Lung cancer

Lung cancer is the leading cause of cancer death worldwide [1]. Due to a decline in smoking habits the incidence of lung cancer has been declining recently in many countries [2]. Even though smoking is responsible for over 90% of the cases of lung cancer, some other etiologic factors, like exposure to asbestos, radon or to heavy metals also plays some role [1]. In lung cancer treated by surgery the five year survival is approximately 10-70% depending on the stage of the tumors [2]. These figures may, however, change in the future due to the appearance of more targeted therapy options based on molecular biology [2].

Lung carcinoma is divided in two main groups; non-small cell (NSCC) and small cell carcinoma (SCC) [1]. The NSCC consists of two main histologic types, squamous cell carcinoma and adenocarcinoma [1, 2]. Due to changes in smoking habits the frequency of adenocarcinoma has increased in relation to squamous cell carcinoma so that it is now the most common histologic type of lung tumors [2]. A rarer type of NSCC is large cell carcinoma, a tumor type not expressing features of keratinisation or mucin production characteristic of squamous or adenocarcinomas, respectively [1, 2].

SCC represent an aggressive neuroendocrine tumor of the lung which is strongly associated with smoking [1, 3]. It has a dismal prognosis and patients suffering from the disease cannot be operated on due to the rapid spread of the tumor [3]. It consists of small tumor cells with round or elongated nuclei, finely dispersed chromatin and frequent mitoses and apoptotic figures [1, 3]. The tumor expresses cytokeratins and neuroendocrine markers like chromogranin and synaptophysin which is in line with the epithelial and neuroendocrine differentiation of the tumor cells [3].

Due to accumulation of data on lung cancer...
there is a constant need to re-evaluate the classification of lung tumors. Recently, the classification of adenocarcinomas have been changed. Previously, the so called bronchioloalveolar carcinoma was classified as a special subtype of lung adenocarcinoma with tumor cells spreading along the alveolar septa [1]. According to recent concepts, the term bronchioloalveolar carcinoma has been rejected and bronchioloalveolar growth is now called a lepidiform growth pattern [2]. A pure lepidiform growth pattern without invasion is regarded as an in situ type of growth representing a precancerous or local noninvasive cancerous growth [2]. In case of a small area of invasion (< 5mm), the tumor is called a minimally invasive adenocarcinoma [2]. Other growth patterns, such as tubular, papillary, micropapillary and solid, always represent invasive growth and adenocarcinomas are now classified according to which growth pattern predominates in the tumor [2]. Tumors may also be pneumocytic or columnar type depending on whether they derive from the alveolar pneumocytes or epithelium of the bronchi [2]. Finally, some specific histologic types of adenocarcinoma, like enteric, colloid or fetal type are classified as separate entities [2].

There is also an increased knowledge on the molecular biologic features of lung carcinoma. In adenocarcinoma EGFR and Ras mutations are found in 10-30% of cases. EGFR mutations can be used in targeted treatment by blocking kinase activation induced by constant EGFR activation with specific tyrosine kinase inhibitors [2]. BRAF mutations in codon B600E commonly found in melanocytic lesions are found in adenocarcinomas as well as EML1-ALK translocation which both are found in 5% of the cases [2]. Mutations in RB and NF1 are found in 10% of cases and p53 mutations in 30-50% of the cases [4].

It is important to distinguish squamous cell carcinoma from adenocarcinoma since the former does not respond to EGFR antikinase treatment and patients may suffer from fatal complications [2]. Ras mutations are found in 15-30% and MET amplifications in 10% of non small cell carcinomas [5]. Some non-small cell carcinomas have PTEN mutations [5, 6]. Small cell carcinomas and large cell neuroendocrine carcinomas express c-kit, and they harbor c-myc amplifications and p53 and ras mutations, and a loss of p16 and RB expression [6, 7].

**Tight junctions**

Tight junctions are cellular structures located at the apicobasal region of epithelial cell membranes [8]. In freeze fraction electron microscopy they can be seen to form a beltlike structure around the cell [9]. Tight junctions have several functions. They regulate the electrical and solute permeability of the paracellular space and are thus important for the fluid and electrolyte balance in the body [8]. They also have a fence function segregating the apicobasal part of the cell membrane from other regions [8]. They also contribute to the polarity of the cell [8]. Tight junctions and their permeability are important in the formation of the blood brain barrier, blood retinal barrier and blood testis barrier [4]. In this way they also form secluded areas in the body which are not reached by the immunological system [4]. Breakage of these barriers may lead to autoimmune reactions. Tight junctions can also be considered as parts of the innate immune system. They also form barriers against bacteria and viruses and prevent them from invading the subepithelial tissues [4]. On the other hand, some bacteria and viruses may use components of tight junctions as gates for the entry to deeper tissues [4].

