<td>21q22.3</td>
<td>25.7</td>
<td>Sensorineural, deafness</td>
<td>Reduced mineral bone density, kidney stone development, knockout causes deafness</td>
</tr>
<tr>
<td>CLD15</td>
<td>7q11.22</td>
<td>24.3</td>
<td></td>
<td>Megaintestine development in knockout mice</td>
</tr>
<tr>
<td>CLD16</td>
<td>3q28</td>
<td>33.8</td>
<td>Hypomagnesemia type 3</td>
<td></td>
</tr>
<tr>
<td>CLD18</td>
<td>3q22.3</td>
<td>27.7-27.9</td>
<td></td>
<td>Present in lung and gastrointestinal tract, expression in GI and lung tumors</td>
</tr>
<tr>
<td>CLD19</td>
<td>1p34.2</td>
<td>22-23.2</td>
<td>Hypomagnesemia with ocular involvement</td>
<td>Knockout influences nerve conduction</td>
</tr>
<tr>
<td>CLD23</td>
<td>8p23.1</td>
<td>31.9</td>
<td></td>
<td>Detected in germinal center B cells, stomach, placenta</td>
</tr>
</tbody>
</table>
In line with their varied expression in different types of tissues containing tight junctions, the expression of claudins may vary in tumors of different sites (Soini 2005). There may also be differences in the expression of claudins between different histological types of tumors in the same location. In gastric cancer, the diffuse type of adenocarcinoma shows a lower claudin expression compared with the intestinal type (Soini 2005). This difference in claudin expression can in some cases be used in differential diagnosis of tumors. The expression of claudins 3 and 4 is lower in malignant mesothelioma compared to metastatic adenocarcinoma and this can be used for differential diagnosis between these two entities (Soini et al. 2006).

According to previous reports claudin 1 seems to have high expression in squamous cell carcinoma and low expression in adenocarcinomas. Claudin 2 expression is decreased in different lung cancers (adenocarcinoma, squamous cell lung cancer, small cell lung cancer and carcinoids). Paschoud et al. noticed that expression of claudins 3 and 4 mRNA indicated a decrease in lung squamous cell carcinoma and adenocarcinoma. In another work of Moldway et al. (Moldvay et al. 2007) these claudins were significantly increased in small cell lung cancer and in adenocarcinomas. There are differences in the samples in which the mRNA was isolated. Moldway et al have isolated RNA from paraffin-embedded samples while Paschoud et al performed the mRNA isolation from fresh material. There were also differences between the normal lung tissue samples. These circumstances might be behind the different results concerning adenocarcinoma in these studies. Claudin 5 has an opposite expression pattern to claudin 1. Claudin 5 is highly expressed in adenocarcinomas but has low expression in squamous cell carcinomas (Paschoud et al. 2007). Expression on claudin 7 is different in the two studies. Moldway et al. observed that expression of claudin 7 is increased and showed strong immunopositivity in almost all their studied cases. Four cases had negative expression and these cases were all neuroendocrine lung cancers (1 SCC, 2 TC and 1 AC). Paschoud et al. found in their work that qPCR indicated a decreased expression of claudin 7 in adenocarcinomas and squamous cell carcinomas.

2.3 Epitheliomesenchymal transition

Epitheliomesenchymal transition (EMT) is a process where cells lose some of their epithelial traits, such as cell adhesion, and become more mesenchymal with an increase in cell motility. Their expression of E-cadherin is repressed and cells
start to express vimentin and alpha smooth muscle actin. EMT occurs in various steps in normal development, including mesoderm and neural crest formation. It is also present in fibrotic processes. EMT is classified in three categories type I, type II and type III. EMT type I occurs during fetal development while type II is adult-related EMT, such as tissue injury repair. Type III EMT involves cancer, including cell invasion, a growing resistance to apoptosis and gain of stem cell features. EMT proceeds through two steps: decreased intercellular adhesion to dissociate from the epithelial cellular sheets and increased cell motility to migrate into connective tissues. These steps are associated with total loss of epithelial cell polarity (Ikenouchi et al. 2003).

EMT attached to carcinogenesis is known for the loss of cell-cell adhesion and cell migration. Loss of cell-cell adhesion is directly linked to claudins as TJ proteins. TJs are important for maintaining barrier function and cell polarity. Dysfunction or loss of claudins in epithelial cells has an impact on TJs. Down-regulation of claudins is found in several carcinoma types including lung carcinomas (Singh et al. 2010).

**2.3.1 Transcription factors in EMT**

Transcription factor is a protein that binds to specific DNA sequences and controls the transformation of genetic information from DNA to mRNA. Genes can be up- or down-regulated. There are several different transcription factor families. As mentioned earlier, the zinc finger family and basic helix-loop-helix family are involved in EMT (Shih et al. 2005).

Transcription factor snail (snail) is a member of the zinc-finger transcription factor family. It is a repressor of e-cadherin and plays a central role in EMT by inducing the epithelial cells to lose their polarity, e.g. in tissue repair in adults. Snail is also essential during early developmental stages (Cano et al. 2000). It also down-regulates the expression of TJ proteins such as occluding and claudins (Ikenouchi et al. 2003, Martinez-Estrada et al. 2006, Ohkubo & Ozawa 2004). Snail and slug are both unstable proteins with a short half-life (<1h) (Zhou et al. 2005).

Transcription factor slug is also a zinc-finger transcription factor which, along with snail, is activated at the blastocyst stage of the early mouse embryo stage, taking part in segregation of cellular layers at an early stage of development (Bell & Watson 2009). In addition to directing EMT in embryogenesis, slug influences hematopoietic stem cell maturation and protects hematopoietic stem
cells from radiation-induced apoptosis. Inhibition of slug expression delays wound healing due to the failure of basal keratinocyte activation and disturbed loosening of cellular adhesion in mice (Arnoux et al. 2008). In EMT, slug expression is activated by the Notch- and TGF-β-induced signaling pathways leading to a loosening of cellular cohesion, proliferation, repression of E-cadherin expression and increased resistance to apoptosis (Leong et al. 2007, Morita et al. 2007).

Twist is essential during embryonic development of the neural tissues being especially important for head and limb development (Pena et al. 2010). Transcription factor twist is a helix-loop-helix transcriptional factor which regulates cell movement and tissue reorganization during early embryogenesis. It down-regulates E-cadherin and it can also induce expression of transcription factor snail. In addition to this, twist can also induce expression of mesenchymal markers such as fibronectin and N-cadherin during EMT in mice (Yang et al. 2004). In adult humans twist is mainly expressed in precursor cells such as osteoblastic cells and chondroblastic cells maintaining their undifferentiated state. Twist also acts as B cell activator in an inflammatory environment and it can inhibit interferons in T helper 1 lymphocytes (Niesner et al. 2008). Ectopic expression of twist results in loss of E-cadherin mediated cell-cell adhesion, activation of mesenchymal markers and induction of cell motility, suggesting that it contributes to cancer metastasis by promoting EMT in mice (Yang et al. 2004).

2.4 Matrix metalloproteases

There are 23 human matrix metalloproteases (MMP), including 17 soluble, secreted enzymes and 6 membrane-associated enzymes. They play an important role in various physiological processes including tissue remodeling and organ development, in the regulation of inflammatory processes and in diseases such as cancer (Kessenbrock et al. 2010). MMPs are proteolytic enzymes that are associated with tumorigenesis and EMT. MMPs are zinc-dependent endopeptidases that are capable of degrading all kinds of extracellular matrix proteins (Kessenbrock et al. 2010, Lin et al. 2011). MMPs are secreted in enzymatically inactive state and activated by a mechanism called cysteine switch. MMP 2 and MMP 9 are gelatinases which can disturb cell adhesion by processing the components of cell-cell and cell-extracellular matrix contacts. This enables the
tumor cells to migrate, invade and spread to neighboring tissues (Orlichenko & Radisky 2008).
3 Aims of the study

The aims of the study were:

I To study the expression and significance of tight junction proteins claudin 1, 2, 3, 4, 5 and 7 in different histological subtypes of lung carcinomas, their associations to patient survival, TNM-status and smoking and to find out the effect of EMT-related transcription factor snail on the expression of claudins, invasion and patient prognosis in lung carcinomas in vivo.

