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Review

The membrane-bound mucins: From cell signalling to transcriptional regulation and expression in epithelial cancers

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ARTICLE INFO

ABSTRACT

The membrane-bound mucins belong to an ever-increasing family of O-glycoproteins. Based on their structure and localization at the cell surface they are thought to play important biological roles in cell–cell and cell–matrix interactions, in cell signalling and in modulating biological properties of cancer cells. Among them, MUC1 and MUC4 mucins are best characterized. Their altered expression in cancer (overexpression in the respiratory, gastro-intestinal, urogenital and hepato-biliary tracts) indicates an important role for these membrane-bound mucins in tumour progression, metastasis, cancer cell resistance to chemotherapeutics drugs and as specific markers of epithelial cancer cells. Some mechanisms responsible for MUC1 and MUC4 role in tumour cell properties have been deciphered recently. However, much remains to be done in order to understand the molecular mechanisms and signalling pathways that control the expression of membrane-bound mucins during the different steps of tumour progression toward adenocarcinoma and evaluate their potential as prognostic/diagnostic markers and as therapeutic tools. In this review we focus on the molecular mechanisms and signalling pathways known to control the expression of membrane-bound mucins in cancer. We will discuss the mechanisms of regulation at the promoter level (including genetic and epigenetic modifications) that may be responsible for the mucin altered pattern of expression in epithelial cancers.

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1. Introduction

Mucins belong to a heterogeneous family of large O-glycoproteins composed of a long peptidic chain called apomucin on which are linked hundreds of oligosaccharidic chains. Based on biochemical studies, mucins were initially defined as high-molecular weight (MW) molecules secreted by epithelia, able to form viscoelastic gels and responsible for rheological properties of mucus. Advent of molecular biology in the nineties allowed identification of high MW O-glycoproteins with structural characteristics of mucins which contained a transmembrane (TM) domain. Today, the family of membrane-bound mucins includes MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20 and MUC21 [1]. Among them, MUC1 and MUC4 are best characterized. Based on their structure and localization they are thought to play important biological roles in cell–cell and cell-extracellular matrix interactions, in cell signalling and in biological properties of cancer cells [2–6]. Moreover, their specific pattern of expression during the different steps of tumour progression toward adenocarcinoma suggests that they play important roles in tumourigenesis and that they are specific markers of epithelial cancer cells. For all these reasons, membrane-bound mucins stay under intense investigation as both potent new biomarkers and therapeutic targets in epithelial cancers. On another hand, the family of secreted mucins, gel-forming components of viscoelastic mucus gels protecting the epithelia, includes mucins MUC2, MUC5AC, MUC5B, MUC6, and MUC19 [7]. Their main function is to participate in mucus formation by forming a tridimensional network *via* oligomerization domains to protect underlying epithelia against various injuries (inflammation, bacteria, virus, pollutants, pH, etc). MUC7 and MUC9 are smaller secreted mucins that do not oligomerize [8,9].

A better understanding of the molecular structure of regulatory regions as well as mechanisms governing mucin expression is also mandatory if one wants to assign direct roles to mucins in carcinogenesis and better understand their influence on the biological properties of the tumour cell. Studies aiming at deciphering the signalling pathways will allow identification of potential therapeutic targets with the ultimate goal to restore normal mucin expression at the cell surface. Moreover the use of mucin promoters in gene-based therapy is under investigation and may provide new biological tools [2]. Animal models will also help define *in vivo* the

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roles of membrane-bound mucins in pathophysiological situations and help the scientific community determine whether mucin altered expression is a consequence of epithelium alteration or actively contributes to histological changes that promote carcinogenesis.

2. MUC1 and MUC4 have a cell- and tissue-specific pattern of expression

In this part of the review we will only describe the pattern of expression of membrane-bound mucins (Table 1). For expression of secreted mucins in the same cancers, see excellent following reviews [10–12].

2.1. Respiratory tract

The mucin gene expression pattern in normal airways and lung is complex [13]. Mucin genes are expressed in an array of epithelial cells exhibiting various phenotypes: MUC1 in submucosal glands and MUC1, MUC4, and MUC13 in the surface epithelium. In distal bronchioles, Clara cells express MUC1 and MUC4. The alveolar type II epithelial cells express the MUC1 glycoprotein whereas MUC4 gene expression is not detected in normal type II pneumocytes but is found in type II pneumocyte hyperplasia [13].

In atypical adenomatous hyperplasia (AAH), the preinvasive lesion of peripheral lung adenocarcinomas, high level of MUC1 expression and very low levels of other mucins have been shown by immunohistochemistry (IHC) (Table 1). MUC1 expression was significantly decreased in the progression from AAH through nonmucinous bronchioloalveolar carcinoma (BAC) to invasive adenocarcinoma while depolarized MUC1 was significantly increased [14].

In hyperplasia (basal cell/goblet cell), metaplasia and dysplasia, the pattern of qualitative expression of mucin genes is similar to

that determined for normal mucosae. Nevertheless quantitative variations of MUC4 expression levels are observed. No expression of MUC3 is found in squamous lesions as in the normal respiratory surface epithelium [15].

Lung cancers are largely classified into two major groups based on their histopathologic differences: non-small-cell lung cancer (NSCLC), which is further divided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma; and small-cell lung carcinoma. The recent studies on mucin expression in lung cancer have been conducted to establish a relationship between the expression of any particular mucin and the histo-logic subtype [10,15–17]. Lung adenocarcinomas express mucin mRNAs which are expressed in normal respiratory mucosa (MUC1, MUC4) and MUC3 mRNAs which are not detected in normal lung by in situ hybridization (ISH) [16]. Nonmucinous type of BAC and non BAC type adenocarcinomas share the constant expression of MUC1 and MUC4, and variable expression of MUC3 [16,18]. Among adenocarcinomas, the mucinous type of BAC (m-BAC) has a particular pattern of mucin gene expression since all mucin genes are expressed. Coexpression of MUC1 and MUC3 is constant. Coexpression of MUC4 is very frequent. This complex but homogeneous expression pattern in m-BAC is in agreement with the great cellular homogeneity of this type of adenocarcinoma [16,18].

2.2. Gastro-intestinal tract

2.2.1. Oesophagus

The normal oesophageal epithelium is considered as nonmucus-secreting. MUC1 and MUC4 are the main mucins expressed both at the mRNA and protein levels in the stratified squamous epithelium [19,20]. Recently, expression of the membrane-bound mucin MUC20 has also been found, without any precision concerning staining location [21].

