THE ROLE OF METHYLATION IN UROLOGICAL TUMOURS

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Summary.- Alterations in DNA methylation have been described in human cancer for more than thirty years now. Since the last decade DNA methylation gets more and more important in cancer research. In this review the different alterations of DNA methylation are discussed in testicular germ cell tumours, Wilms’ tumours, renal cell carcinomas, urothelial cell carcinomas of the bladder and adenocarcinomas of the prostate. Eventually, the potential use in diagnostics, prognostics and therapy are discussed.


DNA Methylation

Epigenetics is defined as the cellular information, other than the DNA sequence itself, which is heritable during cell division (1). Almost all cancer cells have multiple epigenetic abnormalities, which combine with genetic changes to affect many cellular processes, including cell proliferation and invasion, by silencing tumour-suppressor genes. Epigenetic alterations in cancer cells include DNA methylation, histone modification and RNA interference. However, the most studied epigenetic alteration is DNA methylation that can occur at the cytosine that precedes the guanine in a CpG dinucleotide. While CpG dinucleotides are generally underrepresented in the human genome sequence, the promoter regions of around half of the human genes contain CpG-rich areas termed CpG islands that are generally unmethylated in normal cells (2).

Three different types of DNA methylation have been observed in cancer, global hypomethylation, localized hypermethylation and loss of imprinting (3).
Hypomethylation represents a decrease of the overall methylcytosine content compared to normal tissue and is often more pronounced in advanced stages of malignant disease [4]. Methylcytosine levels can be substantially diminished, often to less than 50% of the normal level. To date, there are few reports on hypomethylation of human oncogenes associated with activation by overexpression (5). In contrast with hypomethylation, hypermethylation is not a genome-wide event, but it is found at specific sites (6). Hypermethylation has often been observed in the promoter regions of many tumour suppressor genes and is correlated with decreased gene expression. Since hypermethylation is concentrated at the promoter region it often does not become evident when overall DNA methylation is being measured. It has to be studied on a gene-by-gene basis (7). Hypermethylation is considered a key mechanism involved in silencing of cancer-associated genes and occurs at genes with diverse functions related to tumorigenesis and tumour progression and thereby hypermethylation needs to be considered as an alternative to gene mutation and deletion.

The third alteration related to DNA methylation is loss of imprinting (LOI). LOI includes partial or complete extinction of the difference between the expression levels of the maternal and paternal alleles at imprinted loci, which results in an increased or decreased overall expression. In other words, imprinted genes display a characteristic parent-of-origin-specific DNA methylation pattern that results in only a single allele being expressed (either the paternal or maternal allele) (8). Since the differences in the expression of imprinted alleles are associated with differential methylation, LOI is associated with alterations in DNA methylation. LOI has been observed most often in tumours with an embryonic phenotype (9) and has been implicated in the progression of several tumours (10).

In this review the different alterations of DNA methylation in urological tumours are discussed and eventually followed by the potential use in diagnostics, prognostics and therapy.

**Testicular germ cell tumour**

Testicular germ cell tumour (TGCT) is the most common malignancy among young males and can be classified into two main histological subgroups: seminomas and non-seminomas. Seminomas are generally undifferentiated, and morphologically they resemble the precursor stage intratubular germ cell neoplasia. Non-seminomas, on the other hand, include several histological subtypes. Among these, the embryonal carcinoma is undifferentiated and cells may differentiate along an embryonal lineage into teratomas, containing tissues from all three germ layers, or they may differentiate along extra-embryonal lineages into yolk sac tumours or choriocarcinomas [11]. In general, DNA methylation seems to increase with differentiation.