II To study the expression and importance of claudins 3 and 4 in bronchi and bronchioli of non-smoker’s, smoker’s and COPD patient’s lungs as well as their associations with lung function and smoking history and to examine the expression of transcription factors slug and twist in airways as well as their association with claudin expression and smoking history.

III To study the expression of claudins in vitro in normal bronchial cell line and lung carcinoma cell lines. To study the effect of transcription factor snail on cells invasion, claudin expressions and MMP expression in non-malignant and malignant cell lines. Also to study the effect of transcription factors slug and twist on claudins 3 and 4 in non-malignant cells line as well as the effect of cigarette smoke extract on claudins and modified cell lines.
4 Material and Methods

4.1 Study material

The tumor material in original articles I and II consisted of 343 samples of lung cancer which were retrieved from the archives of the Department of Pathology, University of Oulu. The diagnosis of the cases was based on the WHO classification of lung and pleural tumors (Travis et al. 2004). The material consisted of 128 squamous cell carcinomas, 108 adenocarcinomas, 11 small cell carcinomas, 12 large cell carcinomas, 6 carcinoid tumors, 10 bronchioloalveolar carcinomas, 14 adenosquamous carcinomas and 54 metastases to the lung. All cases were re-evaluated and from each sample two representative tumor regions were chosen and integrated into multitissue microarray blocks with Beecher Instruments Manual Tissue Arrayer (Beecher Instruments, Silver Spring, MD, USA). The microarray sample diameter was 1300µm. The presence of metastases and stage of the tumor was determined at the time of the operation. The survival data was obtained from the Finnish Cancer Registry which maintains a nationwide database on all cancer cases in Finland since 1953. Table 5 shows the clinical characteristics of the patients.

Table 5. Characteristics of the patients in studies I and II.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Age (SD)</th>
<th>Sex (M/F)</th>
<th>Pack-years(SD)</th>
<th>FEV 1 (SD)</th>
<th>FEV/FCV (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td>64 (8)</td>
<td>119 / 9</td>
<td>41.1 (18.7)</td>
<td>2.52 (0.7)</td>
<td>71 (11.7)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>63 (9)</td>
<td>77 / 31</td>
<td>31.3 (21.1)</td>
<td>2.50 (0.75)</td>
<td>72.3 (12.65)</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>66 (8)</td>
<td>9 / 2</td>
<td>35.8 (11.9)</td>
<td>2.26 (0.6)</td>
<td>66.91 (13)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>62 (11)</td>
<td>10 / 2</td>
<td>46.8 (25.6)</td>
<td>2.78 (0.63)</td>
<td>68.62 (12.16)</td>
</tr>
<tr>
<td>Carcinoid</td>
<td>47 (10)</td>
<td>4 / 2</td>
<td>10.6 (10.1)</td>
<td>3.43 (0.73)</td>
<td>73.86 (9.66)</td>
</tr>
<tr>
<td>Bronchioloalveolar carcinoma</td>
<td>64 (10)</td>
<td>6 / 4</td>
<td>23 (28)</td>
<td>2.82 (0.92)</td>
<td>79.6 (9.52)</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>70 (11)</td>
<td>12 / 2</td>
<td>40.5 (16.5)</td>
<td>2.25 (0.68)</td>
<td>66.76 (9.1)</td>
</tr>
<tr>
<td>Metastases</td>
<td>54 (20)</td>
<td>27 / 27</td>
<td>7.7 (15.3)</td>
<td>2.63 (1.18)</td>
<td>73.86 (9.66)</td>
</tr>
</tbody>
</table>

Tissue material for original article III consisted of 24 current smokers with COPD, 24 current smokers with normal lung function and 17 life-long non-smokers. Lung tissue specimens from patients undergoing resection for lung tumor were collected from the archives of the Department of Pathology, Oulu University Hospital. Tissue specimens from tumor-free central bronchi and peripheral lung tissue were selected. Lung tumor was considered so small that it did not have any
effect on lung function or COPD stage. The patients did not receive corticosteroid therapy (either inhaled or systemic) and did not suffer from asbestos-related disease. All smokers were current smokers. The resected lungs were fixed in inflation. The size of each lung tissue specimen was approximately 1–2 cm². COPD was defined on the basis of preoperative lung function: post-bronchodilatation FEV₁/FVC less than 70% and no reversibility (bronchodilatation effect less than 12%). The clinical characteristics were obtained from the patient records and are presented in Table 6.

Table 6. Characteristics of the patients in study III.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Non-Smokers</th>
<th>Smokers</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>64 (13)</td>
<td>62 (4)</td>
<td>65 (6)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>9 / 8</td>
<td>19 / 5</td>
<td>20 / 4</td>
</tr>
<tr>
<td>Pack-Years (SD)</td>
<td>0</td>
<td>49 (19)</td>
<td>38 (13)</td>
</tr>
<tr>
<td>FEV1 l/s (SD)</td>
<td>3.01 (1.17)</td>
<td>2.99 (0.65)</td>
<td>2.08 (0.45)</td>
</tr>
<tr>
<td>FEV1 %predicted (SD)</td>
<td>98 (15)</td>
<td>88 (10)</td>
<td>65 (14)</td>
</tr>
<tr>
<td>FEV1/FVC % (SD)</td>
<td>86 (9)</td>
<td>83 (11)</td>
<td>60 (7)</td>
</tr>
<tr>
<td>DCO %predicted (SD)</td>
<td>92 (15)</td>
<td>76 (14)</td>
<td>73 (25)</td>
</tr>
<tr>
<td>DCO/VA %predicted (SD)</td>
<td>91 (12)</td>
<td>82 (12)</td>
<td>78 (24)</td>
</tr>
</tbody>
</table>

4.2 Immunohistochemical stainings

Four-µm thick sections were cut from the tumor array slides, deparaffinized with xylene and rehydrated in a descending ethanol series. The primary antibodies used in the immunostaining were designed to be used in formalin-fixed paraffin-embedded tissues. The antibodies used are shown in Table 7. Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 10 minutes. After an hour incubation with the primary antibody (a biotinylated secondary anti-rabbit or anti-mouse antibody and Histostain-SP kit (Zymed Laboratories Inc, South San Francisco, CA) was used. For all the immunostainings the color was developed using diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were carried out by substituting non-immune rabbit, or mouse serum and PBS for the primary antibodies. Non-neoplastic kidney, breast, skin and liver samples were used as positive controls.
Table 7. Antibodies used in Immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin 1 (clone JAY.8)</td>
<td>1:50</td>
<td>Zymed laboratories</td>
</tr>
<tr>
<td>Claudin 2 (clone 12H12)</td>
<td>1:50</td>
<td>Zymed laboratories</td>
</tr>
<tr>
<td>Claudin 3 (clone Z23.JM)</td>
<td>1:50</td>
<td>Zymed laboratories</td>
</tr>
<tr>
<td>Claudin 4 (clone 3E2C1)</td>
<td>1:50</td>
<td>Zymed laboratories</td>
</tr>
<tr>
<td>Claudin 5 (clone 4C3C2)</td>
<td>1:50</td>
<td>Zymed laboratories</td>
</tr>
<tr>
<td>Claudin 7 (clone ZMD.241)</td>
<td>1:50</td>
<td>Zymed laboratories</td>
</tr>
<tr>
<td>Snail</td>
<td>1:1000</td>
<td>Dept.of Anatomy, University of Helsinki</td>
</tr>
<tr>
<td>Slug (RB 1398)</td>
<td>1:100</td>
<td>Abgent</td>
</tr>
<tr>
<td>Twist (ab50887)</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>MMP2 (CA-4001)</td>
<td>24 µg/ml</td>
<td>Diabor, Oulu Finland</td>
</tr>
<tr>
<td>MMP9 (GE-231)</td>
<td>10 µg/ml</td>
<td>Diabor, Oulu Finland</td>
</tr>
</tbody>
</table>

4.2.1 Evaluation of the immunohistochemical stainings

In the evaluation of claudins and MMPs membrane-bound positivity and cytoplasmic positivity was considered significant. The evaluation was performed independently by two experienced pathologists without knowledge of clinical data. The immunostaining for claudin was assessed as follows: 0 = no immunostaining present, 1 = less than 25% of cells positive, 2 = 25–50% of cells positive, 3 = 50–75% of cells positive and 4 = 75–100% of cells positive. For evaluation survival and other factors, the cases were further divided into two groups with weak or no positivity (0,<1) or strong positivity (≥1). The immunostaining for MMPs was evaluated as follows: <5% = negative (-), 5–25% = weakly (+), 25–50% = intermediately (++) and >50% = strongly positive (+++).