Table 1
Expression pattern of the membrane-bound mucins MUC1, MUC3 and MUC4 in normal, premalignant and malignant epithelia. + = expressed; ++/+++ = overexpressed; ↓ = decrease of expression. All data presented are at the protein level unless RT-PCR (mRNA) is indicated.

	Normal	Metaplasia/Hyperplasia/Dysplasia	Cancer
Lung	Surface epithelial cells MUC1+, MUC4+ Submucosal gland MUC1+ Clara cells MUC1+, MUC4+ Alveolar type II MUC1+	Atypical adenomatous hyperplasia (AAH) MUC1+++	Lung adenocarcinoma MUC1+, MUC4+ Nonmucinous bronchoalveolar carcinoma (BAC) MUC1↓, MUC4+
Oesophagus	Squamous epithelial cells MUC1+, MUC4+	Barrett's oesophagus MUC4↓, MUC1 n.d. (conflicting reports)	Squamous cell cancer MUC1+, MUC4+ Adenocarcinoma MUC1+++ (conflicting reports), MUC4+++
Stomach	MUC1+	Intestinal metaplasia; MUC1↓ (good prognosis), MUC3+++ (bad prognosis)	
Small intestine	Brünner's gland MUC1+, MUC4+ Duodenum MUC1+ (RT-PCR) Columnar cells MUC4+ Vater's ampulla MUC1+, MUC4+		Small intestine adenocarcinoma MUC1+++ (poor differentiation) Ampullary adenocarcinoma MUC1+++ (poor prognosis)
Colon	Columnar cells MUC4+ Goblet cells MUC4+	Low-grade dysplasia MUC3+++ (early stage marker)	Adenocarcinoma MUC1+++ (late stage marker), MUC4↓
Pancreas	Ductal cells MUC1+	Pancreatic intraepithelial neoplasia; PanIN1A MUC1++, MUC4+; PanIN3 MUC1++; MUC4++ Intraductal papillary mucinous neoplasm MUC1+++ (bad prognosis)	Adenocarcinoma MUC1+++, MUC4+++
Hepatobiliary tract	MUC1 weak, MUC3+++		Gallbladder carcinoma MUC1+ Cholangiocarcinoma MUC1+, MUC4+ Intrahepatic cholangiocarcinoma (ICC) MUC1++

MUC1 and MUC4 were found in the mid layers of the stratified squamous epithelium, whereas their relevant protein core was predominantly found in the more superficial layers. MUC1 and MUC4 are predominantly found at the membrane epithelial cells. However cytoplasmic expression can be found. In squamous cell carcinoma, MUC1 and MUC4 expression is maintained as in normal tissues, mainly on the cell membrane of surface epithelial cells, but also in the cytoplasm in several cases [22–26]. Mucin gene expression greatly varies and accompanies cell differentiation in the process of Barrett's oesophagus characterized by incomplete intestinal metaplasia (IM) with presence of goblet cells, and expresses a mixture of gastric (MUC5AC, MUC6) and intestinal (MUC1, MUC2, MUC3) mucins [20,27–30]. MUC1 and MUC4 mucin genes are also expressed in cardiac and fundic metaplasia whereas MUC3 is scarcely detected. MUC1 is expressed in both superficial and in deeper glands of Barrett's metaplasia. MUC1 gene expression is not found in IM. MUC3 is focally expressed in the superficial epithelium in non-goblet cells adjacent to goblet cells. Mild MUC4 expression is detected in the basal region of columnar cells of the surface epithelium and in the crypt epithelium [19,20]. In our experience this staining concerns both goblet and non-goblet cells and is predominantly cytoplasmic [31,32]. To sum up, between normal mucosa and Barrett's oesophagus, mRNA and protein studies conversely reported higher expression of MUC3 and lower expression of MUC4 [20,33,34]. Reports concerning MUC1 mucin gene expression are conflicting. Both over/neo-expression and decrease of expression have been described [19,20,33,35,36]. During the progression from dysplasia to adenocarcinoma, down-regulation of MUC3 is observed whereas MUC4 is raised [19,20,29,33,35]. Reports regarding MUC1 are still conflicting. Immunohistochemical expression of MUC1 and MUC3 showed that both were absent in high-grade dysplasia, whereas they were reexpressed in adenocarcinoma. In most studies, increase of MUC1 between dysplasia and adenocarcinoma was reported [20,35,37,38]. However, Endo et al. [30] found that patients expressing MUC1 showed a decreased level of MUC1 between dysplasia and adenocarcinoma. Between metaplasia and adeno-carcinoma, MUC3 was mostly down-regulated. MUC4 expression mostly rose from metaplasia to dysplasia and adenocarcinoma. To sum up, MUC1, MUC3 and MUC4 are observed in 22–100%, 33–66% and 42–78% of oesophageal adenocarcinoma [19,20,29,30,35–37,39]. MUC1 immunohistochemical staining was either membra-nous [29,37], cytoplasmic [39] or both [31]. Several authors have now proposed that mucin genes may be considered as reliable phenotypic markers to follow the metaplastic process that leads to Barrett's adenocarcinoma [19,20,34,40]. However, clinical impact of MUC1 and MUC4 expression has been recently evaluated and showed no prognostic value in Barrett's associated oesophageal adenocarcinoma [32]. Recent works in our laboratory showed that MUC1 and MUC4 are targets of bile acids (taurocholic (TC), deoxy-cholic (DC), taurodeoxycholic (TDC), taurochenodeoxycholic (TCDC) and glycocholic (GC) acids, and sodium glycocholate (GNa)) which high gastric concentration has been observed in patients with Barrett's oesophagus. It has been suggested that incomplete IM may be a response to reflux of gastroduodenal contents and in particular to bile acids. This effect is mediated via PI3K signalling pathway and involves HNF-1α and HNF-4α transcription factors direct interaction with MUC1 and MUC4 promoters [31,41,42]

2.2.2. Stomach

Normal stomach mucosa is characterized by expression of MUC1, MUC5AC and MUC6 mucins [12,43,44]. Apical MUC1 expression occurs in the superficial and foveolar epithelium and mucous neck zone cells of gastric mucosa. MUC3 and MUC4 are generally absent from normal gastric mucosa [45]. Mucins appeared as key molecules for the classification of IM of the stomach. Complete IM is characterized by expression of MUC2 in goblet cells and decreased expression of MUC1, MUC5AC and MUC6 [46]. Patients who maintain high immunoreactivity for anti-MUC1 antibody have a better prognosis, whereas those with an increase in anti-MUC3 immunoreactivity have a poorer prognosis, as judged by tumour size, serosal invasion, and metastasis [47] (Table 1).