**Tight junction components**

Occludin was the first molecular component discovered in tight junctions. It is a 65 kDa protein with two extracellular loops and two splice variants and was first suspected to be responsible for tight junctional formation and permeability [10]. In fact, occludin transfected in human fibroblasts induces a weak cellular adhesion and its overexpression in tight junction containing cells increases the number of paracellular strands, influences transepithelial resistance and paracellular flux of molecules between cells [11]. However, embryonic stem cells lacking occludin are able to form tight junctional structures indicating that occludin is not a substantial component required for their assembly [8]. Occludin knockout mice, however, display a number of deficiencies and diseases such as testicular atrophy, atrophic gastritis, male infertility, salivary gland dysfunction, osteoporosis and brain calcifications [12]. Occludin has several phosphorylation sites and it is the phosphorylated molecule which locates to the tight junctions [10]. Phosphorylation is induced by
several mechanisms, such as protein kinases [10]. Extracellular domains of occludin attach it to the tight junction and to other tight junction proteins and influences the tight junctional permeability to some extent. The fact that occluding null mice do not have problems related to paracellular permeability is due to the redundancy of other proteins to substitute for its loss [10, 12].

Tricellulin is a 63.6 kDa tight junctional protein mainly present in contacts between three epithelial cell membranes but it is also found in bicellular contacts but to a lesser degree [11, 13]. In bicellular junctions it decreases solute and electrical permeability while in tricellular junctions there is no influence on electrical permeability but there is a diminished permeability for macromolecules [14]. Tricellulin mutations are associated with hearing loss but do not influence renal, respiratory or gastrointestinal tight junctions to a significant degree [12]. Knockdown of tricellulin results in derangements of tight junctional assembly and occludin influences the localization of tricellulin at tight junctions [11]. Occludin and tricellulin have structural similarities, both bind ZO1 at their structurally similar carboxyterminal domain, and they also contain a MARVEL domain like MarvelD3, yet another tight junctional protein which associates with both tricellulin and occludins [12].

The junctional adhesion molecules (JAMs) belong to an immunoglobulin superfamily. They are 36-41 kDa proteins and can be divided in three groups; JAM-A, JAM-B and JAM-C [15, 16]. All contain one transmembrane domain, an intracellular carboxyterminal part containing the PDZ domain for attachments for ZO-1, for instance, and extracellular immunoglobulin like domains [17]. JAMs are present in epithelial and endothelial cells, on leucocytes and platelets. They are able to interact with integrins influencing interactions between endothelial cells and leucocytes and transmigration of leucocytes [16-18]. JAM-A may also be present as a soluble form in serum inhibiting transmigration of blood cells from the circulation to tissues [18]. Down-regulation of JAMs increases invasion and metastatic potential of cancer cells and they are downregulated in EMT [19, 20]. They also modulate paracellular permeability and contribute to the formation of blood brain barrier or blood testis barrier [21, 22]. Some viruses, like reovirus, may use JAMs as receptors for cell invasion [17].

Claudins

Claudins are essential components of tight junctions. There are 27 types of claudins in mammals, but claudin 13 is missing from humans [23, 24]. Claudins are divided in classic and non-classic claudins which is based on their sequence similarity [23]. Classic claudins include claudins 1-10, 14, 15, 17 and 19 and non-classic claudins 11-13, 16, 18 and 20-24 [23]. Claudins are responsible for the solute and electrolyte permeability of the tight junctions, consequently, their expression may vary in different epithelia [4]. They regulate the solute permeability in nephrons, for example, thus claudin expression varies in different parts of kidney tubules [25]. Similarly, there is a variation in the expression of claudins in different parts of the gut [26].

Claudins are found in epithelial, mesothelial, glial and endothelial cells [27-29]. Their molecular weight is about 20 kDa and in cell membranes they are composed of two extracellular loops, EL1 and EL2, four transmembrane domains, one small 20 amino acid long intracellular part between the two extracellular loops and the intracellular aminoterminal and carboxyterminal ends [4, 23]. In the carboxyterminal ends there are domains recognizing the PDZ domains of ZO1, ZO2 and ZO3 [4]. The larger EL1 loop influences paracellular charge selectivity and harbours the co-receptor site for hepatitis C [4]. The smaller EL2 binds the claudin molecule to the corresponding one of the neighbouring cell [4]. It also contains the oligomerisation site and receptor site for clostridium perfringens enterotoxin (CPE) [4].