With respect to transcription factors, nuclear expression for tumor cells was calculated in the array samples thus giving a value of positivity per array area. In each case, two separate array samples from each case were studied. We also recorded positivity or negativity based on the presence of transcription factor positive nuclei in tumors.

4.3 Cell lines

Human non-malignant bronchial BEAS-2B cells, which are SV40 transformed cells, and human lung carcinoma A427, A594, CALU-6, SK-MES-1 and SK-LU-1 cell lines were obtained from American Type Culture Collection (Rockville,
MD). SK-LU-1 express adenocarcinomatous features and SK-MES-1 show squamous cell features. BEAS-2B cells were cultured in serum-free bronchial epithelial growth medium BEGM (Lonza, Walkersville, MD). Lung carcinoma cell lines were cultured according to supplier’s guidelines.

4.4 shRNA transfection in cell lines

Lentiviruses were produced in Phoenix-GP packaging cells (PhGP-cells) as virusvectors which transport the viralvector into the cell genome to prevent the proper function of the transcription factor protein. The transcription factor is knocked down in cells. PhGP cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were given low glucose medium just before transfection. For transfection a mix of Optimem (Invitrogen, Carlsbad, CA, USA) was prepared containing lipofectamine 2000, the wanted retroviral vector (Sigma-Aldrich, St. Louis, MO, USA) and core protein plasmids (REV, pVSV-G and Gag/Pol). The mix was added to the cells. 24 hours post transfection the medium was removed and replaced by 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, St. Louis, MO, USA) was added in each infection to enhance the efficiency of the infection. After the infections the infected cells were separated from the uninfected cells by antibiotic selection. Puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added to the normal mediums of the cell lines to exterminate cells that were not infected.

4.5 Cigarette smoke exposure

Original cell lines and knockdown cell lines were exposed to cigarette smoke extract (CSE) at different time points. CSE was made by bubbling the smoke of two cigarettes (Red Marlboro, without filter) through 50 ml of RPMI-1640 medium (Sigma-Aldrich, Steinheim, Germany). Fresh CSE was syringe-filtered (Minisart, pore size 0.20 µm, Sartorius Stedim Biotech, Goettingen, Germany) before adding to the cells. Toxicity of CSE was studied with MTT assay kit (Sigma. Aldrich, Steinheim, Germany) and 15% CSE was found to be ideal for exposure experiments. This concentration was not toxic for the cells, but strong enough to make them react. At each time point cells and culture medium were collected for analysis.
4.6 Real time-PCR analysis

Total RNA was extracted from the cells with Qiagen RNeasy mini kit (QiagenGmbH, Hilden, Germany) with QiaShredder columns (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. Samples containing 0.5 µg of total cellular RNA were converted to cDNA with Revert Aid First Strand cDNA Synthesis kit (Fermentas, Ontario, Canada) using Oligo dT-primers.

Real Time-PCR was performed with 5 µl cDNA template in a total volume of 25 µl, using the iQ5 Optical system (Bio-Rad Laboratories, Hercules, CA). DNA-binding dye used in Real Time-PCR was SYBR Green I (iQ Custom SYBR green supermix, Bio-Rad Laboratories) which binds nonspecifically to double-stranded DNA. The primers, used in PCR are shown in Table 8. PCR reactions were performed in duplicates in 96-well plates. Cycling conditions were: one cycle at 95°C for three minutes, 40 cycles of amplification (each 95°C for 10 seconds and 56°C/58°C/59°C for 30 seconds), one cycle at 95°C for one minute, one cycle at 55°C for one minute and melt data acquisition.

Relative quantification analysis was performed with the iQ5 optical system software version 2.0 (Bio-Rad) using the housekeeping gene GAPDH as reference. BEAS-2B cells were considered as the normal situation. The expression of claudins was normalized with the housekeeping gene GAPDH and the effects of CSE on the claudin expression were calculated with the Livak method (Livak & Schmittgen 2001) by comparing control cells to exposed cells in each cell line.
Table 8. Primers used in the Real Time-PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence 1</th>
<th>Tm (°C)</th>
<th>Sequence 2</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin 1</td>
<td>Antisense</td>
<td>5´-CGGGTTGCTTGCAATGTGC-3´</td>
<td>56</td>
<td>5´-CCGGCGACAACATCGTGAC-3´</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>5´-CCGGCGACAACATCGTGAC-3´</td>
<td>56</td>
<td>5´-CCGGCGACAACATCGTGAC-3´</td>
<td>56</td>
</tr>
<tr>
<td>Claudin 2</td>
<td>Antisense</td>
<td>5´-TGGCAGAAAGACTGTGCATCTC-3´</td>
<td>56</td>
<td>5´-CAGCATTGACGACGAGTGG-3´</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>5´-CAGCATTGACGACGAGTGG-3´</td>
<td>56</td>
<td>5´-CAGCATTGACGACGAGTGG-3´</td>
<td>56</td>
</tr>
<tr>
<td>Claudin 3</td>
<td>Antisense</td>
<td>5´-CCTTAGACGTAGTCCTTGCGG-3´</td>
<td>56</td>
<td>5´-CGCGAGAAGAAGTACACGG-3´</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>5´-CGCGAGAAGAAGTACACGG-3´</td>
<td>56</td>
<td>5´-CGCGAGAAGAAGTACACGG-3´</td>
<td>56</td>
</tr>
<tr>
<td>Claudin 4</td>
<td>Antisense</td>
<td>5´-CAGGCGAGATGCCCATTA-3´</td>
<td>56</td>
<td>5´-CGCATCAGGACTGGCTTTATCTC-3´</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>5´-CGCATCAGGACTGGCTTTATCTC-3´</td>
<td>56</td>
<td>5´-CGCATCAGGACTGGCTTTATCTC-3´</td>
<td>56</td>
</tr>
<tr>
<td>Claudin 7</td>
<td>Antisense</td>
<td>5´-CAGGATGACTAGGGCAGACC-3´</td>
<td>58</td>
<td>5´-AGGCATAATTTTCATCGTG-3´</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>5´-AGGCATAATTTTCATCGTG-3´</td>
<td>58</td>
<td>5´-AGGCATAATTTTCATCGTG-3´</td>
<td>58</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Antisense</td>
<td>5´-GACAAGCTTCCCGTTTCAG-3´</td>
<td>56</td>
<td>5´-CCGGCGACAACATCGTGAC-3´</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>5´-CCGGCGACAACATCGTGAC-3´</td>
<td>56</td>
<td>5´-CCGGCGACAACATCGTGAC-3´</td>
<td>56</td>
</tr>
</tbody>
</table>

4.7 Invasion assays

Invasion of the cells was studied by two different assays, the gel matrix assay and myoma organotypic model developed by Professor Salo (Nurmenniemi et al. 2009) Both original and knockdown cell lines were studied in these models. The Culturex BME cell invasion assay from Trevigen (Gaithersburg, MD) was used as the gel matrix assay, and carried out according to the instructions of the manufacturer.

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. The 4-µm thick slides were stained with hematoxylin and eosin and with anti-human cytokeratin (DakoCytomation, 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) by light microscopy.