2.2.3. Small intestine

MUC1 is detected in Brünner's glands [48] (Table 1). By RT-PCR, all mucins including MUC1 are detected in normal duodenum, whereas all mucins but MUC2 are found in Vater's ampulla [49]. In the normal duodenum, MUC1 apomucin is not detected whereas MUC4 is found in columnar cells and Brünner's glands [49]. In Vater's ampulla, MUC1 mucin is absent whereas MUC4 is expressed in columnar cells [49]. MUC17 is expressed throughout the entire intestinal tract with the highest expression in the duodenum [50,51] and was shown to be expressed at the membrane surface in an N-glycosylation dependent manner [52]. In mouse jejunum, Malmberg and collaborators showed a strong MUC17 expression in the brushborder that is dependent of Pdzk1 protein via a PDZ interaction motif in the C-terminal tail of MUC17 [53]

MUC1 is differentially expressed in ampullary adenocarcinoma compared to normal duodenum and its overexpression was confirmed by IHC on tissue microarrays [54]. MUC1 was reported in 26/38 ampullary adenocarcinomas and was related to lymph node metastasis, advanced stage of TNM (T: tumour, N: nodes, M: metastasis) and to a poorer prognosis [55]. As in other localizations, invasive micropapillary carcinomas of the ampullo-pan-creatobiliary region display a reversal of cell polarity with MUC1 labelling observed in the stroma-facing surfaces of the micro-papillary clusters [56]. A recent study on 30 small intestinal adenocarcinomas (SIAs) showed frequent focal staining of MUC1. Moreover, poorly differentiated SIAs tend to express MUC1 more frequently than well- and moderately-differentiated SIAs [57].

2.2.4. Colon

The two membrane-bound mucins MUC3 and MUC4 are expressed by columnar and colonic goblet cells [58–60] (Table 1). MUC3 is expressed in the supra- and perinuclear cytoplasm of goblet cells and also in columnar cells, being more abundant in the superficial epithelium compared with the crypt bases of the colon [58,59]. According to an mRNA analysis, MUC3 is expressed to a lower level in colon compared to small intestine [59]. MUC1 was inconsistently observed at the apical membrane within the crypt base in normal colon, its detection being enhanced by periodate oxydation [61]. MUC12 membrane mucin is also expressed in normal colon [62]. A recent study about expression of membrane-bound mucins in normal colon confirmed high expression of MUC1, MUC3, MUC4, MUC13 and MUC12 [38]. They were also found in normal and tumour rectum with similar expression levels.

Analysis of human tissues demonstrated that some mucin-associated peptides were significantly decreased or increased during the adenoma-carcinoma sequence (Table 1). Indeed, MUC1 is a late stage marker of the adenoma-carcinoma sequence, being highly expressed in high-grade dysplasia, whereas MUC3 is an early stage marker, being mostly detected in low-grade dysplasia [38,63]. Interestingly a study also showed that MUC1 expression did not differ between de novo carcinomas and those developing from adenomas and that its expression was found in the course of colorectal carcinoma development when p53 is overexpressed and apoptosis prominent [64]. MUC12 membrane-bound mucin was found consistently down-regulated in colon carcinomas (6/15) [62] as well as MUC4 and MUC13 [38,65]. MUC17 was shown to be expressed in colon cancer cells [53].

2.3. Pancreas

MUC1 is the main membrane-bound mucin expressed in normal pancreas (Table 1). The labelling concerns acinar cells, at the cytoplasmic level, and ductal cells at the apical level [66]. Therefore, MUC1 has been used as a marker of ductal cells in several studies [67,68].

Despite controversial initial results, no MUC4 transcript or apomucin are found in normal pancreatic tissues [66]. However a number of nonneoplastic duct lesions such as atrophic ducts, or ducts in the setting of an inflammatory reaction, show expression of MUC4.

Clinical, pathological and genetics studies have identified three different preneoplastic lesions of the duct as precursors of pancreatic ductal adenocarcinoma (PDAC). These lesions are pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). Among these lesions PanIN are the most frequent and best characterized both at the morphological and molecular levels [69].

Pancreatic carcinogenesis is characterized by an altered pattern of mucin expression at different stages of tumour progression. Membrane-bound mucin production has been detected as early as PanIN1A stage: neoexpression of MUC4, increase of MUC1 expression (Table 1). The PanIN3 stage, previously named in situ carcinoma, is characterized by an increase of MUC4 and MUC1 expression and occurrence of MUC3 [70].

A gradual expression of MUC4 during pancreatic carcinogenesis has been demonstrated by IHC: 17% of PanIN1A, 36% of PanIN2 and 85% of PanIN3 express MUC4 [71]. In true PDAC, the prevalence of MUC4 apomucin expression reaches 83–89% [71,72]. These results are in agreement with gene expression profile studies which showed that MUC4 belongs to the most differentially expressed genes in PDAC [73]. The MUC1 mucin is more frequently expressed by PanIN lesions (around 70% of the cases) compared to normal pancreas. Around 80% of PDAC samples still express MUC1 [74]. IPMN is thought to be another precursor of PDAC. It originates in the main pancreatic duct and is characterized by a massive dilatation of the ducts or cyst formation and hypersecretion of mucins. Usually IPMN has a good clinical course but 10–20% of IPMN are invasive carcinomas with worse prognosis [75]. IPMN expressing MUC1 correspond to invasive carcinoma with a short survival rate and bad prognosis whereas those that express MUC5AC correspond to slow-growing adenoma with good prognosis.