Claudins associate to one and other laterally and vertically [23]. Vertical association takes place between claudins of neighbouring cells and lateral binding between claudins in the same cell [23]. The exact mechanisms of the association is not known, but claudins may form either homo- or heterodimers. However, not every claudin can associate with each other; claudins 1 and 2, for instance, cannot heterodimerize [23]. The association of claudins between neighbouring cells in tight junctions determines the paracellular permeability of cell layers [23]. Claudin 4, for instance, has a sealing function.
while claudin 2 generally increases the paracellular permeability [23].

**Claudins and tight junctional proteins in the lung**

In lung tissue, mRNA for several claudins has been detected. Claudins 1, 2, 3, 4, 5, 7, 8 and 18 have been detected in bronchial cells, the same claudins except for claudin 2 in type 1 alveolar and the same except for claudin 8 in type 2 alveolar cells [4]. Mesothelial cells are found to express claudins 1, 2, 3, 5 and 7 [4]. Lung endothelial cells express claudin 5 and ZO-1 [30]. In lung embryonic development bronchial epithelial cells express claudins 1, 3, 4, 5 and 7 [31]. During lung tissue maturation expression of claudin 5 decreases in bronchial epithelium [31]. In mature bronchial epithelium claudin 1 and 4 are found in the basal epithelium while claudin 4 and 5 are found in cylindrical cells [30]. Matured alveolar cells lack claudin 1 protein expression [31]. The relative expression of claudin 1 mRNA, however, increases during lung maturation in the whole lung tissue while expression of claudin 3 and 4 mRNAs are highest in the canalicular period [31]. Even though protein expression of claudin 1 and 5 cannot be detected in mature alveolar pneumocytes of the lung, cells displaying squamous or bronchial metaplasia in interstitial lung diseases express claudin 1 and 5 and also show increased expression of claudins 2, 3, 4 and 7 [32]. Claudin expression in bronchial squamous metaplasia has not, however, been studied.

Expression of claudins in cells does not necessarily indicate the presence of tight junctions in cells. The presence of occludin usually indicates a tight junctional structure [8]. Occludin is present in bronchial airway cells but may dissociate from the membranous location due to irritation caused by hydrogen peroxide [33]. Bronchial epithelium also expresses cingulin, ZO-1, ZO-2, and ZO-3 [30, 34]. Occludin, ZO-1, ZO-2, ZO-3, cingulin and JAM-1 are expressed in alveolar cells [30, 35, 36].

**Tight junction proteins in lung tumors and metastatic disease**

**Occludin, tricellulin and JAMs**

In an immunohistochemical study of lung tumors occludin was present in adenocarcinomas but it was not present in squamous cell carcinomas, small or large cell carcinomas [37]. Another study on mRNA expression of occludin indicated an increased mRNA expression in adenocarcinomas and a decreased expression of mRNA in squamous cell carcinomas compared to normal lung or bronchial tissue [30]. This discrepancy may be due to the fact that tight junctions are not formed in squamous cell carcinomas even though occludin mRNA is synthesized, alternatively immunohistochemistry might fail to detect trace concentrations of occludin in a narrowed tight junctional structure. In favor of the last interpretation an ultrastructural study indicated presence of tight junctional structures in small cell carcinoma in addition to adenocarcinoma [38].

Underexpression of occludin has been shown to lead to an elevated level of progression and metastatic potential in breast, ovarian, endometrial and liver carcinoma [39-42]. Downregulation of occludin might not be the reason for the heightened metastatic potential but be due to activation of EMT and a consequent downregulation of adhesion associated proteins. In colon carcinoma cells, for instance, activation of EMT by MEK-1 lead to activation of EMT related transcription factors and downregulation of E-cadherin, occludin and ZO1 [43]. Similar observations were also seen in ovarian carcinoma [40]. In metastatic lung carcinoma, occludin expression has not been studied. In lung adenocarcinoma, overexpression of claudin 1 leads to an increase in occludin along with ZO1 indicating a less invasive phenotype [44].

There are no studies on tricellulin expression in pulmonary tumors. In hepatic fibrolamellar carcinoma tricellulin expression was diminished compared to normal liver [45]. Similarly, tricellulin was downregulated in tonsillar squamous cell carcinoma [46]. Loss of tricellulin expression may be associated with development of an EMT phenotype as has been shown in gastric carcinoma [47].

JAMs have been detected in lung alveolar and bronchial cells in the basolateral membranes [48]. There are few studies on JAM expression in lung carcinoma. JAM-1 mRNA was on about the same level in squamous cell carcinoma compared to lung parenchyma but lower than in bronchial cells [40]. On the other hand, expres-
sion was higher in adenocarcinoma than in lung parenchyma but slightly lower than in bronchial cells [40]. In a study on several malignant cell lines JAM-C was found to be highly expressed in highly metastatic cell lines while JAM-A or JAM-B did not show such tendency [49]. Knockdown of JAM-C leads to a suppression of invasive and metastatic potential of the JAM-C transfected HT1080 fibrosarcoma cell line [49].