Smiraglia and co-workers demonstrated significant epigenetic differences between seminomas and non-seminomas by restriction landmark genomic scanning (12). Seminomas showed almost no CpG island methylation, in contrast to nonseminomas that show CpG island methylation at a level similar to other solid tumours. Furthermore, they demonstrated a higher level of general hypomethylation in seminomas compared to nonseminomas. The same year the group of Smith-Sørensen endorsed the significantly higher methylation frequency found in nonseminomas compared to seminomas, respectively 67% and 24%; $P=0.0003$ (13). Furthermore, they discovered a high frequency of MGMT promoter methylation (32/69) in patients with TGCT. Their conclusion was MGMT contributes to the development of nonseminoma TGCT. DNA repair genes MGMT and RASSF1A became potential target genes also due to the studies of Koul and Honorio (14,15). Subsequently, several studies on novel hypermethylated target genes have been published (16-18). PRSS21, SCGB3A1, HIC1, BRCA1, HOXA9, APC, FHIT, HOXB5, CDH13 and CDH1 were presented as novel target genes hypermethylated at high frequencies among nonseminomas. Nevertheless, only a limited number of tumour suppressor genes showed to be inactivated by DNA promoter hypermethylation in more than a minor percentage of TGCTs, including MGMT, SCGB3A1, RASSF1A, HIC1, and PRSS21. In addition, imprinting defects, DNA hypomethylation, and the presence of the unmethylated XIST gene are often observed in TGCTs.

Aberrant DNA methylation has the potential to improve current TGCT diagnostics. The PRSS21 gene can be a useful biomarker. Initially PRSS21 showed to be hypermethylated in 8 out of 8 TGCTs (16). Nevertheless, these findings have not been validated in a larger cohort of patients since. DNA methylation might also serve as a prognostic marker for treatment response. Cell line research suggests an association between differentiation, global DNA methylation status and response to chemotherapy (19). The degree of methylation might explain the chemotherapy sensitivity of TGCT. But what is the underlying mechanism for this? Normally primordial germ cells and embryonic stem cells have an open chromatin structure that provides a good DNA access. If TGCT carry a similar chromatic structure, this might
explain the extreme chemotherapy sensitivity; the state of the chromatin might determine how efficiently DNA targeting drugs can access the DNA. Subsequently, increased methylation results in a more condensed chromatin structure and a decreased chemo sensitivity (20). Koul and co-workers showed a more frequent promoter hypermethylation of RASSF1a and HIC1 among chemo resistant tumours (17). These findings did not reach statistical significance due to the limited number of patients but suggest that the promoter methylation status may serve as prognostic marker for chemo sensitivity.

Wilms’ tumour

Wilms’ tumour or nephroblastoma is a variety of kidney cancer that typically occurs in children. Wilms’ tumours are characterized by proliferation of immature renal cells, mesenchymal nephroblasts, which are hampered in their ability to differentiate into tubular epithelial cells. Furthermore, the growing tumour compresses the normal kidney parenchyma. Genetically, Wilms’ tumours are heterogeneous. The best-known tumour suppressor gene involved is the WT1 gene, which encodes a transcriptional repressor, expressed in maturing nephroblasts. WT1 mutations are observed in approximately 20% of Wilms’ tumours (21).

A further important change in many Wilms’ tumours is loss of imprinting (LOI) resulting in aberrant expression of the imprinted allele in cancer cells (22). LOI at the IGF2/H19 locus on chromosome 11p15.5 is associated with an overexpression of IGF2, to stimulate proliferation and enhance survival of mesenchymal and epithelial cells, and diminished expression of H19 with a potential tumour suppressor function. More recently, a somatic deletion targeting a previously uncharacterized gene on the X chromosome was discovered. This gene called WTX is inactivated by a monoallelic inactivation of autosomal tumour-suppressor genes, WTX is inactivated by a monoallelic “single-hit” event targeting the single X chromosome in tumours from males and the active X chromosome in tumours from females.

Despite the efforts to identify a marker useful for prognosis, tumour stage remains the most significant prognostic variable widely used in treatment regimens, separating Wilms’ tumours in two distinct subgroups: patients with either low or intermediate risk of relapse. Currently, the only molecular analysis for this stratification is based on genomic observation. Genomic losses of both 1p and 16q are considered adverse risk factors (24). Additional molecular markers are being studied to offer patients with low risk of relapse a therapy that minimizes the potential late toxicities, without decreasing the survival rates. Maschietto and co-workers found five genes, TSPAN3, NCOA6, MMP2, CD01 and MCM2, which were down regulated in patients with relapse (25).

