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Expert Opinion

1. Introduction
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Mucins: a new family of epigenetic biomarkers in epithelial cancers

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Background: Epigenetic regulation of gene expression is a common feature of cancer development and progression. The search for new biomarkers and tools to detect cancer in its early stages has unveiled the usefulness of epigenetics and genes epigenetically regulated as potential targets. Among them, genes encoding mucins has been shown to be regulated by DNA methylation and histone modifications in epithelial cancer cells. These genes encode either secreted glycoproteins necessary for epithelial homeostasis or membrane-bound glycoproteins that participate in tumor progression. **Objective:** The important biological functions played by these large molecules in pathophysiology of the epithelia make them key genes to target to propose new therapeutic strategies and new diagnostic and/or prognostic tools in cancer. **Results:** In that context, the recent data regarding the epigenetic regulation of these genes are reported and their potential as biomarkers in cancer is discussed. Mucin genes are also potentially interesting to study as they may be regulated by miRNAs but also regulate miRNA activity. **Conclusion:** Epigenetic regulation of mucin genes is at its dawn, but there is great potential in that research to (with new technologies and high-throughput methods) provide quickly new biomarkers (diagnostic and/or prognostic), help tumor identification/classification and propose new therapeutic targets to the clinician and pathologist.

Keywords: biomarker, cancer, epigenetics, mucin

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1. Introduction

The term 'epigenetic' refers to a heritable change in the pattern of gene expression that is driven by mechanisms other than alterations in the primary nucleotide sequence of the gene [1,2]. Epigenetic changes in the genome include DNA methylation and histone modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation), two mechanisms that are often tightly linked in the regulation of gene expression and involved in many cellular processes [3]. For example, DNA methylation is a crucial epigenetic modification that drives embryonic development, X-chromosome inactivation, genomic imprinting and chromosome stability, whereas histone modifications govern rearrangement of chromatin structure.

DNA methylation occurs on CpG dinucleotides and follows a cell-specific profile that is established during development by DNA methyltransferases DNMT3A and DNMT3B [4] and is maintained through cell division by DNMT1 [5]. In the human genome, CpG sites are statistically underrepresented but are clustered in GC-rich regions termed CpG islands. These CpG islands are often associated with promoter regions or first exons of half the genes in our genome. In normal cells, 80% of CpG dinucleotides that are not associated with

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55 CpG islands (predominantly in intergenic and intronic
regions of DNA, repeat sequences and transposable elements)
are heavily methylated, whereas CpG islands are usually
60 unmethylated, whether or not the gene is transcriptionally
active [6]. On the contrary, the cancer genome is frequently
characterized by both global hypomethylation of intergenic
regions resulting in chromosomal instability [5] and specific
hypermethylation of CpG islands in promoters resulting in
aberrantly silenced tumor suppressor genes [6].

65 DNA methylation by itself does not directly repress
transcription. The process of silencing needs the involvement
of the epigenetic machinery composed of chromatin modi-
fier enzymes, such as histone deacetylases (HDACs) and
histone methyltransferases (HMTs), and proteins that are
70 able to read methylation marks, namely the methylCpG
binding proteins (MBPs). MBPs recognize methylated DNA
and recruit multiprotein complexes containing HDACs that
remove acetyl residues from protruding histone tails, hence
tightly compacting chromatin and suppressing promoter
access to transcription machinery [7].

75 In the collaboration between the two epigenetic mechanisms
that silences gene expression in cancer it is unclear at the
moment whether DNA methylation or histone modification
is dominant. Studies using drugs that inhibit epigenetic
mechanisms showed that HDAC inhibitors alone cannot
80 induce the expression of hypermethylated genes, indicating
that DNA methylation may be preponderant. However, in
some cases, histone (de)acetylation can drive DNA (de)
methylation [8,9], suggesting that DNA methylation may
simply be a marker of silent chromatin. Nevertheless, stable
85 gene repression by epigenetics in cancers seems to involve
both mechanisms and appears to be a specific mark of cancer
cells. The recent discovery of small non-protein-coding RNA,
named micro-RNAs (miRNAs), which target 3'-untranslated
regions (3'-UTR) of genes and repress either their maturation
90 or lead them to their degradation, added a new layer to
epigenetic regulation by means of RNA interference [10-12].

As altered patterns of epigenetic modifications are observed
in the early stage of carcinogenesis, it was suggested several
95 years ago that aberrant DNA methylation may be used as a
biological tool for early detection and prognosis of cancer [13-15].
Moreover, the potential reversibility of epigenetic modifications
gives new opportunities for the clinical treatment of cancer [16].
Thus, identifying new pharmacological inhibitors of epigenetic
100 modifications has become the focus of research in many
laboratories, and both DNMT and HDAC inhibitors have
shown promising results in clinical trials [17,18].

Next to pharmacological inhibitors, growing interest has
been shown by researchers in controlling gene expression
during the early steps of carcinogenesis and/or using altered
105 pattern of expression of these genes as diagnostic/prognostic
markers. Epigenetically marked genes offer great and exciting
possibilities in that sense because methylation occurs during
the early stages of cancer. Among them, genes encoding
secreted and membrane-bound mucins are often aberrantly
109

expressed in tumors and this finding has led to the hypothe- 110
sis that mucins may be used as diagnostic and/or prognos-
tic factors in epithelial cancers of different origins [19-22].
Alterations of expression include both silencing and overex-
pression of mucin genes while their promoters share a GC-rich
115 structure and, for most of them, contain a CpG island [20].
Moreover, the four genes *MUC2*, *MUC5AC*, *MUC5B* and
MUC6, encoding the gel-forming mucins, are clustered on
the p15.5 region of chromosome 11 (11p15 MUC genes) [23],
in an area known to be a hot spot of abnormal methy- 120
lation in cancer [24]. This explains why most of the studies
regarding epigenetic regulation of mucin genes focused on
the 11p15 MUC cluster.

In this paper, the studies that were conducted by the
authors' laboratory are reviewed, as well as others about the
epigenetic regulation of the 11p15 MUC genes and those 125
about *MUC1* and *MUC4* genes that encode two membrane-
bound mucins known to participate in tumor progression
and alter cancer cell biological properties [25-27]. The second
part of the review opens up to the recent development of
new technologies to study epigenetic regulation, and the 130
exciting new avenues that mucin gene regulation by miRNAs
may provide to the clinicians in terms of therapeutic tools
are discussed. No data exist about mucin gene regulation by
miRNAs in the literature, so for the first time miRNA data- 135
bases were searched and the (long) lists of potential regulatory
miRNAs targeting 3'-UTRs of mucin genes established. The
paper concludes with a discussion of what the detection of
mucin gene methylation could bring to the clinician and/or
pathologist as a diagnostic/prognostic tool for early detection
140 of cancer and tumor identification/classification.