2.4. Hepatobiliary tract

Strong expression of MUC3 mRNA in all biliary epithelial cells and hepatocytes, and weak expression of MUC1 in biliary epithelial cells has been described [48,70,76] (Table 1). No expression of MUC4 mRNA was detected. By IHC, expression of MUC1 and MUC4 apomucins, was found in cholangiocarcinomas whereas gallbladder carcinomas express MUC1. The increased expression of MUC1 and MUC4 is related to the aggressive behaviour of carcinoma cells and poorer prognosis [77]. Intrahepatic cholangiocarcinoma (ICC) can be classified into mass-forming type, periductal-infiltrating type or intraductal-growth type, with a better prognosis for the intraductal-growth type [77,78]. MUC1 is expressed in all types in more than 70% of tumours [78]. Mass-forming type ICC is associated with higher MUC1 expression [79]. In biliary tract carcinoma (BTC), high expression of MUC1 is associated with positive detection of carcinomatous cells by bile juice cytology [80]. In patients with biliary obstruction, MUC4 expression in bile and MUC5AC expression in serum are highly specific markers for BTC [81]. In gallbladder carcinomas, MUC1 expression increases with histological dedifferentiation [82]. Biliary papillomatosis is a rare disease that is characterized by multiple numerous papillary adenomas in the biliary tree, in majority of cases associated with biliary papillary or mucinous adenocarcinoma [83]. Marked expression of MUC1 is rather frequent in biliary papillomatosis with carcinoma and intrahepatic biliary papillary carcinoma compared with biliary papillomatosis alone [84].

3. Mucins as diagnostic and/or prognostic factors in epithelial cancers

From expression studies described in the first part of this review it is obvious that mucin gene and protein regulation is profoundly altered in epithelial cancers (Table 1). Early abnormal detection of mucins during carcinogenetic sequence or mucins showing onco-foetal pattern of expression raises the question of whether these molecules may be useful diagnostic markers for the clinician. Moreover, prognostic value of mucins has been evaluated in many carcinomas and in some cases already proved useful [85]. However, their prognostic value is often better when included in a panel of biomarkers. Mucins are also used as phenotypic markers to classify tumours as well as to identify primary site of metastatic tumours.

MUC1 and MUC4 are also often associated with tumour progression and metastasis, even though opposite activity has been described in upper and lower aerodigestive tracts. For most of the cancers prospective studies are required to decipher some conflicting data about the utility of mucins as biomarkers. The problems of the specificity of the antibodies, of the standardized immunostaining protocols and strict criteria for staining interpretation are also important issues still to improve. Use of gene expression profiling, which allows investigation of thousands of genes at once, in the clinic is also being debated at this time and the relevance of molecular signatures to patient care still questionable [86]. The interpretation of these molecular signatures remains a challenge but their use to phenotype tumours is very promising although still in its early-age [87]. Next to studies about molecular pathogenesis of cancer, pre-clinical mouse models of cancer potentially represent effective strategies for cancer diagnosis and treatment [88]. The biggest challenge with animal models being to find the best model that reproduces all the morphological and molecular events found in humans.

Overexpression of MUC1 represents a marker of aggressive biological behaviour in nonsmall lung, gastric, colorectal carcinomas. Some studies already use MUC1 as a therapeutic target in immunotherapy or gene-based therapy [2,89,90]. MUC1 could also be used as a prognostic marker. In gastric cancer, patients who maintain a high reactivity for MUC1 have a better prognosis [91]. Moreover, MUC1 expression in IPMN is related to a worse prognosis whereas MUC5AC secreted mucin is associated with a better survival rate [92].

In general MUC4 expression in carcinomas depends on histological type and differentiation grade of the tumour. MUC4 overexpression is associated with more aggressiveness and increased metastases in breast cancer, extrahepatic bile duct carcinoma, and cholangiocarcinoma [93–95]. Conversely, improved patient survival was associated with MUC4 expression in ovarian cancer, mucoepidermoid carcinoma of the salivary glands, and squamous cell carcinoma of the upper aerodigestive tract [96–98]. In NSCLCs, MUC4 expression was associated with an improved survival in early stage adenocarcinomas. In the lung its diagnostic value was also demonstrated to distinguish between epithelial mesothelioma and lung adenocarcinoma [99]. Thus the relationship between MUC4 expression and tumour behaviour is organ-dependent. This means that correlation to patient survival will have to be assessed in every type of carcinoma where MUC4 is overexpressed.

4. Transcriptional regulation of MUC1 in epithelial cancer cells

Because membrane-bound mucins are often overexpressed in carcinomas as described in the first part of this review, investigators have started to study their transcriptional regulation in order to use them (i) as targets and control their expression and (ii) as biological tools in human gene therapy.

MUC1 transcriptional regulation has been extensively studied in mammary cancer cells, an organ in which its overexpression is routinely used as a tumour marker and thought to contribute to disease progression.

The MUC1 promoter is GC-rich and contains a typical TATA box located 25 nucleotides upstream of the transcriptional start site (Fig. 1) [100]. 2.9 kb of the 5′-flanking region has been published so far (GenBank accession numbers: L06162 and X69118). It is characterized by numerous binding sites for specificity protein-1 (Sp1), activator protein-1 (AP-1), AP-2, AP-3, nuclear factor -1 (NF-1), Estrogen Receptor (ER), "milk binding protein factor" and STAT (Signal Transducers and Activators of Transcription) transcription factors [100–102]. In MCF-7 breast cancer cell line, a 45 kDa unidentified protein interacts with the −505/−485 region and is necessary for MUC1 transcription [101].

The −743/−1 region was shown to be sufficient to drive transcriptional activity of a reporter gene in MUC1-expressing epithelial cell lines. The −150/−60 region, which contains one Sp1 cis-element (−99/−90) and one E-box called E-MUC1 (−86/−64), is crucial to observe MUC1 tissue-specific expression (Fig. 1) [103].

The −101/−89 region interacts with two transcription factors: Sp1 and SpA. SpA recognizes Sp members consensus sequence GGGCGG but is distinct from the other members (Sp3, Sp4). In breast epithelial cancer cells, SpA, acts as a transcription inhibitor and regulates MUC1 expression level. In carcinomas, increase of MUC1 expression could be the result of an increase of Sp1 binding. Sp1/SpA ratio seems important in MUC1 transcriptional regulation since these two transcription factors compete for the same binding site [104].

Three regions called M-PMR1 (Mucin-Purin pyrimidine Mirror Repeat, −641/−615), M-PMR2 (−253/−237) and M-PMR3 (−133/−102) have been identified in MUC1 promoter [105,106]. These purine-rich regions (G + A) on one strand and pyrimidine-rich (T + C) on the other strand are characterized by a perfect mirror repeat element and are found to be associated with the formation of an H-DNA helix which could be involved in transcription regulation. A 27 kDa protein interacting with these regions was identified [107,108]. SpA binding specificity is influenced by M-PMR3 element which adjoins it but M-PMR3 had a very modest inhibitory effect on MUC1 transcriptional activity [106].