4.8 Zymograms

Cells were grown in serum-free media and the media were collected from the cell culture flasks, centrifuged and the supernatants were concentrated with 10.000 cut-off concentration tubes (Millipore) to a final volume of 150 µL. Proteins were measured with DC protein assay-kit (BIO-RAD) and 3 µg of each sample was applied to 1.5 mm 10% SDS polyacrylamide slab gels cast in the presence of 1
mg/mL fluorescently (2-methoxy-2,4-diphenyl-3-[2H]furanone) labeled gelatin (Fluka Ronkonkoma, NY) according to the method described by O’Grady et al. 1984. After electrophoresis, SDS was removed by 2.5% Triton X-100 to reactivate 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 as negative control. The gelatin degradation was visualized under long-wave ultraviolet illumination followed by 0.5% Coomassie Blue R-250 staining of the gels.

4.9 Transepithelial resistance measurement

Transepithelial resistance (TER) was measured from BEAS-2B cells, BEAS-2B slug knockdown cells and BEAS-2B twist knockdown cells. 400,000 cells were applied to wells on a 8W10E- plate (Applied Biophysics). Measurements were made three times with duplicate samples for each cell line (BEAS-2B, BEAS-2B slug knockdown and BEAS-2B twist knockdown). Cells were applied in their own medium on the plate and the plate was placed on a detector in cell culture cupboard. The ECIS 1600R program was started, measuring impedance on each well continually. Medium was changed once in 24 hours. Impedance was measured for 50 hours.

4.10 Statistical methods

The statistical analyses were performed with SPSS for Windows software (SPSS, Chicago, IL, USA). Continuous data were compared using analysis of variance (ANOVA) followed by two-tailed t-tests. Categorical data were compared using Fisher’s exact test. Survival-data were analyzed using the Kaplan-Meier method with the use of the log-rank test and only deaths with lung tumor as the primary cause of death were considered. P-values less than 0.05 were considered statistically significant.

Comparisons between groups were made using Mann-Whitney U-test, Kruskall-Wallis test and t-test. The significance of the associations was determined by Fisher’s exact probability test and correlation analysis. Survival data were studied by Kaplan-Meier method and Cox regression model. Probability values <0.05 were considered significant.
5 Results

5.1 In vivo

5.1.1 Claudins in lung carcinomas

The immunoreactivity of claudins varied in different histological types of lung tumors. Claudin 1 was significantly stronger in the three primary lung epithelial tumors (squamous cell carcinoma, adenocarcinoma and small cell carcinoma) than in the other types of lung tumors and metastases \((p \leq 0.001\) and \(p = 0.003\), respectively). Adenocarcinomas expressed significantly more claudin 2 than small cell carcinomas or carcinoid tumors \((p = 0.05\) and \(p = 0.04\), respectively.) Squamous cell carcinomas expressed also significantly more claudin 2 than small cell carcinomas \((p = 0.05)\). Adenocarcinomas and small cell carcinomas showed significantly stronger claudin 3 positivity than squamous cell carcinomas \((p = \leq 0.001,\) for both) and carcinoid tumors \((p = 0.001\) and \(p = 0.002\), respectively). Claudin 4 was expressed strongly in small cell carcinomas, squamous cell carcinomas and adenocarcinomas. Carcinoid tumors were least positive to claudin 4 compared to small cell carcinomas, adenocarcinomas-, or squamous cell carcinomas \((p = \leq 0.001,\) for all). Compared to other claudins, claudin 5 was clearly the most weakly expressed of all the claudins studied. Strongest positivity was observed for claudin 7, with 91% positive cases in lung tumors. Most of the tumors showed over 90% of strong positivity except for large cell carcinomas and other lung tumors, containing mesenchymal tumors and metastatic tumors to the lung had a lower expression of claudin 7. (Table 9).

Table 9. Immunoreactivity of claudins in different lung carcinomas.

<table>
<thead>
<tr>
<th>Carcinoma Type</th>
<th>Claudin 1</th>
<th>Claudin 2</th>
<th>Claudin 3</th>
<th>Claudin 4</th>
<th>Claudin 5</th>
<th>Claudin 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td>+++</td>
<td>+½</td>
<td>½⁺</td>
<td>+⁺⁺½</td>
<td>+</td>
<td>+++½</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++⁺⁺½</td>
<td>+</td>
<td>+++⁺⁺⁺⁺</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>++</td>
<td>+½</td>
<td>+++</td>
<td>+++⁺⁺½</td>
<td>+½</td>
<td>+++⁺⁺⁺⁺</td>
</tr>
<tr>
<td>Bronchioloalveolar carcinoma</td>
<td>+++⁺½</td>
<td>+++⁺⁺⁺⁺</td>
<td>+++⁺⁺⁺⁺</td>
<td>+++⁺⁺⁺⁺</td>
<td>+</td>
<td>+++⁺⁺⁺⁺</td>
</tr>
<tr>
<td>Small Cell Carcinoma</td>
<td>+++</td>
<td>-</td>
<td>+++⁺⁺⁺⁺</td>
<td>+</td>
<td>+++⁺⁺⁺⁺</td>
<td></td>
</tr>
<tr>
<td>Large cell Carcinoma</td>
<td>+++⁺⁺⁺⁺</td>
<td>-</td>
<td>+++⁺⁺⁺⁺</td>
<td>+</td>
<td>+++⁺⁺⁺⁺</td>
<td></td>
</tr>
<tr>
<td>Carcinoid tumors</td>
<td>-</td>
<td>-</td>
<td>++⁺½</td>
<td>+++⁺⁺⁺⁺</td>
<td>½⁺⁺</td>
<td>+++⁺⁺⁺⁺</td>
</tr>
<tr>
<td>Metastases of other carcinomas</td>
<td>½⁺⁺⁺⁺</td>
<td>+⁺⁺⁺⁺</td>
<td>+⁺⁺⁺⁺</td>
<td>+⁺⁺⁺⁺</td>
<td>-</td>
<td>+++⁺⁺⁺⁺</td>
</tr>
</tbody>
</table>
Associations with tumor size, grade or the presence of regional or distant metastases of any carcinoma type and claudin expressions were not found. Intense claudin 1 and 4 positivity was found more often in carcinoma samples of smokers and ex-smokers compared to non-smokers in whole tumor material (p≤0.001 and p=0.003, respectively). Heavy smoking with more than 40 pack-years was associated with intense claudin 1 (p=0.011), claudin 4 (p=0.050) or claudin 7 (p=0.058, approaching significance) expression compared to non-smokers or smokers with less pack-years. Interestingly in adenocarcinoma, there was a trend for more intense claudin 2 expression with less pack-years and weak or negative expression for those with more pack-years (p=0.053). Impaired lung function was associated with positive claudin 1 expression (p=0.032 for FEV1 and p=0.006 for DCO/VA) and claudin 7 expression (p=0.032 for FEV1).

5.1.2 Survival of lung carcinoma patients

Patients with negative claudin 1 expression had better survival in adenocarcinoma (p=0.044) but in the squamous cell carcinoma group positive claudin 1 expression predicted better survival (p=0.027). Positive claudin 4 staining predicted better survival in patients with adenocarcinomas (p=0.048) but not with squamous cell carcinomas. In squamous cell carcinomas, but not in adenocarcinomas, claudin 7 positivity was associated with a better survival (p=0.011). Other claudins were not associated with survival of the patients (See Paper I Figure 5).

5.1.3 Claudins in COPD

Claudins 3 and 4 were studied in the central bronchi and peripheral bronchioli in the tissue samples. These claudins were studied for their sealing properties in tight junctions. In central bronchi and peripheral bronchioli claudin 3 showed to be strongly expressed in most cases studied. Claudin 4 expression was slightly more often weak than strong in all the cases studied. Claudin 3 was more often strongly expressed in the central bronchus and peripheral bronchioli than claudin 4 (p≤0.001 and p=0.004, respectively). In central airways strong claudin 3 expression was associated with COPD (p=0.049). Claudin 4 did not have significant association with COPD. In peripheral bronchioli there was no association between COPD and claudins 3 or 4.
There was no association between increasing pack-years and the studied claudins in central bronchi or in peripheral bronchioli. In central bronchus smokers, COPD patients and non-smoker had similar claudin 3 and 4 expression. A stronger expression of claudin 3 and 4 in peripheral bronchioli was found in smokers and COPD patients compared to non-smokers (p=0.05 and 0.044, respectively).