2. Mucins and epigenetics

2.1 DNA methylation and histone marks

2.1.1 The 11p15 mucin genes

2.1.1.1 The 11p15.5 chromosomal region

The 11p15 chromosomal region is of particular interest for
studying epigenetic mechanisms of regulation. Aberrant
DNA methylation in this region is associated with severe
developmental abnormalities [28,29] and cancer [24,30]. This 150
chromosomal region is characterized by one of the highest
concentration of CpG islands in the genome, being separated
from each other by 15 – 100 kb (Figure 1) [31]. Within this
region lies a 1 Mb imprinted domain, containing several 155
genes involved in growth and development, and potential
tumor suppressor genes that are aberrantly methylated in
cancer [24,32-34]. Interestingly, *MUC2*, one of the 11p15 mucins,
was recently proposed to have tumor suppressor activity in
colorectal cancer [35].

The 11p15 MUC cluster is 400 kb in length and is situated 160
~ 700 kb from the imprinted domain, between insulin-like
growth factor-2 (*IGF-2*) and Harvey rat sarcoma viral oncogene
homolog (*H-RAS*) genes, within a region containing 15 CpG
islands (Figure 1) [23]. Within the cluster, mucin genes are 164

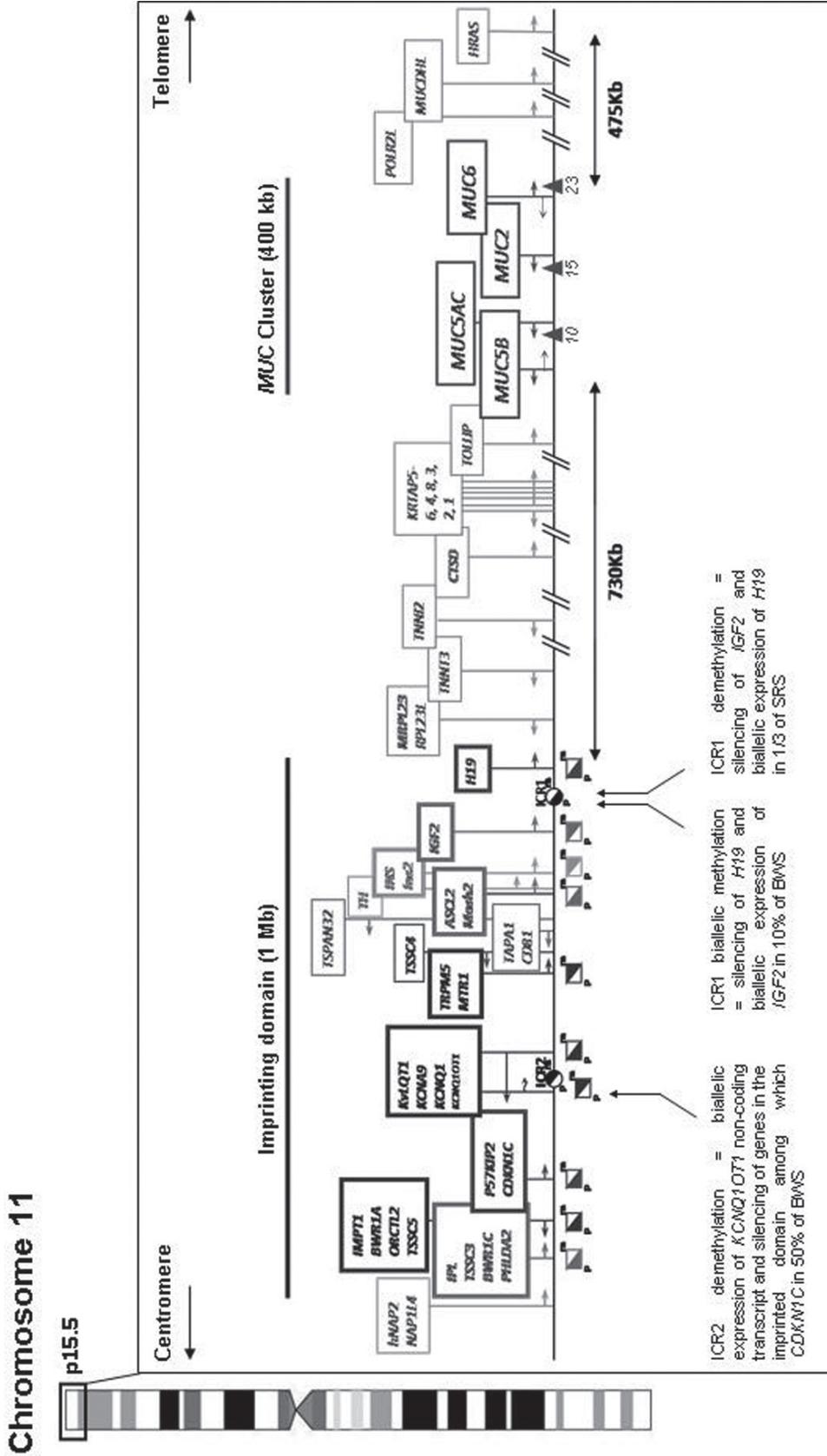


Figure 1. The 11p15 chromosomal region. Positions of the imprinting domain, the two imprinting control regions (ICR) and the mucin gene cluster (MUC) are indicated (black boxes). Full arrows indicate the transcription orientation for each gene. Dashed arrows indicate an antisense transcript (MUC5B, MUC6). Imprinted genes are boxed in gray and bold. Other known designations for each gene are indicated in the same box. Allele-specific methylation of imprinted genes is indicated by half-filled rectangles, p: paternal allele, m: maternal allele. Epigenetic abnormalities frequently described in Beckwith-Wiedemann Syndrome (BWS) and Silver-Russell Syndrome (SRS) are indicated. Triangles represent putative target regions for post-transcriptional regulation of mucin transcripts by miRNAs (MUC2, MUC5AC, MUC6). The number of putative miRNAs targeting these regions is indicated and is detailed in **Table 2**.

165 organized as follows, *MUC6–MUC2–MUC5AC–MUC5B*
 from telomere to centromere, *MUC2*, *MUC5AC* and *MUC5B*
 being transcribed from telomere to centromere, whereas *MUC6*
 is transcribed in the opposite direction (Figure 1).

170 2.1.1.2 Epigenetic regulation of the 11p15 mucin genes

The first studies that aimed at studying epigenetic regulation
 of mucin genes focused on *MUC2*. Based on the observation
 that *MUC2* mucin expression was repressed in non-mucinous
 colorectal cancers and their metastasis, Hanski and collaborators
 were the first to investigate the role of DNA methylation in
 175 *MUC2* silencing [36]. To this end, they used the methylation-
 sensitive enzyme HpaII and the methylation-independent
 enzyme MspI to digest DNA from colon carcinoma cell lines
 expressing variable levels of *MUC2*, followed by Southern
 blotting. They showed hypermethylation of *MUC2* promoter
 in non-mucinous carcinoma cell lines [36,37].