MUC1 promoter contains cis-elements for inflammatory signalling pathways such as interferon-γ or cytokines. In T47D breast cancer cell line, inside the promoter region, four transcriptional STAT sites (−90, −75, −55 and −35) were characterized close to the TATA- and GC-boxes. STAT sites are downstream elements of interferon signalling pathway. In breast cancer cell lines, proinflammatory factors interferon-γ (IFN-γ) and interleukin-6 (IL-6) stimulated transcription of a luciferase reporter construct under
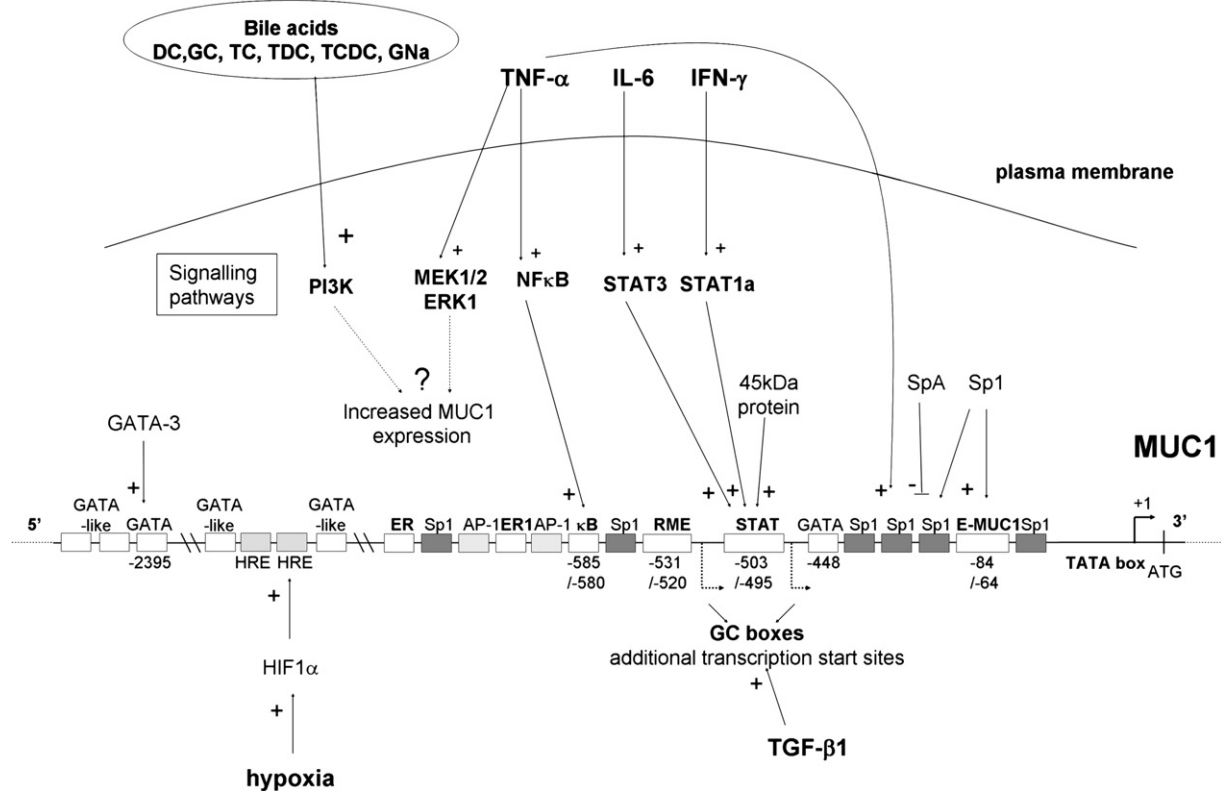

Fig. 1. Schematic representation of the promoter of MUC1. Factors, signalling pathways and identified cis-elements that participate in MUC1 transcriptional regulation are shown. "?" denotes lack of knowledge about the functional mechanism mediating MUC1 overexpression induced by PI3K or MEK1/2-ERK1 pathways. DC: deoxycholic acid, GC: glycocholic acid, TC: taurocholic acid, TDC: taurodeoxycholic acid, TCDC: taurochenodeoxycholic acid, GNa: sodium glycocholate, TNF: tumour necrosis factor, IL: interleukin, IFN: interferon, TGF: transforming growth factor, PI3K: phosphatidylinositol 3-kinase, MEK: mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase, ERK: extracellular signal regulated kinase, NFκB: nuclear factor-kappa B, STAT: signal transducers and activators of transcription, Sp: specificity protein, HRE: hypoxia response element, ER: estrogen receptor, AP: activator protein, RME: responsive mucin element.

the control of the −722/−1 region of MUC1 promoter (Fig. 1) [102]. An STAT cis-element localized at −503 mediates this activation since its mutation abolished stimulation of the reporter by these cytokines. Furthermore, STAT mutation decreased promoter basal activity in unstimulated cells. IFN-γ and IL-6 were shown to respectively activate STAT1 and STAT3, which then, interact with their cis-element at −503/−485. These results strongly suggest that STAT binding is an important mechanism in the overexpression of MUC1 in tumour cells [102].

Regulation of MUC1 by TGF-β1 growth factor is driven by two additional transcriptional start sites (−520 and −130) [109]. Authors suggested that some GC-boxes positioned at the 3′-end of the promoter may drive transcription independently of the TATA box. Knockdown experiments showed that GATA-3 transcription factor (TF) is a mediator of transcriptional upregulation of MUC1 in MCF7 and T47D cells via a GATA-3 functional binding site [110].

Shirotani et al. have shown that a region between nucleotides −531 and −520 of 5′-flanking region of MUC1 promoter was sufficient to observe transcriptional activity under normal colon conditioned medium (NCCM) conditions, a situation known to stimulate mucin production by human colon carcinoma cells [111]. This sequence was called RME (Responsive Mucin Element), and seems to interact with an unknown 70 kDa protein [112].

In A549 human lung adenocarcinoma cells proinflammatory cytokine tumour necrosis factor-α (TNF-α) stimulates expression of MUC1 by increasing de novo transcription in a mitogen-activated protein kinase kinase (MEK)1/2-extracellular signal-regulated kinase-1 (ERK1) dependent manner. MUC1 promoter activation also requires an Sp1 element located between nucleotides −99 and −90 [113]. In clear renal cell carcinomas (cRCC), MUC1 is directly regulated by Hypoxia Inducible Factor (HIF)-1α via HIF responsible elements (HRE) located at −1488/−1485 and at −1510/−1507. That upregulation affects the invasive and migration properties of renal cancer cells [114].