Recently, methylation of the RASSF1A promoter was found predictive for poor outcome in Wilms’ tumours (26). Methylation was more frequent in tumours of older compared to younger children, and in advanced-stage compared to early stage tumours. However, multivariate analysis could not confirm the prognostic significance of RASSF1A methylation, possibly because of the small number of advanced stage tumours examined. Nevertheless, the methylation status of RASSF1A might be a novel biomarker to predict outcome of patients with a Wilms’ tumour.

Renal cell carcinoma

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney. Compared to four decades ago, there has been a five-fold increase in the incidence of, and a two-fold increase in mortality from, RCC (27). The disease is histopathologically heterogeneous, comprising several subtypes of which the most common (75-80%) is the clear cell subtype. Papillary (10–15%), chromophobe (5%) and other more rare forms such as collecting duct carcinoma (<1%) comprise the remainder (28).

The most frequent form of familial RCC occurs in individuals with inherited von-Hippel Lindau (VHL) syndrome. The identification of the VHL tumour suppressor gene located on chromosome 3p led to the finding that, in addition to inactivation by point mutation (50-80% of the clear cell RCCs) or deletion, the VHL gene also showed allelic inactivation by hypermethylation of the promoter region associated with transcriptional silencing in 10-15% of familial and sporadic RCC (29). VHL inactivation occurs only in clear cell tumours and similarly, methylation of VHL has been found only in clear cell RCC. Since VHL inactivation is the initiating event in familial clear cell renal tumours, it is likely an early event in sporadic clear cell renal tumorigenesis.

Fiona and co-workers studied DNA methylation profiling in both VHL-related and unrelated RCC and have shown that the RASSF1, TWIST1, PITX2, CDH13, HS3ST2, TAL1, WT1, MMP2, DCC, ICA1 and TUSC3 genes are more frequently methylated in RCC patients with wild-type VHL than in RCC patients with mutant VHL (30). These findings demonstrate
different patterns of CpG methylation in VHL and non-VHL clear cell RCC.

Recently, Girgis and co-workers conducted an integrated analysis of copy number, gene expression, protein expression, and methylation changes in clear cell RCC (31). They identified genes located in peak copy-number aberrations with concordant changes in methylation; hypomethylated in copy-number gains such as STC2 and CCND1 or hypermethylated in deletions such as CLCNKB, VHL and CDKN2A/2B. Furthermore, they found that CA-IX expression is significantly increased in CA-IX hypomethylated tumours and these patients revealed to have a better prognosis than the non-hypomethylated. Moreover, the copy number loss of CA-IX in conduction with methylation status synergistically worsens patient survival among CA-IX non-hypomethylated patients.

An extensive study on 11 RCC cell lines identified eight genes (BNC1, PDLIM4, RPRM, CST6, SFRP1, GREM1, COL14A1 and COL15A1) that were frequently (>30%) methylated in primary RCC (32). Hypermethylation and re-expression of BNC1, CST6, RPRM and SFRP1, suppressed the growth of RCC cell lines. Subsequently, in tissue samples, methylation of BNC1 and COL14A1 was associated with a poorer prognosis independent of tumour size, stage or grade. Another cell line study reported UCHL1 as hypermethylated in around a third of primary RCC (33). Hypermethylation of UCHL1 is also correlated with a poor prognosis in RCC patients (34).

With respect to Wnt antagonist family genes in RCCs, DNA hypermethylation has been observed in SFRP1, SFRP2, SFRP5, WNT, WIF1 and DKK3 genes (35). SFRP1 showed to be a significant independent predictor of RCC. The multigene methylation score showed a sensitivity of 79% and a specificity of 76% as diagnostic biomarker. Furthermore, the methylation score could significantly distinguish grade, T stage and overall survival, which makes Wnt antagonist genes an excellent epigenetic biomarker panel for detection, staging and prognosis of RCC.

A mass spectrometry analysis of a set of genes down-regulated in RCC compared to normal renal tissue in RCC identified besides SFRP1 also SCNN1B, SYT6, DACH1, TFAP2A and MT1G hypermethylated in RCC (36).

A first generation methylation array analysis of RCC identified many genes (33 out of 42) not previously reported to be methylated in >20% of primary RCC patients (37). Further characterization of these genes and their pathways may lead to the development of potential epigenetic biomarkers for diagnosis and prognosis of RCC, and in the longer term may enable more individualized treatments.