Thereafter, arising bisulfite-treated genomic DNA-based
 methods allowed more precise analysis of methylation profiles
 of CpG sites involved in *MUC2* silencing. These studies
 showed the involvement of 9 CpG sites situated directly
 185 upstream of the transcriptional start site of *MUC2*, at -289,
 -274, -246, -192, -160, -126, -118, -6 and -2, in the silencing
 of the gene in non-expressing normal columnar cells and in
 the non-mucinous carcinoma tissues of the colon [37] as well
 as in pancreatic cancer cell lines [38]. The use of methylation-
 sensitive single nucleotide primer extension (Ms-SNuPE)
 allowed Mesquita and co-workers to demonstrate the role of
 site-specific promoter hypomethylation in aberrant *MUC2*
 mucin expression in mucinous gastric carcinoma [39].

In 2005, mapping of the methylation status of the 59 CpG
 sites of *MUC2* promoter lying from position -1989 to position
 +288 in pancreatic cancer cell lines confirmed the role of
 site-specific methylation in *MUC2* silencing, and authors
 described methylation of DNA binding sites for AP-2 and
 Sp1 transcription factors. However, the authors did not
 200 study the influence of methylation on *MUC2* activation by
 these two transcription factors [40]. They also showed that
 histone H3 modification status at *MUC2* promoter, and
 especially demethylation of lysine 4, methylation of lysines 9
 and 27 and deacetylation of lysines 9 and 27, tightly related
 to DNA methylation, played an important role in *MUC2*
 gene silencing [41].

Very few studies have been conducted concerning the
 three other genes of the cluster. The first study concerning
 epigenetic regulation of *MUC5B* was carried out at the
 authors' laboratory and showed, using HpaII and MspI
 enzymes combined with Southern blotting and cell treatment
 with DNA methylation inhibitor 5-aza-22deoxycytidine
 (5-aza), that the -1095/-695 region of *MUC5B* proximal
 promoter was hypermethylated in gastric *MUC5B*-expressing
 KATO-III cells, whereas methylation occurred throughout
 the 55-flanking region in gastric low-expressing AGS
 cells [20,42]. This pattern of methylation corresponded to the
 215 downregulation of *MUC5B* apomucin expression observed
 219

in undifferentiated gastric carcinomas [42]. The only study
 carried out on *MUC5AC* and more particularly on one
 CpG site in pancreatic cancer cell lines did not show any
 correlation between epigenetic regulation of the gene and
 expression of the apomucin [43].

To assess whether epigenetic regulation of the four 11p15 MUC
 genes was concerted and associated with cancer, a simultaneous
 study was undertaken of the four genes in a wide panel of
 human epithelial cancer cell lines with different epithelial
 origins and different status of differentiation [44]. The analysis
 was carried out on large portions of the promoters, that is,
 225 3.3 kb for *MUC2*, 1.3 kb for *MUC5AC*, 2.3 kb for *MUC5B*
 and 1.8 kb for *MUC6*. *In silico* analysis showed that *MUC2*,
MUC5B and *MUC6* promoters were particularly rich in
 G+C nucleotides, up to 70, 80 and 75%, respectively, and
 contained CpG islands of 115, 104 and 166 bp in length,
 230 respectively, situated at -4647/-4533, -976/-873 and -160/+6,
 235 respectively, whereas relatively few CpG sites were found in *MUC5AC* [44].

The studies indicate that regulation by methylation (after
 treatment with 5-aza) and by histone acetylation (after
 treatment with HDAC inhibitor trichostatin A [TSA]) is
 240 both gene- and cell-specific. In some cases, TSA induced
 repression of mucin gene expression, especially in confluent
 differentiated cells expressing high levels of the mucin gene
 of interest [44]. The same inhibitory mechanism by the
 HDAC inhibitor sodium butyrate had previously been
 245 found in *MUC2* high-expressing intestinal cancer cell
 lines [45]. This may reflect a mechanism of definitive
 function of the secretory lineage in order to maintain
 epithelial homeostasis [46].

Bisulfite-treated genomic DNA sequencing and chromatin
 250 immunoprecipitation (ChIP) assays confirmed that *MUC2*
 was regulated by site-specific DNA methylation associated
 with establishment of a repressive histone code, including
 histone H3 deacetylation and methylation of lysine 9. Moreover,
 our study covering the -3269/-1791 region of *MUC2* promoter
 255 allowed us to identify 4 more relevant CpG sites at -3269,
 -3199, -2331 and -1912. It was also shown that hypermethyl-
 ation of *MUC5B* promoter was the main mechanism
 responsible for its silencing in epithelial cancer cells and
 involved a wide region of its distal promoter at -2677/-2163,
 260 as well as cytosines at -434 and -421 in the proximal promoter.
 Thus, we and others have identified key methylated cyto-
 sines in *MUC2* and *MUC5B* promoters that should provide
 useful tools, which are easy to detect, to screen mucin gene
 repression by methylation in epithelial cancers and identify
 265 tumors in which their repression may indicate good
 or poor prognosis.

On the contrary, the expression of *MUC5AC* was rarely
 influenced by epigenetic mechanisms and it was not possible
 to identify CpG sites in its promoter which hypermethylation
 270 would be associated with its silencing. Hypermethylation
 of *MUC6* promoter was not correlated to its repression
 because DNMT and HDAC inhibitors had no influence on
 its expression in the cancer cell lines studied. Thus, epigenetic
 274

275 regulation of 11p15 MUC genes is not concerted and
involves gene-specific mechanisms [44].

280 Interestingly, our study also indicates that 11p15 mucin
genes share a common feature regarding the methylation
pattern of their promoters, which is that their CpG islands
are heavily methylated regardless of their level of expression
in the cancer cells studied [44]. However, these results will
have to be confirmed in normal tissues compared with
tumors because it is known that cancer cell lines often harbor
an altered methylation pattern of their genome and excessive
CpG island hypermethylation [47].

285 DNMT1, by a small interfering RNA (siRNA) approach,
was identified as a potent regulator of *MUC2* and *MUC5B*
epigenetic regulation. The DNMT1 effect was potentiated
by HDAC2. In the same studies it was found that methylation
of the two promoters dramatically impaired their activation
by Sp1 transcription factor. Finally, one of the most important
findings in this study was that *MUC2* and *MUC5B* epigenetic
regulation, and particularly the methylation profile of their
promoters, was directly dependent on the cell differentiation
status, leading to the hypothesis that hypermethylation of
MUC2 and *MUC5B* may be a specific mechanism used by
epithelial cancer cells to maintain a proliferating undiffer-
entiated state or by normal cells to maintain pluripotency
during development [44].