In vivo studies using transgenic mice have confirmed results obtained in vitro. To this aim, the group of Taylor-Papadimitriou has developed two strains of transgenic mice containing (i) a 10.6 kb genomic fragment consisting of the exons and introns of MUC1 flanked by 1.6 kb of the 5′-sequence and 1.9 kb of the 3′-sequence of the gene [115] and (ii) a transgene in which 1.4 kb of MUC1 promoter was fused to MUC1 cDNA [116]. The results showed that MUC1 is a compact gene and that the first 1400 nucleotides of MUC1 promoter contain all the cis-elements necessary (i) for epithelial-specific expression of the gene in vivo and (ii) for the upregulation of MUC1 expression observed at lactation and in tumourigenic process.

5. MUC1 epigenetic regulation in cancer cells

The term of epigenetics refers to a heritable change in gene expression that is driven by mechanisms other than alteration of nucleotidic sequence. Epigenetic changes include methylation of DNA, methylation and acetylation of histones and very recently regulation by miRNA. These mechanisms are often altered in pathological processes. Alterations of expression include both silencing and overexpression of mucin genes while promoters of mucin genes share a GC-rich structure and, for most of them, contain a CpG island, two structures characteristics in favour of epigenetic regulation [117]. MUC1 gene is characterized by the presence of a CpG island in a large transcribed region that consists of 60-bp tandemly repeated units, each containing one SmaI restriction site. Evaluation of the methylation status of various regions of the MUC1 gene by Zrihan-Licht and collaborators using the methylation-sensitive enzymes SmaI and XmaI showed that, in peripheral blood leukocytes that do not express MUC1, the 60-bp

repeat array was completely methylated, whereas the same sequences were entirely unmethylated in DNA from MUC1-overexpressing breast tumour tissue. Hypomethylation within the repeat array was also observed in other epithelial tissues that express MUC1 at much lower levels than those seen in breast cancer tissue, demonstrating that hypomethylation of the tandem repeat array was an absolute requirement for MUC1 gene expression in epithelial tissues [118]. Influence of methylation in MUC1 silencing was also showed in prostate cancer cells by using inhibitor of methylation 5-aza-2′-deoxycitidine [119]. In the same study, influence of histone deacetylation was observed after cell treatment with sodium butyrate but no information regarding direct involvement of histone modifications in MUC1 gene regulation is available at this time. Lately, Yamada and collaborators have identified 9 hypermethylated cytosine residues tightly associated with H3 lysine 9 (H3-K9) dimethylation in MUC1 non-expressing cell lines [120].

6. Transcriptional regulation of MUC4 in epithelial cancer cells

3.7 kb of MUC4 5′-flanking region have been characterized so far (GenBank accession number: AF241535) and four transcriptional initiation sites have been described: one localized in the proximal promoter at −199 and three in the distal promoter at −2603, −2604 and −2605 from the translational start site (Fig. 2) [121]. The proximal promoter is TATA-less and mainly composed of GC-rich domains that are potential binding sites for Sp1 but also for the CACCC box binding protein [121]. Furthermore, a very high density of binding sites for factors known to initiate transcription in TATA-less promoter (Sp1, CACCC box, glucocorticoid receptor element, AP-1, polyomavirus Enhancer Activator-3 (PEA3) and Med-1) was found inside the proximal promoter [122–125]. The distal promoter is characterized by a TATA box located at −2672/−2668. Numerous putative binding sites for Sp1, AP-1, AP-4, GATA and cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) transcription factors were found. MUC4 promoters also contain numerous putative binding sites for transcription factors involved in proteine kinase A (PKA), PKC, cAMP signalling pathways, in inflammation and in intestinal and respiratory differentiation.

Pancreatic cancer cells have been the major model for MUC4 regulation since MUC4 is not expressed in normal pancreas, is neoexpressed in early neoplastic precursor stage of pancreatic carcinogenesis with constant increase till adenorcarcinoma (see Section 2.3). In that model, MUC4 was shown to be regulated by a wide range of specific factors (Fig. 2). Tumour suppressor AP-2 down-regulates MUC4 expression at both protein and transcriptional levels via two AP-2 cis-elements located in the −475/−238 region of the promoter [72]. The Ets family member PEA3 is implicated in a wide range of cellular processes such as differentiation, proliferation and transformation [126] and was shown to upregulate MUC4 expression in synergy with c-Jun and Sp1 whereas PEA3 represses the transcriptional activity of ErbB2 promoter [127]. Inflammatory pathway IFN-γ increases MUC4 expression via STAT-1 upregulation [128]. TGF-β which possesses both tumour-suppressive and oncogenic activities [129] is a strong activator of MUC4 expression. TGF-β regulation involves a cooperation between Smad2 and Smad4 transcription factors, and Smad4 binding to seven Smad Binding Elements (SBE) located along proximal and distal promoters [130]. When Smad4 is mutated and inactive, TGF-β is still able to activate MUC4 expression via mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K) and PKA signalling pathways [130]. All-trans-retinoic acid (RA) treatment increases MUC4 expression via retinoic acid
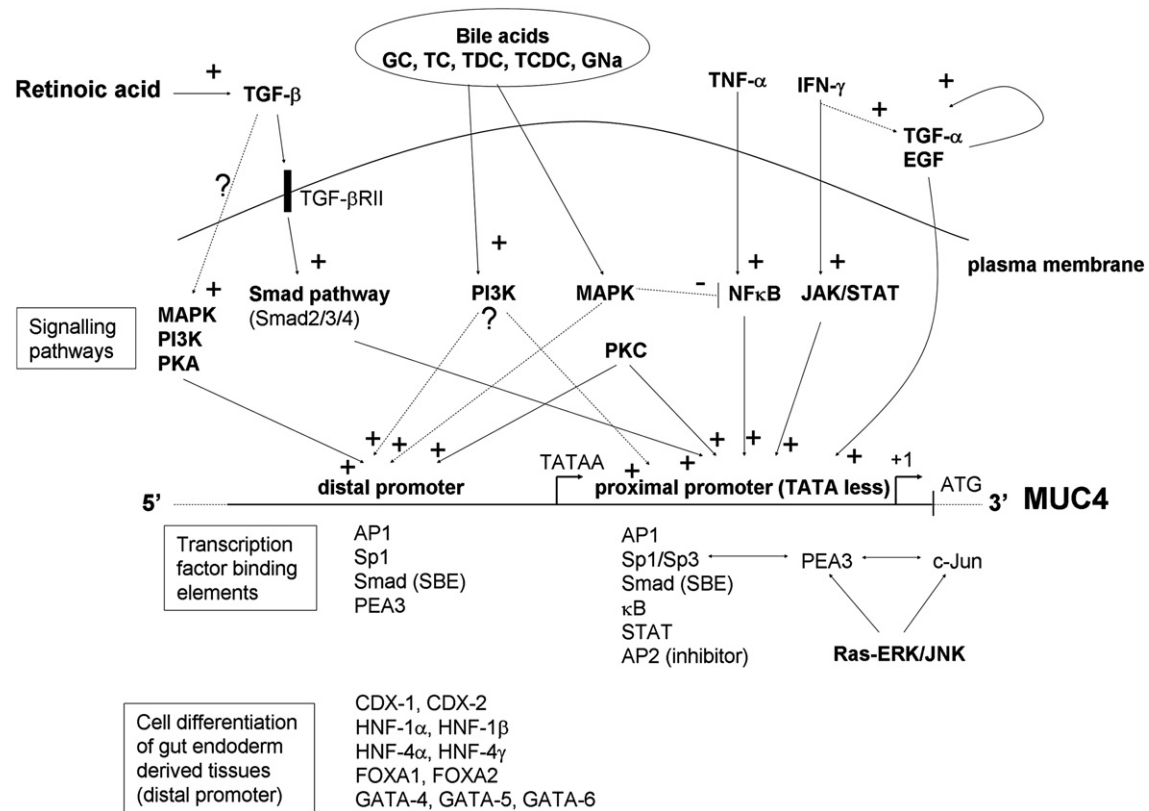
Fig. 2. Schematic representation of promoters of *MUC4*. Factors, signalling pathways known to regulate *MUC4* expression are shown. Transcription factors binding elements are listed. "?" denotes lack of knowledge about the functional mechanism mediating activation of MAPK, PI3K and PKA pathways by TGF-β. GC: glycocholic acid, TC: taurocholic acid, TDC: taurodeoxycholic acid, TCDC: taurochenodeoxycholic acid, GNa: sodium glycocholate, TGF: transforming growth factor, EGF: epidermal growth factor, TNF: tumour necrosis factor, IL: interleukin, IFN: interferon, PI3K: phosphatidylinositol 3-kinase, MAPK: mitogen-activated protein kinase, MEK: MAPK-extracellular signal regulated kinase, PKA: protein kinae A, NFκB: nuclear factor-kappa B, STAT: signal transducers and activators of transcription, JAK: Janus kinase, Sp: specificity protein, AP: activator protein, SBE: Smad binding element, PEA3: polyomavirus Enhancer Activator-3, JNK: Jun N-terminal kinase, Cdx: caudal-related homeobox, HNF: hepatocyte nuclear factor, FOXA: forkhead box A.