Claudins

Claudins show a diverse expression pattern and have a variable influence on tumor behavior in different types of epithelial neoplasia. In breast carcinoma, lowered claudin 1, 2 and 7 expression associates with a more aggressive breast carcinomas [50-53]. On the other hand, high claudin 4 expression was found to be associated with a worse survival and aggressive disease in breast carcinoma [54]. In gastric carcinoma, low claudin 4 expression was associated with a worse survival of the patients [55]. In colon cancer, increased claudin 2 is associated with tumor progression and tumor growth [56]. In nasopharyngeal carcinoma decreased claudin 4 and increased claudin 7 were significantly associated with metastatic disease [57]. In ovarian carcinoma high claudin 3 and 7 expression in cells of the effusion fluid predicted a poor survival [58].

In lung carcinomas claudin expression has been studied by immunohistochemistry and mRNA expression. In the study of Paschoud et al., squamous cell carcinomas and adenocarcinomas differed in their expression of claudin 1 and 5, the latter expressing claudin 5 but not claudin 1 while the former was negative for claudin 5 and positive for claudin 1 [40]. In the qRT-PCR experiments similar trends were observed.

In the study of Moldway et al., significant differences in claudin expression could be found between small cell lung carcinoma and adenocarcinoma and squamous cell carcinoma in immunohistochemical claudin 2 expression [59]. There were also significant differences between squamous cell carcinoma and adenocarcinoma, the former expressing less claudin 3, and squamous cell carcinoma and small cell carcinoma with claudins 3 and 4, and between squamous cell carcinoma and adenocarcinoma with claudin 7 [59] which were in agreement with some previous studies [60, 61]. Additionally, carcinoid tumors generally showed a low expression of claudins and in this respect were different from other lung tumors [59, 61].

In the study of Moldway et al, the qRT-PCR results on tumor tissue appeared similar to those observed in immunohistochemical analyses [59]. Small cell lung carcinoma had a 16 fold higher level of claudin 3 mRNA expression than normal lung tissue, while claudin 4 mRNA was upregulated to 3.4 fold level in squamous, adenocarcinoma and small cell carcinoma [59]. Both adeno- and squamous cell carcinoma showed a slight downregulation of claudin 1 mRNA compared to normal lung and squamous cell carcinomas had a 2.7 fold level of claudin 1 mRNA than adenocarcinomas [59].

Chao et al. studied claudin 1 expression in adenocarcinoma and found that a low expression of claudin 1 was associated with a worse survival in these tumors both by immunohistochemistry and mRNA expression [44]. Transfection experiments in lung carcinoma cell lines changed them to less invasive and aggressive [44]. Regardless of this claudin 1 overexpressing cells were able to activate MMP2 [44]. Also claudins 2-5 have been shown to activate MMPs in some studies [62, 63].

In immunohistochemical expression of claudins 1, 2, 3, 4, 5 and 7 on lung carcinomas based on our studies the three primary lung epithelial tumors (SQCC, AC and SCC) showed significantly stronger claudin 1 positivity than other types of lung tumors [60, 61] (Figure 1 and 2). Squamous cell carcinomas and adenocarcinomas (including bronchioloalveolar carcinomas) had significantly more cases with claudin 2 positivity than small cell carcinomas or carcinoid tumors. Squamous cell and large cell carcinomas showed a lower claudin 3 positivity compared to adenocarcinomas, small cell carcinomas, bronchioloalveolar carcinomas, carcinoid tumors and adenosquamous carcinomas.

Small cell carcinomas and adenocarcinomas showed a stronger claudin 4 positivity compared to squamous cell carcinomas, large cell carcinomas, carcinoid tumors, large cell carcinomas, however, showing a higher frequency of positivity compared with carcinoid tumors (Figure 3).
Adenocarcinomas displayed most claudin 5 positive cases, followed by squamous cell carcinomas and small cell carcinomas (Figure 4). Others tumors were mainly weak or negative in regard to claudin 5 expression. Compared to other claudins studied, claudin 5 was clearly the most weakly expressed and expression was significantly lower compared to the expression of other claudins. Interestingly also, bronchioloalveolar carcinomas had a similar expression of claudins as adenocarcinomas in general except for the fact that they had a lower expression of claudin 5. This indicates that in situ type of adenocarcinomas as bronchioloalveolar carcinomas mostly are according to a modern classification [1, 2] show a lower expression of this claudin than invasive tumors suggesting that upregulation of claudin 5 might indicate lung adenocarcinoma progression.