300 2.1.2 Membrane-bound mucins

300 2.1.2.1 *MUC4*

305 The 5'-flanking region of *MUC4* is characterized by a high
G+C content, up to 72% concentrated almost exclusively in the
proximal promoter and the 5'-UTR, and the presence of two
CpG islands of 135 and 146 bp, respectively, both located in
the proximal promoter at -2370/-2236 and -738/-593 [48].

310 The influence of histone deacetylation on *MUC4* silencing
was first assessed by the authors' laboratory in the pancreatic
cancer cell line PANC-1 using cell treatment with TSA [49]
and later shown in prostate cancer cells treated with sodium
butyrate by Singh and co-workers [50]. This group also
showed the influence of DNA methylation in regulating
MUC4 in these cells by using 5-aza.

315 The studies in epithelial cancer cells that express variable
levels of *MUC4* showed that *MUC4* proximal and distal
promoters, including both CpG islands, were heavily methy-
lated whether or not the gene was expressed in the cells. On
the contrary, *MUC4* 5'-UTR, and particularly cytosines at
320 -81, -93, -102, -113 and -121, showed a methylation profile
that was directly correlated to the level of *MUC4* expression
in the cells [48]. Hypermethylation of *MUC4* 5'-UTR was
associated with the establishment of a repressive histone
code, including deacetylation of histone H3 and methylation
of lysines 9 and 27 of histone H3 [48]. As four Sp1 binding
sites had previously been identified and located, just
upstream of and within the 5'-UTR [51], the influence of
methylation of these Sp1 *cis*-elements on the regulation of
325 *MUC4* by this transcription factor after *in vitro* methylation

of the promoter coupled to site-directed mutagenesis of the
Sp1 sites was investigated further. It was found that methy-
lation of Sp1 binding sites located in the proximal promoter
and the 5'-UTR at -276/-271 and -166/-156 dramatically
impaired *MUC4* activation by Sp1, whereas methylation of
the adjacent CpG site upstream of the 5'-UTR had no
effect. Finally, siRNA approach and ChIP assays allowed us
to identify DNMT3A, DNMT3B and HDAC3 as potent
regulators of *MUC4* endogenous expression that directly
bind to its 5'-UTR [48].

330 From these results, a model was proposed of *MUC4*
epigenetic regulation in epithelial cells [48]. In normal pancreatic
cells that do not express *MUC4* [52], *MUC4* silencing would
be mediated by DNA methylation and histone deacetyla-
tion at the 5'-UTR, whereas in early steps of pancreatic
carcinogenesis progressive demethylation would allow low
expression of *MUC4*, and finally in adenocarcinomatous
high-expressing cells, selective hypomethylation and permissive
chromatin would allow binding of essential transcription
factors such as Sp1 and full transcription of the *MUC4*
gene. Recently, Yamada and co-workers showed that the
methylation level of cytosines at -170 and -160 was associ-
ated with *MUC4* gene expression in 10 cancer cell lines using
the MassARRAY compact system, and therefore confirmed
the critical contribution of promoter CpG methylation in
MUC4 silencing [53].

330 2.1.2.2 *MUC1*

335 The *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 1 SmaI restriction
site. The first evaluation of the methylation status of various
regions of the *MUC1* gene by Zrihan-Licht and co-workers
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 methy-
lated, whereas the same sequences were entirely unmethy-
lated in DNA from *MUC1*-overexpressing breast tumor
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 expres-
sion in epithelial tissues [54]. The influence of epigenetics
in *MUC1* silencing was then confirmed in prostate cancer
cells by using cell treatment with 5-aza and sodium
butyrate [50]. The precise mechanisms of epigenetic regula-
tion at *MUC1* promoter had not been deciphered until
recently, when Yamada and co-workers found hypermethyla-
tion of cytosines at -70, -55, -51, -47, -31, -21, -14, -4 and +20,
tightly associated with dimethylation of H3-K9, in most
MUC1 non-expressing cell lines, providing the first proof
that *MUC1* gene expression was regulated by DNA methylation
of the promoter and associated histone H3 lysine 9 (H3-K9)
modification [55].

385 **2.2 Moving towards using mucin methylation as a diagnostic tool**

390 Bisulfite-treated DNA sequencing of single clones is a gold standard and has been a very useful tool in our comprehensive studies to analyse precisely the methylation profile of mucin gene promoters and to determine the key CpG sites to use mucin gene methylation as a biomarker. However, applica-
 395 tion of this method in routine procedures for molecular diagnosis is constrained by several issues, as it is complex, time-consuming, expensive, and requires a fair number of sequenced clones to be considered quantitative. Therefore, our laboratory is currently validating the pyrosequencing technology to analyse the methylation status of mucin promoters in human tissues and validate the use of mucin gene methylation as a diagnostic and/or prognostic tool in
 400 epithelial cancers. Pyrosequencing is a fast, robust and flexible sequencing-by-synthesis-based method that offers a reproducible quantification of individual, consecutive CpG sites (up to 100 bp amplicon length), enabling discrimination of small changes in methylation levels, from single cytosines to
 405 global methylation of a promoter region, in a large number of samples [56].

This switch to pyrosequencing seems very promising, as our first data for *MUC5B* (this review), *MUC5AC* and *MUC4* (unpublished data) already allowed us to confirm
 410 the potent status of CpG methylation as a biological tool in lung, colon and pancreatic cancer, respectively. The high level of methylation of *MUC5B* distal promoter seen in a *MUC5B* non-expressing normal lung tissue by bisulfite DNA sequencing is even more important in the correspond-
 415 ing *MUC5B* non-expressing lung adenocarcinoma (Figure 2A). In another histologic type of lung cancer, a *MUC5B* non-expressing squamous cell lung carcinoma, it was also found, using pyrosequencing, that *MUC5B* distal promoter was hypermethylated at cytosines -2430, -2424, and -2421 when
 420 compared with normal lung tissue (Figure 2B). Regarding *MUC4*, our first analysis of its pattern of methylation in normal and cancerous pancreatic tissues confirmed that demethylation of cytosines at -93, -102 and -113 occurs in pancreatic adenocarcinoma when compared with *MUC4*-
 425 non-expressing normal pancreas (not shown). Although these preliminary data need further confirmation on larger panels of tissues, it is hoped to provide a rapid way of evaluating mucin gene methylation in cohorts and use it as a specific biomarker. As mucin genes show altered expres-
 430 sion in various types of epithelial cancer (adenocarcinoma, squamous carcinoma), and also in numerous organs, tissues (respiratory tract, gastrointestinal tract, urogenital tract, etc.) and fluids (tears, saliva, sperm, milk, etc.), a lot of work as yet to be done. Moreover, in the future this method should
 435 be of great help to determine the prevalence of mucin gene methylation in genomic DNA released from tumors in biological fluids such as plasma or serum, bronchoalveolar lavages for lung cancer, and pancreatic juice for pancreatic cancer.
 439