5.1.4 Transcription factor snail in lung carcinomas

Nuclear positivity for transcription factor snail was found in (63/279) 22.6% of the cases. The number of snail positive cells was quantitatively assessed in the array samples. The mean number of positive nuclei in tumor cells in all array samples was 6.5 (range 0–703). In squamous cell carcinomas of the lung, the mean number with nuclear positivity was 2.5 (range 0–58), in adenocarcinoma it was 8.1 (range 0–624), in small cell carcinoma 31.2 (range 0–326) and in large cell carcinoma 19.4 (range 0–183). In contrast, carcinoid tumors showed no positivity. In primary lung tumors, the difference in the amount of nuclear positivity was significant between squamous cell carcinoma and small cell carcinoma (p=0.047), and between adenocarcinoma and small cell carcinoma (p=0.013).

Nuclear expression of snail was high in cases with low claudin 1 expression compared to cases with high claudin 1 expression (p=0.023). A similar tendency was seen with claudin 3 expression but it was not statistically significant. Claudin 5 expression was increased when there was no snail expression (p=0.034) and this tendency was also seen with claudin 7 (p=0.062). There were no significant associations between snail and other claudins.

Snail expression was associated with patient survival (see Paper II Figure 2). In whole tumor material patients with negative snail expression had better predicted survival than patients with strong snail expression (p≤0.001). Patents with strong snail expression had only half the life expectancy of patients without snail expression (p=0.001). In two major subgroups, adenocarcinomas and squamous cell carcinomas, the survival of the patients was similar as in whole material (p=0.004 and p=0.005, respectively). Snail had an independent prognostic value in the whole tumor group and in two major subgroups according to Cox multivariate regression analysis. Smoking and presence of metastases did not change the risk factor. Large size of tumor increased the risk of shorter survival (p≤0.001).
5.1.5 Transcription factors in COPD

Transcription factors slug and twist were evaluated by nuclear expression from the tissue samples. Transcription factors expression was mainly weak in central bronchi and in peripheral bronchioli in all the cases studied. Slug and twist were inversely associated with claudin 3 in central bronchi and in peripheral bronchioli ($p \leq 0.001$ for both in both cases). Claudin 4 was also inversely associated with slug and twist in central bronchi ($p=0.009$ and $p=0.001$, respectively) and in peripheral bronchioli ($p=0.018$ and $p \leq 0.001$, respectively).

Weak slug expression was found in smokers and COPD patients compared to non-smokers ($p=0.026$). Reduced slug expression was also associated with increasing pack-years in peripheral bronchioli ($p=0.026$). This was not seen in central bronchi. Twist was not associated with smoking.

5.1.6 Matrix metalloproteinases in lung carcinomas

Matrix metalloproteinase (MMP) 2 and 9 were studied in the histological sample of lung carcinomas (Figure 3). MMP 2 was found to be expressed more in squamous cell carcinomas than in adenocarcinomas and bronchioalveolar carcinomas ($p=0.02$ and $p=0.01$, respectively). Metastatic carcinomas express significantly lower amounts of MMP 2 than squamous cell carcinomas ($p=0.002$). These kinds of differences in MMP2 expression were not seen with other carcinoma groups. MMP 9 expression was not associated with any of the carcinoma groups.

MMPs did not have any associations with the TNM status. MMP 2 positivity was associated with worse prognosis ($p=0.022$): the same tendency was seen with MMP 9. In adenocarcinomas weak MMP 2 expression predicted better survival compared to strong expression of MMP 2 ($p=0.030$).

Patients with lung tumors and metastases showing strong MMP 2 expression had longer smoking histories ($p=0.006$) and more pack-years ($p=0.031$). These patients were also somewhat older compared to the non-smokers ($p=0.028$). Smoking history did not have any significant influence on MMP 9 expression, although patients with tumors strongly expressing MMP 9 had more pack-years ($p=0.030$).

There was a positive association between the levels of MMP2 and claudin 1 ($p=0.016$) and claudin 2 ($p=0.015$) and between MMP9 and claudin 2 ($p=0.001$).
MMP2 and MMP9 also showed a positive association with slug (p=0.033, p=0.006, respectively).

Fig. 4. MMP immunoreactivity in lung carcinomas. A. MMP 2 immunoreactivity in squamous cell carcinoma and B. MMP 9 immunoreactivity in adenocarcinoma.

5.2 In vitro

5.2.1 Claudins in cell lines

Claudin expressions were studied by Real Time -PCR in different cell lines. BEAS-2B line was considered as a non-neoplastic human bronchial epithelial cell line and other cell lines were compared to it. The expression of claudin 1 was strong in most of the cell lines studied. Relative to BEAS-2B cell lines, only A427 showed stronger claudin 1 mRNA expression. Claudin 2 expression was very weak in the BEAS-2B cells. In cell lines A549 and SK-MES-1 the expression of claudin 2 was strong. Equally strong claudin 3 mRNA expression was found in A427 and in SK-MES-1 cell lines. SK-MES-1 expressed claudin 4 mRNA more strongly than BEAS-2B and the A427 line exhibited stronger expression of claudin 7 mRNA than BEAS-2B cells. Claudin 5 was not found in these cell lines despite several attempts. Two malignant cells lines (SK-MES-1 and SK-LU-1) were chosen for further studies, because these cell lines were close to the squamous cell carcinoma and adenocarcinoma, respectively. These two lines had similar differences in claudin expression as was found in the human squamous cell carcinoma and adenocarcinoma samples.
5.2.2 Smoking and claudins

Chosen cell lines were exposed to cigarette smoke extract (CSE) for different time periods. In BEAS 2-B cells levels of claudins 2, 3, 4 and 7 increased after 2 hours of exposure. At the 2-hour time point a major elevation of all studied claudins was seen in SK-LU1 cells whereas SK-MES1 cells did not display any evident changes. After 6 hours’ exposure, there was reduced expression of all claudins in BEAS-2B and SK-LU1 cells, except for claudin 2 expression which declined more slowly in the latter cell line. Again, SK-MES1 cells did not reveal any evident changes. Expression levels of claudins 2, 3, 4 and 7 increased again in BEAS-2B cells at the 24-hour time point but declined at the 48-hour time point, except, for claudin 4 expression which continued to increase. In SK-LU1 cells, there were no such changes except for claudin 4 at the 24-hour time point, showing a decline, as did the other claudins at the 48-hour time point. In SK-MES1 cells claudin 1 and 3 decreased slightly at the 24- and 48-hour time points but there was a slight increase in claudin 4 and 7 expression at the 48-hour time point.

5.2.3 Effect of the transcription factor knockdown in cell lines

Snail knockdown

After knockdown the expression and functioning of snail protein was prevented in the cell lines. Snail knockdown cells did not express snail protein in immunohistochemical dyeing. Knockdown of snail changed the growth time of the cells into twice that of the original cell lines, and the cells were more easily detached from the culture surfaces than the original cell lines. The cells were more star-shaped than flat and round epithelial cells. The effects on cells’ physical changes were not dependent on the malignancy of the cells. Snail knockdown in BEAS-2B cells decreased the expressions of claudins 2 and 7 (p=0.001). Knockdown of snail did not have any effect on claudin 1, 3 and 4 in knockdown cells. Snail knockdown did not alter the expression of claudins in the malignant SK-LU-1 cell line. In the other malignant cell line, SK-MES-1, the snail knockdown increased claudin 3, 4 (p=0.011) and 7 (p=0.005) mRNA expression. Compared to the other malignant cell line, SK-LU-1, claudin 1 mRNA expression was decreased in snail knockdown cells (p=0.005).
Slug knockdown

Slug knockdown in the cell lines changed the cell growth pattern similarly as in snail knockdown cells. Slug knockdown cells were more easily detached from culture surfaces than snail knockdown cells. Slug knockdown was studied only in BEAS-2B cells. Silenced slug protein expression was detected in the cells by immunocytochemistry. The level of claudin 3 mRNA expression was doubled in the slug knockdown cell line when compared to normal BEAS-2B cells (p=0.001). Claudin 4 expression was three-fold higher in knockdown cell lines than in normal BEAS-2B cells (p≤0.001). The slug knockdown cell line reached confluence slowly and when growing on microscope slides the cells formed islets instead of a confluent cell layer.