**Urothelial cell carcinoma of the bladder**

In the western world bladder cancer is the fourth most common cancer in men and the eighth most common in women (38). The most important histological subtype of bladder cancer is urothelial cell carcinoma. It accounts for 90% of all bladder tumours. The remaining 10% consists of squamous cell carcinoma and adenocarcinoma. Since these tumours are in direct contact with urine, methylation studies can focus on urine instead of tissue. Hoque and co-workers studied nine genes (APC, ARF, CDH1, GSTP1, MGMT, CDKN2A, RARbeta2, RASSF1A, and TIMP3) in urine and tissue samples of 15 bladder cancer patients and 25 controls (39). The methylation pattern in urine matched that in the primary tumour. Four genes (CDKN2A, ARF, MGMT, and GSTP1) displayed 100% specificity. Results were validated on 175 urine samples, which displayed methylation in at least one of these genes (sensitivity 69%; specificity 100%).

To identify primary bladder cancer in urine samples Renard and co-workers performed a methylation-specific polymerase chain reaction (MSP) assay on DNA from bladder tissue (40). Genes with 100% specificity were retained for subsequent MSP analysis on DNA extracted from urine samples. MSP assays performed on 466 urine samples identified TWIST1 and NID2 (sensitivity 90%; specificity 93%) for the primary detection of bladder cancer.

In another study on urine samples, DNA methylation of 10 genes, discovered by microarray analysis, were analysed by MSP (41). A panel of 5 genes (MYO3A, CA10, NKK6-2, DBC1, and SOX11 or PENK) had 85% sensitivity and 95% specificity for detection of bladder cancer (area under curve = 0.939). Analysing the data by cancer invasiveness showed a detection rate of 81% in non-muscle invasive bladder cancer and 90% in muscle invasive disease. For the detection of bladder cancer Costa and co-workers identified novel methylation markers by gene expression microarray analysis of bladder cancer cell lines and tissue samples (42). A panel comprising GDF15, TMEFF2, and VIM correctly identified bladder cancer in tissue with 100% sensitivity and specificity. In urine samples, the panel achieved a sensitivity of 94%, a specificity of 100% and an area under the curve of 0.975.

Another study of Costa focused on a set of biomarkers to identify bladder, renal and prostate cancer simultaneously (43). After gene expression
microarray analysis, PCDH17 and TCF21 were selected for further validation. Methylation levels of both genes were quantified in 12 cancer cell lines and 318 tissue samples. PCDH17 and TCF21 methylation levels provided a sensitivity rate of 92% for bladder cancer, 67% for renal cell cancer and 96% for prostate cancer. Methylation levels were differentially expressed for all tumour types compared to cancer free controls ($p < 0.001$), providing 83% sensitivity and 100% specificity for cancer detection. Although in urine samples the sensitivity was decreased considerably to 60%, 32% and 26% for bladder, renal, and prostate tumours, respectively (39% overall), absolute specificity was retained. Obviously, additional efforts are required to increase the assay’s sensitivity.

Reinert and co-workers performed a gene expression microarray analysis in 56 tissue samples (44). Independent validation was conducted in 63 samples by a PCR-based method and bisulphite sequencing. Subsequently, methylation levels in 174 urine specimens were quantified. A panel of four genes, ZNF154, POU4F2, HOXA9, and EOMES ($p < 0.0001$), achieved 84% sensitivity and 96% specificity for early detection of bladder cancer. Furthermore, they identified a marker for disease progression TBX4 ($p < 0.04$).

Currently, we are investigating a panel of 20 previously identified methylation markers by pyrosequencing analysis. Nine hundred urine samples obtained from patients from four different countries are being analysed. Patients with proven bladder cancer of all stages and grades, patients with a history of bladder cancer or controls without (a history of) bladder cancer are included. One of the objectives is to verify whether methylation analysis can be used in the follow up of patients with non-muscle invasive bladder cancer in order to decrease the number of cystoscopies needed in the follow up. Results of this by the Dutch Cancer Society supported validation study will be published soon.