2.3 Mucins and epigenomics

440 All the techniques that were used to study epigenetic changes at promoters of candidate genes can now be coupled both with genome-wide approaches, such as microarray technologies, and high-throughput methods, such as ultradeep sequencing, for high-resolution mapping of epigenetic profiles in both
 445 normal and cancer cell types [57,58]. *Methods* for genome-wide mapping of DNA methylation patterns include methylated CpG island amplification (MCA), followed by hybridization on microarrays [59], methylated DNA immunoprecipitation (MeDIP)-on-chip [60], or ultradeep bisulfite sequencing [61].
 450 Similarly, histone modification status can be widely studied using chromatin-immunoprecipitation-based methods [13,62] such as ChIP-on-chip or ChIP-seq. These new technologies will allow us to decipher further the mechanisms by which mucin genes are epigenetically regulated as clusters. Indeed,
 455 regulation of the 11p15 MUC cluster at the gene level has been studied previously in the authors' laboratory, but has not yet been completely understood at a more global level. Similarly, regulation of mucin gene clusters at 3q29 (*MUC4*, *MUC20*) and 7q22 (*MUC3*, *MUC12*, *MUC17*) chromosomal
 460 regions [26] remains to be investigated.

These genome-wide approaches will also be essential and necessary if one wants to give an explanation of the never-answered hypothesis as to how the tight coordination works and leads to simultaneous expression of a specific panel of
 465 both secreted and membrane-bound mucins, which follow two different biosynthetic pathways, in each type of epithelium. Finally, these comprehensive studies will allow us to understand better the specific mechanisms responsible for main-
 470 taining epithelium protection, as mucins are key proteins in epithelial homeostasis, and, therefore, the potential impacts of the dysregulation of their genes in epithelial diseases.

Most importantly, as it is now agreed that individual biomarkers are not informative enough for the diagnosis of most tumor types, translational epigenomics will be an
 475 essential tool to determine an ideal set of various biomarkers (tumor ID or tumor signature), which will include specific epigenetic modifications of mucin genes, that may define each neoplasia and therefore be used for diagnosis and prognosis of epithelial cancers.
 480

2.4 Mucins and regulating RNAs

2.4.1 MicroRNAs

485 MicroRNAs are endogenous non-coding RNAs, of 18 – 24 nucleotides in length, typically excised from 60 to 110 nucleotide foldback RNA precursor structures [63], that play important negative regulatory roles in animals and plants by targeting messenger RNA transcripts for degradation or translational repression, involving the RNA interference silencing complex (RISC) [10]. Recently, Gonzalez and co-workers
 490 showed that miRNAs can also trigger transcriptional silencing by means of chromatin remodelling (i.e., trimethylation of H3K9) at DNA regions containing complementary sequences [64].
 494