receptor-α (RAR-α). That upregulation is a consequence of the increase of expression of TGF-β2 regulating MUC4 expression in an autocrine manner [131]. IFN-γ and RA can synergize at the transcriptional level to induce *MUC4* expression in pancreatic tumour cells [132]. MUC4 expression is negatively regulated by chloride channel called cystic fibrosis transmembrane conductance regulator (CFTR) that is implicated in multiple cellular functions. CFTR mutation carrier status is correlated with early-onset of pancreatic adenocarcinoma [133]. It was observed that CFTR was down-regulated in pancreatic cancer cells and negatively correlated with MUC4 [134].

MUC4 regulation has also been studied in oesophageal cancer cells since this mucin was shown to be overexpressed during the metaplasia/dysplasia/adenocarcinoma sequence characteristic of oesophageal adenocarcinoma (see Section 2.2.1). Studies focused on MUC4 regulation by bile acids since they are incriminated to promote tumour progression in oesophageal cancer cells. Bile acids positively regulate *MUC4* expression *via* PI3K signalling pathway and binding of HNF-1α transcription factor to *MUC4* promoter [31,41].

MUC4 regulation has also been studied in intestinal cells since previous works had shown early expression of MUC4 mRNA in embryonic gut [135,136]. *In vitro* studies indeed showed that hepatocyte nuclear factors (HNF-1/-4), forkhead box A (FOXA1/A2), GATA-4/-5/-6, and caudal-related homeobox (CDX-1/-2) TFs which control cell differentiation of gut endoderm derived-tissues during embryonic development, are also regulators of *MUC4* and may act in synergy [137].

Altogether, these results indicate that *MUC4* expression is extremely complex and tightly regulated and involves many signalling pathways including inflammatory- and tumour- associated factors (Fig. 2). This of course is in favors of an important role for MUC4 mucin during development, epithelial differentiation, but also in epithelial repair and cancer.

7. MUC4 epigenetic regulation in cancer cells

The 5′-flanking region of *MUC4* is characterized by a high G + C content, up to 72% almost exclusively concentrated in the proximal promoter and the 5′-Untranslated Region (5′-UTR), and the presence of two CpG islands of 135 and 146 bp, respectively, both located in proximal and distal promoters at −2370/−2236 and −738/−593 [138].

Influence of histone deacetylation on *MUC4* silencing was first assessed in our laboratory in the MUC4 non-expressing pancreatic cancer cell line PANC-1 using cell treatment with trichostatine A (TSA) [130] and later shown in prostate cancer cells using cell treatment with sodium butyrate by Singh and collaborators [119]. This group also showed the influence of DNA methylation in regulating *MUC4* in these cells by using 5-aza-2′-deoxycytidine (5-aza).

In order to better understand how constant increase of MUC4 during the different steps of pancreatic carcinogenesis could be regulated, we undertook a thorough study of *MUC4* chromatin status in pancreatic cancer cells expressing different levels of MUC4 mucin. Our recent studies indicates that *MUC4* proximal and distal

promoters, which possess each a CpG island, are heavily methylated whether the gene is expressed or not in the cells. On the contrary, *MUC4* 5′-UTR shows a methylation profile, that was directly correlated to the level of *MUC4* expression in the cells. Hypermethylation of *MUC4* 5′-UTR is associated with establishment of a repressive histone code, including deacetylation and methylation of histone H3 [138]. Methylation of Sp1 binding sites located in the proximal promoter and the 5′-UTR dramatically impairs *MUC4* activation by Sp1, whereas methylation of the adjacent CpG site upstream of the 5′-UTR has no effect. DNA methyltransferase (DNMT)-3A, DNMT3B and histone deacetylase-3 (HDAC3) were identified as potent regulators of *MUC4* endogenous expression *via* a direct binding to its 5′-UTR [138].