Twist knockdown

As in snail and slug knockdown lines the growth rate was slower and cell were easily detached. The effect of twist knockdown was studied on claudins 3 and 4 only in the BEAS-2B cell line. Claudin 3 mRNA expression was doubled in twist the knockdown cell line compared to normal BEAS-2B cell line (p≤0.001). Claudin 4 expression was three-fold higher in knockdown cell lines compared to normal BEAS-2B cell line (p=0.001).

5.2.4 Expression of MMP 2 and MMP 9 proteins

Wild Type (WT)-cell line BEAS-2B produced proMMP 2 and proMMP 9 proteins. The knockdown cell line produced more proMMP 9 and proMMP2 protein than the original BEAS-2B cell line. In WT-malignant cells proMMP 2 was expressed, but not MMP 9 proteins. More proMMP2 was produced in knockdown cells than in original malignant cell lines. Both malignant knockdown cell lines produced various forms of the proMMP 9 protein. Gelatinolytic activities were studied for negative control after EDTA treatment and gelatinolytic activities disappeared in treatment.

5.2.5 Invasion

The invasion properties were reduced in snail knockdown cells in all the cell lines studied and by both invasion assay models. In the gel matrix assay, the invasion
properties of WT-SK-MES-1 and WT-SK-LU-1 were higher than in the WT-BEAS-2B cells. Snail knockdown in cells made them less invasive than the WT-cell lines. The SK-MES-1 cell line was more invasive than SK-LU-1: this could be demonstrated in both assays. In the myoma invasion assay, the results were similar in BEAS-2B cells and SK-MES-1 cells, i.e., snail knockdown cell lines were less invasive than WT-cell lines, and in BEAS-2B cells invasion depth difference was statistically significant (p=0.003). WT-SK-LU-1 cells were more invasive than snail knockdown cell line in gel matrix assay (p=0.004). Curiously, the WT-SK-LU-1 snail knockdown cell line was slightly more invasive than the original cell line in the myoma invasion assay, but the difference was not statistically significant.

5.2.6 Cigarette smoke exposure

In BEAS-2B cells claudin 4 was 1.5-fold increased in exposed cells compared to unexposed control cells at the 6-h time point. At the 24-h time point expression was increased to 2.6 fold in exposed cells (p≥0.001). Claudin 3 expression was nearly the same in exposed and control cells at the 6-h time point. Expression decreased at 24-h.

Claudin 3 expression was lower in exposed BEAS-2B slug knockdown cells than in control cells at 6h timepoint and it was increased to 2.1-fold at 24-h (p=0.012). Claudin 4 expression was also increased from the 6-h time point to the 24-h time point.

Exposed BEAS-2B twist knockdown cells expressed 1.2 and 1.3 times more claudin 3 and 4, respectively, than control cells at the 6-h time point. Expression of both claudins was increased in exposed cells at the 24-h time point but was not significantly changed.

5.2.7 Transepithelial resistance

Transepithelial resistance (TER) was measured at 50 hours. Cells were attached for a few hours and TER was measured. Confluence was reached in a few hours and barrier functions were developed between 10h and 40h. After 40h the resistance remained at the same level for the rest of the measurement. In WT-BEAS-2B cells TER was lower than in twist knockdown cells. TER for slug knockdown cells could not be used as a reliable measure as the cells did not form
an even cell layer and detached very easily from the plate surface during medium exchange. For TER measurement the cell layer must be confluent; otherwise the measured resistance is not accurate since the device measures the resistance from the bottom of the well itself.
6 Discussion

In this thesis, the WHO classification from 2005 has been used with lung carcinomas divided into non-small cell lung carcinomas and small cell carcinomas (Travis et al. 2004). Lung carcinomas were previously divided into two main groups, small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC). Today they are divided into non-neuroendocrine and neuroendocrine tumors. Two main types of NSCLC as well as non-neuroendocrine lung tumors are adenocarcinomas and squamous cell carcinomas. Small cell carcinoma is the most common type of the neuroendocrine tumors. It is usually found and diagnosed late and patients rarely undergo surgical operations.

Epitheliomesenchymal transition enables the carcinoma cells to move and invade into neighboring tissues. Transcription factors are involved in EMT and they influence the expression of the adhesion proteins in epithelial cells. Up-regulation of the MMPs is also one feature of the EMT. MMP 2 and 9 are important in cancer cell invasion since these enzymes degrade basement membrane components. The effects of the transcription factors have to some extent been studied in lung carcinomas but not much in COPD.

Claudins are tight junction proteins that are localized on the apical site of the epithelial cells. They might play a role in EMT since some claudins, such as claudin 1, are regulated by EMT-related transcription factors (Ikenouchi et al. 2003). These proteins are the backbone of the tight junctions. Claudins are found in tissue type specific compositions on TJs. Normal airway epithelial cells express various claudins (Soini 2005).

In this thesis expression of claudins 1, 2, 3, 4, 5 and 7 as well as transcription factor snail and MMPs 2 and 9 were studied in 344 lung carcinoma samples and the results were associated with clinical and histological data. Claudins 3 and 4 were studied in central bronchi and peripheral bronchioli of 65 patients suffering from COPD. Transcription factors slug and twist were also studied in these samples. In vivo studies were complemented by in vitro experiments with a normal bronchial cell line and two malignant carcinoma cell lines.

6.1 Claudins in lung carcinomas

Claudin expressions showed significant differences between different histological types of lung carcinoma. Our findings are in line with previous studies that have also shown this in lung carcinoma as well as with studies on carcinomas of other
Squamous cell carcinoma and adenocarcinoma express strongly different claudins and differ mainly in their expression of claudin 3, which has also been shown in other studies (Moldvay et al. 2007) and in tumors of other sites such as esophageal carcinoma (Takala et al. 2007). Claudin 2 expression was higher in squamous cell carcinoma and adenocarcinoma than in other types of lung carcinomas. Squamous cell carcinoma, adenocarcinoma and other types of primary lung tumors could roughly be separated with these two claudins. Large cell carcinomas and squamous cell carcinoma can be separated by claudin 7 immunohistochemistry because its expression was significantly lower in large cell carcinoma.

Claudin expression was slightly lower in metastatic carcinomas than in primary pulmonary carcinomas, especially squamous cell carcinomas and adenocarcinomas. Metastases expressed significantly lower amounts of claudin 1 and 7. These results are in line with previous studies showing alteration in adhesion molecules such as claudins 4 and 7 during the metastatic process (Erin et al. 2009). In line with this observation the results of this thesis reflect a tendency of metastatic cells to lose some of their adhesion features in the process of metastatic spread. This observation is related to EMT, which is directed by transcriptional factors that have been shown to down-regulate adhesion molecules and some claudins (Martinez-Estrada et al. 2006). Sarcomas metastatic to the lung had an even lower expression level of claudins than carcinoid tumors. In previous studies it has also been shown that the expression of claudins is very low in mesenchymal tumors (Soini 2005), a finding which is reflected also in this study.