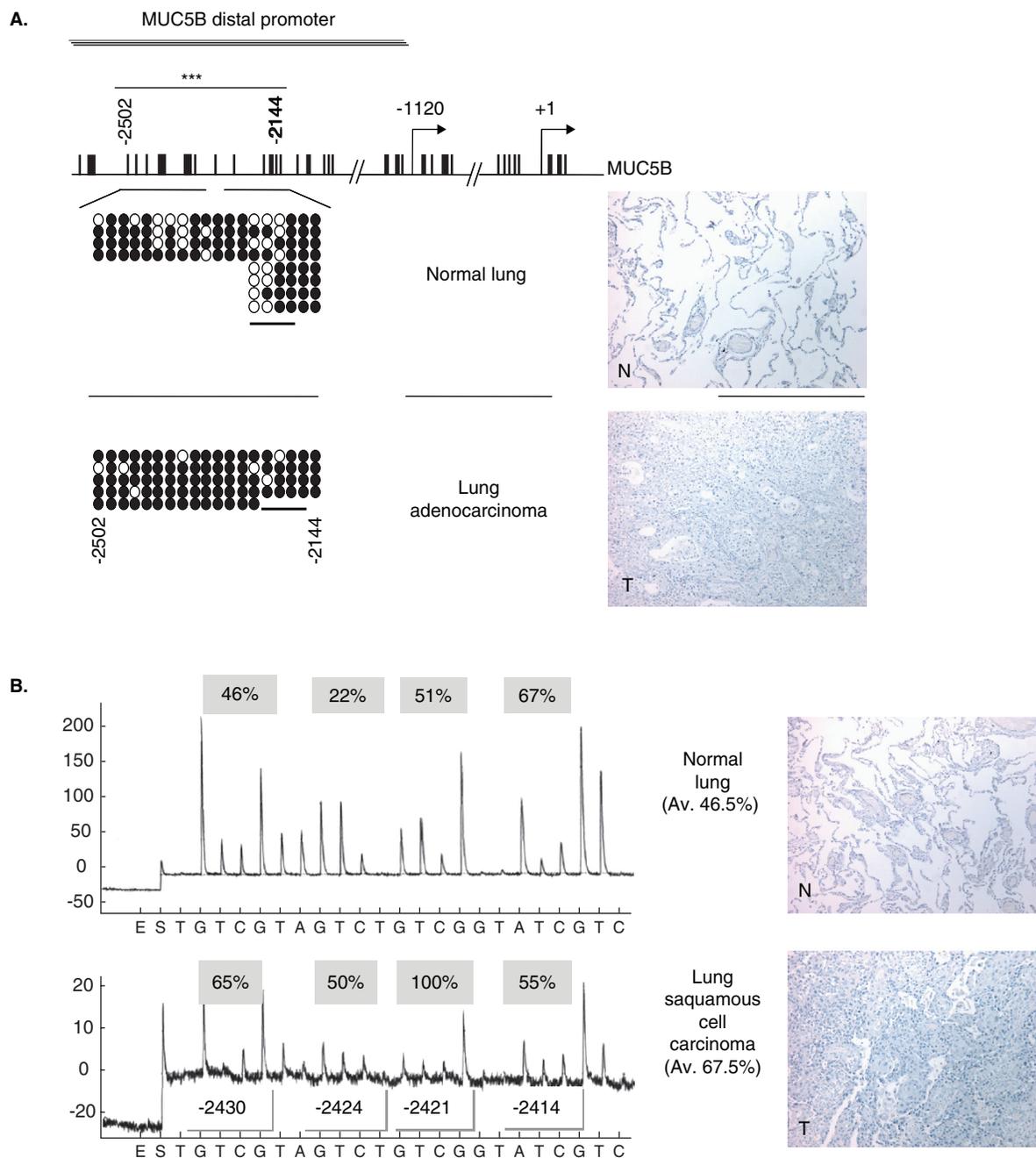


Figure 2. Methylation profile of *MUC5B* distal promoter in *MUC5B* non-expressing lung cancers. **A.** Mapping of methylated cytosines was carried out by bisulfite-treated DNA sequencing in normal lung (upper panel, N) and in a *MUC5B*-negative lung adenocarcinoma (lower panel, T). On the right-hand side is shown immunohistochemical staining of *MUC5B* apomucin in the corresponding tissues (magnification $\times 100$). Partial sequence of *MUC5B* promoter is represented by the horizontal bar. Numbering refers to the proximal transcription start site of *MUC5B* (+1, broken arrow) [42,106]. Vertical bars indicate CpG sites. More than four individual clones were analysed per CpG site and type of tissue (normal lung or lung adenocarcinoma). Black circles represent methylated cytosines and open circles represent unmethylated cytosines. Key CpG methylated sites previously identified in cancer cell lines [44] are topped with a star and their position in the promoter is indicated. The horizontal thick bar indicates the promoter region analysed further using the pyrosequencing technology. **B.** Mapping of methylated cytosines in *MUC5B* promoter by pyrosequencing in normal lung (upper panel, N) and in a *MUC5B*-negative lung squamous cell carcinoma (lower panel, T). On the right-hand side is shown immunohistochemical staining of *MUC5B* apomucin in the corresponding tissues (magnification $\times 100$). The pyrograms show the methylation percentages of the four CpG sites of interest (-2430, -2424, -2421 and -2414) in *MUC5B* distal promoter using the pyrosequencing technology (PyroMark™ MD, Biotage).

Av: The average methylation content of the promoter in normal lung compared with lung squamous cell carcinoma.

495 So far, hundreds of unique, mature miRNAs have been
 identified in humans, with more to be discovered. Their expres-
 sion levels vary greatly among tissues [65] and especially during
 pathological processes [66], including carcinogenesis [12,67,68].
 500 Chromosomal rearrangements, genomic amplifications or
 deletions, epigenetic regulation and mutations at genes
 encoding miRNAs lead to their specific overexpression or
 underexpression in different types of tumor, which contributes
 to cancer development and progression. Changes in miRNA
 expression have been reported by various genome-wide techni-
 505 ques (microarray platforms, bead-based flow cytometry) in col-
 orectal neoplasia [69], pancreatic cancer [70-72], kidney and
 bladder cancers [73], esophageal [74] and gastric carcinomas [75]
 and lung cancer [76], potentially affecting expression levels of
 numerous putative targets (Table 1). This led to the new concept
 510 of miRNA expression signatures [75] associated with cancer
 diagnosis (tissue type), predisposition, prognosis (differentiation
 and aggressiveness, [77]), identification of a primary tumor [78]
 and response to therapy [79]. Moreover, miRNAs are present
 in human plasma in a remarkably stable form and therefore
 515 represent a potentially useful approach for the biological
 fluid-based detection of human cancer [80]. For most miRNAs
 though, identification of their target genes and the biological
 consequences of their repressive activity on cancer progression
 remain to be defined.

520 No evidence for mucin gene regulation by miRNAs has
 been reported so far. However, interrogation of MiRbase and
 MiRanda databases ([81-84]) revealed a large number of
 miRNAs that could potentially target 33-UTR of mucin
 genes encoding either the membrane-bound mucins MUC1,
 525 MUC4, MUC20, MUC3A/3B, MUC17 and MUC13 or
 the secreted mucins MUC2, MUC5AC, MUC6, MUC7
 and MUC19 (Table 2). Interestingly, although miRNAs
 might be responsible for mucin gene regulation, a recent
 study by Ramasamy and co-workers showed, conversely, that
 530 *N*-glycosylation of Asn-36 on MUC1 C-terminal subunit
 suppresses miR-322 expression in breast and prostate cancer
 cells, thereby increasing expression of galectin-3 [85]. This
 very interesting result suggests that, in turn, mucins may
 regulate miRNA expression and activity.

535 **2.4.2 Antisense RNA**

Natural antisense transcripts are endogenous RNA molecules
 containing sequences that are complementary to the protein-
 encoding sense transcript. *Cis* antisense RNAs are transcribed
 540 at the same genomic locus from the opposite strand, whereas
trans antisense RNA are transcribed from separate loci.
 Antisense RNAs are thought to regulate their target genes at
 the genomic level by triggering chromatin remodelling [86], or at
 the transcriptomic level by interfering with mRNA transcription,
 545 processing, stability, translocation and translation [87].

Recently, evidence for antisense transcripts has been
 reported in 2900 – 6400 human genes using the asymmetric
 strand-specific analysis of gene expression (ASSAGE)
 549 technology [88]. The distribution of these antisense RNAs

is non-random across the genome and differs among cell types, 550
 suggesting a fundamental role in regulation of sense tran-
 scription. In this comprehensive study, sense and antisense
 expression of 32,354 Ensembl genes in three cancerous and
 two normal cell lines was assessed, including those of *MUC1*,
 555 *MUC4*, *MUC3A/3B*, *MUC17*, *MUC16*, *MUC2*, *MUC5AC*,
MUC5B, *MUC6* and *MUC7*. The results show that high
 expression of *MUC5B* and *MUC6* antisense RNAs (Figure 1)
 was found in non-epithelial cells that do not express sense
 transcripts of these genes. These preliminary data concerning
 expression of mucin antisense RNAs will need further inves-
 560 tigation. Moreover, as epigenetic silencing of tumor suppressor
 genes by their antisense RNA has already been reported, the
 role of these transcripts in mucin gene silencing by methylation
 and histone modifications will have to be elucidated.

565 **3. Expert opinion**

It is obvious that epigenetic regulation of mucin genes is
 still at its dawn, but *in vitro* studies in cancer cell lines,
 expression studies on human tissue sections and very recent 570
 genome-wide studies using new technologies clearly indicate
 that DNA methylation of their promoters is a key molecular
 mechanism regulating their expression in epithelial cancers.
 Detection of MUC methylation may thus soon provide a
 useful biological tool for the clinician. The greatest challenge 575
 for researchers working in the mucin field is of course
 related to the huge diversity of these glycoproteins and also
 their genes (> 20 known until now), the huge size of the
 genes, their polymorphisms, and the redundancy that such a
 complex family of molecules may create. 580