**Adenocarcinoma of the prostate**

In Europe and the United States of America prostate cancer is the most common type of cancer in men. One man in six will eventually develop prostate cancer during lifetime, and up to 20% will die of this disease. Although prostate cancer can be found early through PSA testing, this analysis is not very accurate. A part of the prostate cancer patients’ progress relatively slow and often these patients die with prostate cancer rather than of prostate cancer. The other part of prostate cancer patients is characterized by a fast and aggressive grow with a high chance of metastasizing. There are two challenges in prostate cancer, early detection and discriminating these aggressive from indolent tumours.

The best single risk stratification tool described so far, Gleason grading, has several limitations. Gleason grading is consistent and highly correlated with outcome, but it is observer dependent and the chance remains that part of the prostate is not sampled correctly. For this reason there is an urgent need for new prostate cancer biomarkers.

The most studied gene affected by de novo methylation during prostatic carcinogenesis is GSTP1, which encodes an enzyme responsible for detoxifying electrophiles and oxidants, including those that threaten cell and genome damage (45). Hypermethylation of GSTP1 transcriptional regulatory sequences has been consistently detected as an early event in prostate carcinogenesis in more than 90% of prostate cancers in more than 50 independent analyses.

In addition to GSTP1, more than 40 genes have been reported to be targets of epigenetic gene silencing in prostate cancers (46). Yegnasubramanian and co-workers reported that although hypermethylation (>85%) of GSTP1, APC, RASSF1a, COX2, and MDR1 could be detected both in localized and in metastatic prostate cancers, hypermethylation of ERα, hMLH1, and p14/INK4a tended to appear only later during disease progression (47).

Jerónimo and co-workers found that methylation levels of GSTP1, APC, RASSF1A, and CRBP1, differed significantly between prostate carcinoma and high grade PIN, and/or high grade PIN or benign prostate hyperplasia ($p < 0.0001$) (48). The combined use of GSTP1 and APC demonstrated a sensitivity of 98.3% for prostate carcinoma, with 100% specificity.

Hypomethylation is less studied in prostate cancer, but it also occurs during prostatic carcinogenesis. Analyses of 5-meC content in genomic DNA have revealed hypomethylation mostly in association with disease progression; metastatic prostate cancer cases express low 5-meC levels (49). More recently, Cho and co-workers found LINE-1 hypomethylation in 53% of all prostate cancer cases, in 67% of cases with lymph node metastases but in only 8% of cases without lymph node metastases (50).

Hoque and co-workers looked at the feasibility of using urine samples of prostate cancer patients (51).
They found that a panel of 4 genes often methylated in prostate cancer (p16, ARF, MGMT, and GSTP1) would theoretically be able to detect 87% of prostate cancers with 100% specificity. This finding underlines the possibility of a non-invasive method to detect prostate cancer. Additional studies are necessary to elucidate the role of detecting aberrant methylation in urine as a tool for early detection and surveillance of prostate cancer.

Conclusions and considerations

Investigating the genetic and epigenetic changes and their biological pathways is crucial for the improvement of current diagnostics, prognostics, and drug development. Several urological tumours have the advantage of being in direct contact with urine, which enables the use of urine in for example methylation analysis. This non-invasive procedure is preferred especially in follow up investigations, but could also be very useful in the primary diagnosis providing that the sensitivity is equal or superior compared to other diagnostics.

Nevertheless, the reported frequency of methylation of genes extracted from tissue or urine DNA shows various discrepancies between different studies. There are several reasons for these discrepancies, such as differences in: the tumours studied; the technology used for analysis, the primer design and location.

The question now raises which technique is best to use and how to score methylation? Quantitative real time PCR provides more information than conventional MSP, but there still is no standardization for reporting results; direct bisulphite sequencing provides a long sequence read, but is only semi quantitative; pyrosequencing is quantitative but gives only a short read. In conclusion, all different techniques have various (dis) advantages and hopefully future studies will help us determine which technique is superior and how to score methylation the best way in order to improve the usability of methylation analysis in diagnostics and prognostics.

REFERENCES AND RECOMMENDED READINGS
(*of special interest, **of outstanding interest)