Claudins 1, 4 and 7 appeared to have prognostic significance in lung carcinomas. Claudin expression has been found to have associations with prognosis also in carcinomas originating from other organs than the lung. Claudin 7 is down-regulated in breast carcinoma, correlating to histological grade and metastasis (Sauer et al. 2005). Claudins 3 and 4 are up-regulated in ovarian cancer (Morin 2005) and this over-expression leads to increased invasion, motility and cell survival (Agarwal et al. 2005). In gastric carcinoma, loss of claudin 3 expression is associated with a poor prognosis (Soini et al. 2006) while in renal clear cell carcinoma, up-regulation of claudin 1 expression leads to a poor prognosis (Fritzsche et al. 2008). Interestingly, these authors found claudin 1 to be a good prognostic marker for renal papillary carcinoma, which was quite opposite to the results of clear cell carcinoma (Fritzsche et al. 2008). According to
our results, strong claudin 1 and 7 expression predicted a better survival in squamous cell carcinoma and strong claudin 4 in adenocarcinoma. Strong claudin 1 expression predicted shorter lifetime in the adenocarcinoma group. Chao et al. found that claudin 1 expression was low in lung adenocarcinoma patients with shorter survival. Chao et al looked at overall survival (Chao et al. 2009); in our study the causes of death from the Finnish cancer register were available and in survival analysis only lung cancer deaths were calculated.

Claudin expressions were compared to the histological data of the samples and low claudin 4 expression was associated with a positive pN-status. This refers to the importance of claudin 4 in determining invasiveness, metastatic potential and survival also in lung adenocarcinoma, as has been discovered to be the case in gastric cancer (Ohtani et al. 2009). Low claudin 7 expression was associated with large tumor size in the whole material and with shorter survival for squamous cell carcinoma. Claudin 7 could play a role in regional growth of lung tumors and in squamous cell carcinoma, but also other factors determine prognosis.

The significance of claudins in the pathogenesis of tobacco-related lung cancer is seen in the correlation between smoked pack-years of the patients and claudin expression in lung tumors. Expression of claudins 1 and 4 was more often found in lung tumors of smokers and ex-smokers: furthermore, expression of claudins 1, 4 and 7 was associated with more pack-years in squamous cell carcinoma. Conversely, low claudin 2 expression was associated with more pack-years in adenocarcinoma.

6.2 Claudins in COPD

Claudins 3 and 4 were studied in the central bronchi and peripheral bronchioli of normal lung, smokers’ lungs and COPD-patients’ lungs. Samples from these patients were collected from the lung cancer resection. These selected TJ proteins are expressed in normal airway epithelia (Kaarteenaho-Wiik & Soini 2009). Both of these claudins also have sealing function in TJs. This is interesting because in the exacerbations of COPD bacteria and inflammatory substances move through airway epithelium inducing destruction in smaller airways and alveoli. Destruction of peripheral lung tissue reduces lung functions. Claudin 3 was strongly expressed in central bronchi and peripheral bronchioli in the whole material. In peripheral bronchioli of the smokers and COPD patients claudin 3 expression was higher than in non-smokers. This result is in line with a recently
published study in mice, where increased claudin 3 was found to be an early marker of smoking-induced epithelial injury (Cuzic et al. 2012). Claudin 4 was generally more weakly expressed than claudin 3, but the expression pattern was similar to claudin 3.

Smoking is the leading cause of COPD and induces the remodelling of the epithelia in airways. In COPD patients there is goblet cell metaplasia and mucous hyperplasia (Thorley & Tetley 2007). Goblet cells are also found in peripheral airways where these cells are rarely found. In the large airways ciliated cells are replaced by serous and bronchiolar exocrine cells. Smoking also has potential to initiate EMT in lungs (Sohal et al. 2011). EMT does not automatically mean invasion. EMT is also present in injury repair. In paper III claudins were increased in non-smokers. It is possible that there is ongoing repair and remodeling in the airways of lungs. This can also mean tighter TJ’s between epithelial cells and a better defense mechanism against foreign matter such as tobacco smoke and bacteria. Smoking inhibits the ability of the mesenchymal cells to promote repair in airway epithelia (Rennard 2006).

6.3 Transcription factors snail, slug and twist

Snail expression was evaluated by nuclear staining. About one fourth of the samples were positive for snail depending on the histology of the tumors. Snail expression was strongest in the small cell carcinoma, which is also the most aggressive type of lung carcinomas. Snail could not be found in carcinoid tumors. Snail down-regulates the expression of E-cadherin and cytokeratin 18. Snail also up-regulates the expression of the vimentin. These changes lead to the mesenchymal phenotype of the tumor cells and decrease the cohesion of the cells (Blechschmidt et al. 2008, Hipp et al. 2008). In this study it was found that snail down-regulated the expressions of claudin 1 and 5. A similar tendency was seen with claudins 3 and 7. Up-regulation of snail expression was associated with poor prognosis and metastatic potential of tumors in ovarian or head and neck carcinomas (Blechschmidt et al. 2008, Yang et al. 2007). Also in this thesis increased expression of snail was associated with poor prognosis in the whole material and in two major carcinoma subgroups, in squamous cell carcinomas and in adenocarcinomas. Snail expression had an independent prognostic value.

Snail is an important part of the EMT and tumor invasion. It has also been found to influence the expression of MMP. Up-regulation of the matrix
metalloproteases (MMPs) such as MMP 2 and MMP 9 is one feature of EMT (De Wever et al. 2008, Orlichenko & Radisky 2008). Association between snail and MMP 2 or 9 was not found in the tissue material. This might due to the small number of snail-positive cases.

Similarly to snail, the expression of slug and twist was evaluated only by nuclear expression. Slug and twist expression was studied in the central bronchi and peripheral bronchioli in the COPD material. These transcription factors were found to be mainly negative or weakly expressed in the material. In peripheral bronchioli the expression of slug was higher in the non-smokers than in smokers and COPD patients. This was not seen in the central bronchi. Transcription factor twist was not associated with smoking status in either central bronchi or peripheral bronchioli. The studied transcription factors, such as snail, are involved in down-regulation of adhesion proteins, such as E-cadherin (Peinado et al. 2004), and TJ proteins such as claudin 1 (Martinez-Estrada et al. 2006). This study showed that both slug and twist were inversely associated with claudin 3 and 4 in central bronchi and peripheral bronchioli. Up-regulation of claudins and down-regulation of slug was more prominent in the cases with COPD but was also found in healthy-smokers. Smoking triggers slug down-regulation leading to permeability changes in the airway epithelium. This could be one component in the cascade leading to the structural changes in TJs in airway epithelia observed in COPD. Acute cigarette smoke exposure has been found to cause short-term increased permeability effect in airways in vitro (Olivera et al. 2007). In vivo long-term smoking might have an effect on the permanent morphological and functional changes in the tight junctions between epithelial cells, EMT, COPD and carcinoma development.

6.4 In vitro studies

Claudins were expressed in the normal BEAS-2B cell line and the two malignant cell lines, SK-LU-1 (adenocarcinomatous features) and SK-MES-1 (originating from squamous cell carcinoma). These cell lines were selected for further study because they expressed all the claudins except for claudin 5. This claudin is more often found in endothelial than in epithelial cells. Consequently, these cell lines did not express this claudin at all. Claudin 2 was weakly expressed in the BEAS-2B cells and SK-LU-1 cells and strongly expressed in SK-MES-1 cells. Other claudins were strongly expressed in the cell lines.
Since smoking is a risk factor for development of lung carcinomas and COPD and COPD is also a risk factor for lung cancer, the effects of cigarette smoke extract were studied in malignant as well as non-malignant cell lines. In a normal cell line, BEAS-2B cells, expression of the claudins was increased and decreased in between different time points. After short exposure to cigarette smoke extract the expression of claudins SK-LU-1 cells increased, but returned to pre-exposure level with longer exposure to cigarette smoke extract. Malignant cells were more resistant than BEAS-2B cells and exposure might not change the survival of these cells. BEAS-2B cells, on the other hand, undergo a change in their tight junction permeability, which is seen in the changes of claudin expression. Change in the tight junction permeability is a defense mechanism in airway epithelia. Epithelial cells are protected against the toxic components in cigarette smoke. Certain claudins were more often found in smokers’ and ex-smokers’ lungs and with more pack-years in squamous cell carcinoma.