We think that systematic comparison of MUC methylation
 in normal and carcinomatous tissues will provide useful
 tools for diagnosis and prognosis of numerous epithelial
 cancers, including those of lung [89], pancreas [90] and
 colon [91], but also esophageal and gastric carcinoma. Altera- 585
 tions in DNA methylation are early events in carcinogenesis
 and, as such, could become specific and sensitive bio-
 markers that could be detected in biological fluids such as
 blood (liver cancer), sputum and bronchoalveolar lavage
 (lung cancer), feces (colon cancer), urine (bladder cancer) or 590
 nipple aspirate (breast cancer) [5,92]. However, so far, methy-
 lation status for one particular gene as a reliable marker has
 been described only in rare cases (*GSTP1* in prostate cancer [93]
 or *APC* in esophageal adenocarcinoma [94]). Most of the
 time, analysis of several genes (methylome signature) is 595
 necessary to distinguish cancers from normal tissues or
 other pathologies [88,95]. Moreover, as methylation is a
 tumor-specific change in DNA, identification of panels of
 complementary biomarkers may help to classify histopatho-
 logic tumor types, tumor grades as well as tumors from 600
 different tissues [13]. Thus, methylation of genes encoding
 mucins, which are markers of cell differentiation in many
 epithelial tissues, may provide a very useful tool to clinicians
 and pathologists. 604

Table 1. Putative target genes of differentially expressed miRNAs identified in aerodigestive cancers.

miRNA	Chromosomal localization	Putative targets	Tumor type
let-7 family	Multiple loci	Ras [‡] , c-myc [‡] , HMGA2, IMP-1, ARID3B, HIC2, GM632	Kidney, colon, lung [§]
miR-100	11q24.1	FZD8, INSM1, SMARCA5, ZZE1, FRAP1, TRIB2, HOXA1, FGF3	Esophagus
miR-103-1	5q35.1	RAB10, ZHX1, OGT, HSH1, BACH2	Bladder
miR-106a	Xq26.2	MAP3K9, MAP3K11, MAPK9, FASTK, ARHGAP12, BCL2L11	Colon, pancreas
miR-107	10q23.31	FGFR2, CCNE1, MAP2K3	Colon, pancreas, stomach
miR-125b	11q24.1	BAK1, MIZF, MAP2K7, RAB6B, MMP11, MADD	Esophagus
miR-143	5q32-33	MAPK7 [‡] , MAP3K7, KRAS, HOXA5	Colon
miR-146a	5q33.3	IRAK1	Pancreas
miR-152	17q21.32	ELF5, TGIF2, DNMT1, CDC2L5	Esophagus
miR-155	21q21.3	PLR3 [‡] , FGF7, GDF6, TP53INP1	Colon, lung [§] , stomach
miR-17-5p	13q31	ZBTB4, ITGB8, EZH1, KIAA1404, GM632	Bladder, colon, lung, pancreas
miR-181b-1	1q31.3	ADARB1 CTDSPL PAWR PHF15 TNFSF11	Pancreas
miR-185	22q11.2	ZNF1A4, SLC16A2, PCDHAC2, PCDHAC1, PCDHA8	Kidney, bladder
miR-191	3p21.31	GAP43, EGR1, MAP3K12, NRCAM, CCND2, FZD5, PLCD1	Colon, lung, pancreas, stomach
miR-192	11q13.1	ATF1, CCNT2, RAB2	Esophagus
miR-194	1q41	CROP, TGIF1	Esophagus
miR-200c	12p13.31	RELN, PDCD10, PCDH8	Esophagus
miR-203	14q32.33	ZNF281, RKHD2, MGC39518, TRPS1, LPIN1	Bladder, esophagus
miR-205	1q32.2	PLCB1, CPEB2, MGRN1, ESRRG, CHN1	Bladder, esophagus
miR-21	17q23.1	BCL2, NTF3, PDCD4, RAB6A, RAB6C, RASA1, RHOB, SKI, TGFB1, TGFB2, TIMP3	Colon, lung, pancreas [§] , stomach, esophagus
miR-214	1q24.3	FGFR2, IGF1R, RAB14, RAB15	Pancreas, stomach
miR-221	Xp11.3	RIMS3, HECTD2, CDKN1B, RBM24, ZFPM2	Bladder, colon, pancreas, stomach
miR-223	Xq12-13.3	MEF2C, ZCCHC14, UE2A, SPRED1, SACS	Bladder, colon, pancreas, stomach
miR-23a	19p13.2	CCNT2, TOP1, SATB1, SEMA6D, NEK6	Bladder
miR-23b	9q22.31	CCNT2, TOP1, SATB1, SEMA6D, NEK6	Bladder

[‡]Identified targets.

[§]Prognostic and/or diagnostic markers.

Adapted from Gottardo *et al.* [73], Volinia *et al.* [75], Feber *et al.* [74] and Calin and Croce [63].

Target prediction is based on miRBase, TargetScan and MiRanda. The listed miRNAs are predicted by at least two of these prediction programs The most likely tumor-related genes are listed.

Table 1. Putative target genes of differentially expressed miRNAs identified in aerodigestive cancers (continued).

MiRNA	Chromosomal localization	Putative targets	Tumor type
miR-24-1	9q22.32	BCL2L11, TRAF7, CREBL2, IGFBP5, PCDH10, TP53INP1, RASA1, MAPK7	Colon, pancreas, stomach
miR-25	7q22.1	PCDH11X, RAB23, FZD10, CDH10, E2F3	Pancreas, stomach
miR-26b	2q35	NHS, HSHIN1, LARP1, LOC153222, DDX3X	Bladder
miR-28	3q28	SSRP1, SFRS5, RHO, GPM6A, EN2	Kidney
miR-29b-2	1q32.2	COL3A1, COL4A1, COL4A5, COL7A1, COL2A1, COL15A1, TFAP2C, CCNYL1, NKRF	colon, pancreas
miR-30c	1p34.2	PCDH10	Colon, pancreas
miR-32	9q31.3	RAB14, RAB23, RAP1B, CREM, MYCBP2, FZD10, CREB1, SMAD7, NOTCH1, PAX3, SMAD6, GAP43	Colon, pancreas
miR-7-2	15q25	SPATA2, OGT, POLE4, RSBN1, KIAA1920	Kidney
miR-93	7q22.1	CREBL1, MYCN, MAPK9	Esophagus

*Identified targets.

§Prognostic and/or diagnostic markers.

Adapted from Gottardo *et al.* [73], Volinia *et al.* [75], Feber *et al.* [74] and Calin and Croce [63].

Target prediction is based on miRBase, TargetScan and MiRanda. The listed miRNAs are predicted by at least two of these prediction programs The most likely tumor-related genes are listed.

605 Yet, this potential future promising clinical application
 depends crucially on the discovery of useful aberrantly
 methylated sequences. In this context, studying the methyl-
 ation status of mucin genes, which are aberrantly expressed in
 cancers, may help to develop these early detection tools. The
 610 development of new powerful, reliable and rapid methods,
 such as pyrosequencing or high-throughput technologies on
 chips [13,96], to screen methylated genes in tissues should
 quickly provide new information regarding mucin gene epi-
 genetic status in tissues from cancer patients and at different
 615 stages of carcinogenesis.