From these studies, we proposed the following model of MUC4 epigenetic regulation: in non-expressing cells, *MUC4* silencing would be mediated by DNA methylation and histone deacetylation at the 5′-UTR, whereas in low expressing cells progressive demethylation would allow low expression of *MUC4*, and finally in high-expressing cells selective hypomethylation and permissive chromatin would allow binding of essential transcription factors such as Sp1 and full transcription of *MUC4* gene [138]. This model may reflect constant upregulation of MUC4 in epithelial carcinogenesis in different organs such as oesophagus, pancreas, lung or colon. Yamada and collaborators recently confirmed the CpG methylation on *MUC4* 5′-UTR using MassARRAY® compact system and showed two additional methylated cytosines associated with control of *MUC4* gene expression in ten cancer cell lines [139].

Globally, regulation of *MUC1* and *MUC4* genes is complex and involves a wide array of signalling pathways. Common growth factors (EGF, TGF-β) or proinflammatory pathways (IFN-γ, TNF-α) are regulators for both genes. In a similar manner, MUC1 and MUC4 are members of the class of epigenetically controlled genes. However, a concerted regulation of the two genes remains hypothetical. Such regulations also raise the questions of whether these two mucins can compensate for each other or if they act separately as they are differentially expressed during carcinogenesis.

8. Regulation of other membrane-bound mucins in cancer cells

To date, very little is known about regulation of the other membrane-bound mucins.

The 5′-flanking region of *MUC3A* gene has been analysed. *MUC3A* transcripts initiate from multiple start sites along a region spanning around 180 nucleotides. *MUC3A* promoter lacks a cognate TATA box. Analysis using TFSEARCH and MatInspector softwares showed that *MUC3A* promoter contains potential binding sites for transcription factors implicated in the regulation of intestinal genes (CDX-2, GATA, USF, CACCC and HNF-1) [140]. Moreover, mRNA level of *MUC3* is increased by overexpression of GATA-5 in both COS-7 and 293T cells [141]. Most of the expression studies do not differentiate *MUC3A* from *MUC3B*. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), which modulate diverse cellular responses through signal transduction pathways like PKC pathway, caused upregulation of mRNA levels of *MUC3* in HM3 human colon cancer cells. The *MUC3* upregulation is inhibited after treatment with protein synthesis inhibitors or Calphostin C, a highly specific inhibitor of PKC. These changes were associated with increase of matrix metalloproteinase (MMP) activity as well as by increase in the invasive and motility properties of cells. Therefore, PKC signalling pathway may regulate *MUC3* expression and modulate the invasive and metastatic properties of colon cancer cells. It was also shown that the secreted thiol-activated cytolysin listeriolysin O (LLO), responsible for *L. monocytogenes*-induced high-molecular

glycoproteins (HMGs) exocytosis, leads to *MUC3* and *MUC12* increased expression in colon cancer cells HT29-MTX [142].

Helicobacter pylori causes gastric preneoplasia and neoplasia. cDNA microarray assays showed that *MUC13* is down-regulated after *H. pylori* infection and upregulated in placebo control group on gastric biopsies suggesting the potential use of *MUC13* as a marker of gastric cancer risk [143].

MUC16 is upregulated at mRNA and protein levels following an RA treatment of human conjunctival epithelial (HCjE) cells. The RA-associated upregulation of MUC16 appears to be mediated by secretory phospholipase A(2) group IIA (sPLA(2)-IIA) [144]. RT-PCR and western blot experiments also showed that *MUC16* gene expression is upregulated 48 h after addition of dexamethasone in human corneal epithelial cells. Treatment with glucocorticoid receptor antagonist RU38486 inhibits the change of *MUC16* by dexamethasone; thus, the effect of dexamethasone on *MUC16* expression is mediated by glucocorticoid receptors [145].

MUC17 upstream region is an intergenic region located in between *MUC12* and *MUC17* genes that contains VDR/RXR, GATA, NFκB and Cdx-2 response elements. That 1146 bp region shows a strong enhancer and a significant promoter activity in AsPC-1 and HPAF pancreatic cancer cell lines [51].

9. Perspectives

In the future, new technologies will bring new clinical tools. In that sense, detection of mucin epitopes or motifs in biological fluids, establishment and access to high throughput studies of mucin epigenetic status are very promising.

To the clinical point of view, an ideal marker implicates non-invasive and rapid procedures of detection. Therefore, several laboratories develop the idea of mucins as circulating markers in the blood or markers associated with biological fluids such as pancreatic juice. In pancreatic cancer, recent work showed that *MUC1* mRNA was detected in pancreatic juice during the development of high-grade PanIN to intraductal carcinoma. That quantification has a high potential for preoperative diagnosis of pancreatic cancer [146]. Moreover, Jhala et al. used MUC4 as a biomarker in fine-needle aspirate (FNA) samples and showed that MUC4 could help distinguish ductal epithelial cells from pancreatic adenocarcinoma [147]. Therefore, Klapman and Malafa proposed that screening high risk individuals (familial pancreatic cancer, hereditary pancreatic cancer syndrome, cigarette smoking or early-age onset of diabetes) by FNA remains so far the only practical approach to detect precancerous or cancerous changes in the pancreas at a phase during which surgery will have a high chance to cure [148].

Rapid and systematic evaluation of epigenetic status of mucin genes in tumour cells by pyrosequencing may also provide clinicians with new early detection tools to screen for the presence of cancer, in which mucin genes are epigenetically expressed.

Recently microRNAs (miRNA) were described as a new epigenetic regulation of mammalian genes. MiRNA are small non-coding RNAs that negatively regulate gene expression in a sequence-specific manner. MiRNAs are essential regulators of various biological processes such as proliferation, differentiation, development or cell death [149]. So far, there is no direct evidence of mucin regulation by miRNA but one cannot exclude a targeting of mucins long 3′-UTRs. Preliminary studies from our laboratory suggest that mucin 3′-UTRs contain conserved miRNA target elements [150]. Thus, further analysis will be conducted to demonstrate the role of miRNA in the ever-increasing complexity of mucin gene regulation in epithelial cancers.

The ultimate goal is to establish tumour identification for each patient that will allow adaptation of treatment and therapy. Mucins are among the specific epithelial markers that may be included in

such ID next to a panel of biomarkers that will become more and more precise as detection technologies improve and will allow rapid analysis of the whole genome.

Acknowledgments

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