Transcription factor snail was studied in the lung carcinoma cell lines and normal bronchial epithelial cells by inhibiting the protein expression with short hairpin-RNA (shRNA). Snail has been found to down-regulate the expression of adhesion protein E-cadherin (Ohkubo & Ozawa 2004) and TJ proteins occludin (Ikenouchi et al. 2003) and claudin 1 (Martinez-Estrada et al. 2006). Snail can regulate the above mentioned proteins independently. Snail protein expression in the cells disappeared after knockdown immucytochemical dyeing. As a control, non-target transfection was made just to check if the vector would change the behavior of the cell. Non-target cells grew and functioned like the original cells. At transcriptional level claudin 2 and 7 expressions were decreased in the BEAS-2B cells after knockdown. Expression of other claudins was increased after knockdown. In the malignant cell line SK-MES-1 inhibiting of the snail did not alter claudin 2 expression, but claudin 3, 4 and 7 expression was increased while that of claudin 1 decreased. Knockdown of the transcription factors did not affect the expression of the claudins in the SK-LU-1 cell line. Increased claudin expression in the carcinoma cell line was in line with the immunohistochemical studies. Decreased expression was surprising, but this might be due to the different conditions in vitro and in vivo. The BEAS-2B cell line is immortalized and this might also have some influence on the behavior of the cells. In mouse cell lines the expressions of the claudins were down-regulated by induced snail expression (Ikenouchi et al. 2003).
Knockdown of the snail reduced the invasion properties in the studied cell lines in both invasion experiments. In the myoma invasion experiment the WT-SK-LU-1 cells were slightly more invasive than the knockdown line. This result was not statistically significant. Also BEAS-2B cells were invasive in this experiment. Myoma tissue in the invasion experiments was fresh human myoma which was washed before use. After washing there might be some substances that attract the cells to move in tissue. Since it is natural tissue there are holes and cracks in the surface of the tissue where the cells can attach when applied on the myomas. BEAS-2B cells are, as mentioned before, immortalized cells that might have some invasion properties in optimal environment. The medium used in the experiments with BEAS-2B cells is serum-free and supports human primary cell growth. Malignant lines were cultured in different media than BEAS-2B. This invasion assay is not the best possible model because it uses fresh human tissue, albeit thoroughly washed, but the interaction with the medium for BEAS-2B cells cannot be predicted. The results of the other matrix invasion assay are in line with the histological data. Strong snail expression was associated with poor prognosis of the patients and significant risk for spread of the tumor. This assay is based on a matrix which is originates from mouse. It might not interact with the medium as the myoma tissue probably does. Strong snail expression is associated with high invasiveness in in vitro cell lines. These findings are in line with patient data where strong snail expression is associated with poor prognosis.

MMPs have been found to be up-regulated in EMT. MMP 2 and 9 are important in cancer cell invasion. Expression of these MMPs did not change much in BEAS-2B cells. ProMMP 2 expression was increased in the knockdown cells compared to the original BEAS-2B cells. In malignant cell lines MMP 9 was not present, but in the knockdown line there were several proMMP 9 forms. In oral tongue carcinoma cells the silencing of the MMP 9 increases their invasion properties (Vilen et al. 2013). This observation is in line with the results of this thesis. In both malignant cell lines proMMP2 expression decreased in knockdown lines compared to the original lines.

Two other transcription factors, slug and twist, were studied only in the BEAS-2B cell line as a reference to the COPD material. BEAS-2B cells formed an epithelial-like confluent monolayer in cell culture and the cells looked like flat epithelial cells. Knockdown of the transcription factors changed the appearance of the cell to star-like. Knockdown cells reached the confluent stage later than normal BEAS-2B cells. Twist knockdown cells also formed a monolayer which detached much more easily from culture pads compared to BEAS-2B cells. Slug
knockdown cells grew in more islet-like monolayers than an epithelial-like confluent monolayer. Slug knockdown could have more influence on cadherins in the adherent junctions than on claudins in TJs (Shih et al. 2005). Cells were stuck together but were very easy to detach from the culture pads. Knockdown of slug and twist increased the claudin 3 and 4 mRNA expression in the BEAS-2B cells. This is in line with the finding of the TER. TER was higher in the twist knockdown cells than in WT- BEAS-2B cells. Claudins 3 and 4 influence the tightness of the TJs between the cells. TER increases when cells are closer together. In slug knockdown this could not be seen perhaps due to the islet-like growth pattern of the cells. TER cannot be measured accurately by ECIS when the cells do not grow in a consistent monolayer. ECIS measures the impedance from the whole area of the measurement chamber and it requires a confluent epithelial layer.

CSE exposure up-regulated the expression of claudin 3 in slug knockdown cells, but not claudin 4 expression to a significant degree. In the twist knockdown cells no differences were seen after exposure.
7 Conclusions

Based on the results of the thesis, the major observations and conclusions are as follows:

1. Expression of claudins 1, 2, 3, 4, 5 and 7 varied in histological subgroups of lung carcinomas. Squamous cell lung carcinoma and adenocarcinoma showed the strongest immunoreactivity of the studied claudins. Large variation in claudin immunoreactivity was seen in other types of lung carcinomas such as carcinoid tumors, large cell carcinoma and small cell carcinoma.

2. Strong claudin 1 expression associated with better survival in two main types of lung carcinomas. This was also found in expression of claudin 4 in adenocarcinomas and in expression of claudin 7 in squamous cell carcinomas.

3. Smoking was associated with claudin 1, 4 and 7 expression. The expression of these claudins was more intense in smokers with a high number of pack-years than in non-smokers or smokers with less pack-years. Intense claudin 1 and 4 expression was also found more often in ex-smokers than in non-smokers.

4. Claudins 3 and 4 were expressed in the epithelial tissue of bronchus and bronchioli of normal lung. Claudin 3 expression was stronger in bronchus than in bronchioli in normal lung of non-smokers compared to smokers and COPD patients. Claudin 4 expression pattern was similar to that of claudin 3, but somewhat weaker. Strong claudin 3 expression on bronchi was associated with COPD.

5. EMT-associated transcription factor snail was strongly expressed in small cell carcinoma and absent in carcinoid tumors. Strong expression of snail was associated with poor prognosis of lung carcinoma.

6. Transcription factor slug was mainly weak in bronchus and bronchioli of normal lung. Slug expression was higher in bronchioli of non-smokers compared to smokers or COPD patients. Slug was inversely associated with claudins 3 and 4 in bronchi and bronchioli.

7. Transcription factor twist was mainly negative in bronchi and bronchioli. Twist was not associated with smoking, but was inversely associated with claudins 3 and 4 in bronchi and bronchioli.

8. Non-malignant and malignant cell lines expressed all other claudins studied, but not claudin 5, which is an endothelial-associated protein. Cigarette smoke exposure first increased and after longer exposure decreased claudin
expression in non-malignant cell line suggesting an active repair mechanism and remodeling of the tight junctions between airway epithelial cells. In adenocarcinoma-like cell line the mRNA expression of claudins increased in short exposure, but not with longer exposure times. Exposure had no effect on squamous cell carcinoma-like malignant cell line.

9. Knockdown of transcription factor snail in non-malignant and malignant cell lines decreased the mRNA of certain claudins (CLD 2 and 7 in non-malignant line and CLD 1 in squamous cell carcinoma-like cell line), but increased the mRNA expression of other claudins. Knockdown had no effect on adenocarcinoma-like cell line.

10. Snail knockdown decreased the invasion in cell lines and increased the proMMP 9 expression in malignant cell lines.

11. Knockdown of transcription factors slug and twist in non-malignant cell line increased the mRNA expression of all claudins studied. CSE exposure had no effect on twist knockdown cells, but up-regulated the mRNA expression of claudin 3 in slug knockdown cells.

12. Transepithelial resistance was higher in twist knockdown cell line than in normal epithelial cell line, indicating that tight junctions are more closed up and thus less permeable.
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