The strongest positivity for all claudins studied, on the other hand, was for claudin 7 (Figure 5). Large cell carcinomas and carcinoid tumors had, however, a lower expression.

Based on these expressions, a schematic presentation displaying a differential diagnosis of lung tumors based on claudin 1, 2, 3, 4, 5 and 7 can be proposed (Figure 6). Such a presenta-
Claudin 18 is expressed in pulmonary tissues and in the gastrointestinal tract. Consequently, it is expressed in GI derived tumors, such as pancreatic carcinomas, gastric carcinomas and colon carcinomas, the latter, however, displaying a low frequency [64-67]. In our study on lung carcinomas, claudin 18 was especially expressed in adenocarcinomas displaying a 20% positivity and the expression was specifically found in tumors of non-smokers [68]. Claudin 10 was reminiscent in its expression to claudin 18 in this respect also being displayed in approximately 20% of cases [68]. Claudin 18 was also associated with a better prognosis [68].

**Regulation of claudin expression in cancer**

EMT is proposed to be detrimental in the invasive and metastatic process [69, 70]. In EMT epithelial tumor cells lose their epithelial traits, such as E-cadherin expression and display fibroblast type traits such as smooth muscle actin and vimentin [69, 70]. In EMT, downregulation of claudins, like claudin 1, has been observed [71]. EMT is regulated by transcription factors, like slug, snail, twist or zeb1 [69, 70]. In fact, claudin 1 has been shown to be downregulated by snail and slug [72].

In a study on lung tumors, epithelial metastases showed a 50-70% expression of claudins 1, 2, 3, 4 and 5 while the expression of claudin 7 was about 90% [60]. In metastases, twist and zeb1 were higher in pulmonary metastases than in primary lung tumors and there was an inverse

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**Figure 5.** Claudin 7 in pulmonary adenocarcinoma with a papillary growth pattern. Carcinoma cells are surrounded by a claudin 7 positive zone.

**Figure 6.** Differential diagnosis of lung tumors based on claudin 1, 2, 3, 4, 5 and 7 immunoreactivity. (SQCC=squamous cell carcinoma, AC = adenocarcinoma, SCC=small cell carcinoma, LCC=large cell carcinoma, EM=Pulmonary epithelial metastases).

**Figure 7.** Expression of claudins 1, 2, 3, 4, 5 and 7 in relation to adenocarcinoma growth pattern. The solid and mucinous type growth pattern shows a reduction in claudin expression. (Lep=lepidiform, Ac=acinar, Pap=papillary, Muc=mucinous, So=solid).
association between zeb1 and claudins 1 and 2 and twist and claudin 5 [73]. Like in breast carcinoma, the expression of twist and zeb1 is low in lung carcinoma representing less than 15% of the cases [73]. However, EMT related transcription factors surely play a role and influence claudin expression in lung tumors.

Many cancer genes, however, also influence claudin expression and are able to modify their presence in tumor cells. Over 30% of lung cancer show EGFR overexpression and EGFR has been found to upregulate claudins 2, 3 and 4 and downregulate claudin 2 [75, 76]. Similarly K-ras which is present in lung carcinoma, upregulates claudins 1, 4 and 7 but downregulates claudin 2 [77]. Claudin 18 has been shown to be regulated by protein kinases (PKC) in pancreatic carcinoma [78]. Expression is also affected by the methylation status of the gene [78]. Similarly, other claudins may be affected by epigenetic mechanisms. In gastric cancer, hypermethylation of claudin 11 promoter region leads to downregulation of claudin 11 [79]. Also histone modification plays a role. Modification of histone proteins H3 and H4 in claudin 3 and 4 promoter regions in ovarian carcinoma leads to a repression of gene expression [80]. Epigenetic modification of claudins in lung tumors have not, however, been studied, neither is there any information on microRNA regulation of claudin gene expression in general.

In conclusion the influence of tight junctions and their proteins on lung carcinoma development and progression is complex. There are some differences in the expression of tight junctional proteins, such as claudins, in different histological types of lung tumors, but it seems not to be of great assistance in the differential diagnosis of special tumor types. During EMT the expression of tight junctional proteins would be expected to decline and even though there are some indications of this in some studies, tight junctional proteins may many times be overexpressed in lung tumors. This could be related to other effects of tight junctional proteins, such as claudins, which may activate matrix metalloproteinases and in this way promote tumor spread. Much research needs still to be done to elucidate the role of tight junctions in lung neoplasia.

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