The studies of epigenetic regulation of mucin genes also
 point out the opposite role of secreted and membrane-bound
 mucins in carcinogenesis. Indeed, genes encoding secreted
 mucins, and especially MUC2 and MUC5B, are often
 620 aberrantly silenced in non-mucinous colon [97] and lung [98]
 carcinomas, respectively, whereas genes encoding MUC1
 and MUC4 membrane-bound mucins are overexpressed
 in cancers particularly in breast [99] and pancreatic [100]
 carcinomas, respectively. Moreover, in cancers, expression of
 625 secreted mucins is often associated with better prognosis [101],
 whereas expression of membrane-bound mucins is associ-
 ated with tumor cell detachment, invasion and metastasis
 and worse prognosis [99,102]. These data are in accordance
 with the findings that MUC2 and MUC5B promoters
 630 are hypermethylated, whereas those of MUC1 and MUC4
 631 are demethylated in cancers. Aberrant silencing of specific

genes by hypermethylation and ectopic onco-fetal expres- 632
 sion of other genes by demethylation are common features
 of cancer cells and lead to selective advantage in term of
 growth and survival. Thus, epigenetics provide a new 635
 generation of tumor suppressor genes and oncogenes [103].
 Therefore, studying epigenetic regulation of mucin genes
 may help clarify their role in carcinogenesis as candidate
 tumor suppressor genes, as has already been suggested for 640
 Muc2 [35], or oncogenes, as has already been proposed for
 MUC1 [104].

Finally, the findings that mucin genes may be epigeneti-
 cally regulated in cancers prompt us to consider them as
 attractive targets for new therapeutic strategies. As epigenetic
 changes are potentially reversible, an increasing number of 645
 specific DNA methylation or histone deacetylation pharma-
 cological inhibitors have been developed over the past few
 years. Among them, the DNMT inhibitor 5-aza (decitabine)
 is the most extensively studied and has been shown to reac-
 tivate MUC2 and MUC5B *in vitro* in numerous cancer cell 650
 lines [44]. On the other hand, the HDAC inhibitor TSA,
 which is known to induce apoptosis and enhance the
 response to chemotherapeutic agents in pancreatic cancer [105],
 has been shown to induce a severe decrease of MUC4
 expression in pancreatic cancer cells [48]. Hence, in the future, 655
 potential application of epigenetic drugs in either reactivating
 expression of secreted mucins or inhibiting membrane-bound
 mucins will have to be investigated. 658

Table 2. Predicted miRNA targeting mucin genes.

Mucin gene	Chromosomal localization	Number of predicted miRNA	List of iRNA
Membrane-bound mucins			
<i>MUC1</i>	1q21	27	miR-183 miR-193b miR-219-2-3p miR-296-5p miR-320 miR-328 miR-331-5p miR-367 miR-371-5p miR-485-5p miR-502-5p miR-509-3p miR-512-5p miR-519e miR-548b-3p miR-562 miR-587 miR-588 miR-612 miR-619 miR-647 miR-652 miR-668 miR-760 miR-885-5p miR-892b miR-923
<i>MUC4</i>	3q29	52 [†]	let-7 family* miR-10a miR-122 miR-138 miR-139-3p miR-145 miR-147, miR-147b miR-16-1 miR-193a-3p miR-197 miR-199a-5p, miR-199b-5p miR-204 miR-20a* miR-210 miR-211 miR-212 miR-219-1-3p miR-297 miR-324-5p miR-335 miR-373 miR-384 miR-411 miR-491-5p miR-500 miR-502-3p miR-548b-5p, miR-548c-5p

[†]Predicted only by miRBase.

Target prediction is based on miRBase and miRanda. The listed miRNAs are predicted by the two prediction programs.

Table 2. Predicted miRNA targeting mucin genes (continued).

Mucin gene	Chromosomal localization	Number of predicted miRNA	List of iRNA
			miR-560 miR-566 miR-574-5p miR-595 miR-611 miR-627 miR-632 miR-648 miR-652 miR-924 miR-933 miR-935 miR-98
<i>MUC20</i>	3q29	15	miR-125a-3p miR-129-3p miR-205* miR-222 miR-326 miR-330-5p miR-455-3p, miR-455-5p miR-486-3p miR-552 miR-588 miR-603 miR-611 miR-622 miR-647
<i>MUC3A/B</i>	7q22	13	let-7 family* miR-154 miR-196a, miR-196b miR-487a miR-98
<i>MUC17</i>	7q22	7	miR-302b, miR-302c, miR-302d miR-450b-3p miR-486-5p miR-769-3p miR-95
<i>MUC13</i>	3q13	16	miR-132 miR-133a, miR-133b miR-181a, miR-181b*, miR-181c , miR-181d miR-211 miR-30a, miR-30d miR-335 miR-485-5p miR-520d-3p, miR-520e miR-543 miR-633

*Predicted only by miRBase.

Target prediction is based on miRBase and miRanda. The listed miRNAs are predicted by the two prediction programs.

Table 2. Predicted miRNA targeting mucin genes (continued).

Mucin gene	Chromosomal localization	Number of predicted miRNA	List of iRNA
			miR-485-5p miR-520d-3p, miR-520e miR-543 miR-633
Secreted mucins			
11p15 mucin genes			
<i>MUC2</i>	11p15	15 [‡]	miR-128a miR-25* miR-27a miR-296-3p miR-32* miR-380 miR-431 miR-448 miR-453 miR-455-5p miR-512-5p miR-557 miR-646 miR-675 miR-92b
<i>MUC5AC</i>	11p15	10	miR-193a-3p, miR-193b miR-214* miR-423-3p miR-612 miR-638 miR-647 miR-663 miR-892b miR-922
<i>MUC5B</i>	11p15	0	-
<i>MUC6</i>	11p15	23 [‡]	miR-10a miR-139-3p miR-146b-3p miR-184 miR-206 miR-298 miR-323-5p miR-342-5p miR-34a miR-370 miR-432 miR-483-5p miR-485-5p miR-486-5p miR-492 miR-574-5p miR-585 miR-592 miR-595 miR-616

[‡]Predicted only by miRBase.

Target prediction is based on miRBase and miRanda. The listed miRNAs are predicted by the two prediction programs.

Table 2. Predicted miRNA targeting mucin genes (continued).

Mucin gene	Chromosomal localization	Number of predicted miRNA	List of iRNA
			miR-765 miR-890 miR-936
Other secreted mucins			
<i>MUC7</i>	4q13-q21	2	miR-199a-3p miR-544
<i>MUC19</i>	12q12	9	miR-140-5p miR-146a*, miR-146b-5p miR-29b

*Predicted only by miRBase.
Target prediction is based on miRBase and miRanda. The listed miRNAs are predicted by the two prediction programs.

659 Deciphering the epigenetic mechanisms regulating mucin
660 gene expression has been very helpful in understanding
mucin gene biology better, but in the future it will provide
clinicians and pathologists with new biological tools to
screen patients and detect cancer at an early stage as well as
classify tumors and propose new therapeutic strategies.

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