Cancer diagnosis, risk assessment and prediction of therapeutic response by means of DNA methylation markers

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Abstract. Epigenetic alterations are heritable changes in gene expression without an accompanying change in primary DNA sequence. Two major mechanisms that cause epigenetic changes are post-translational histone modifications and DNA methylation at cytosine bases within a CpG dinucleotide. Epigenetic defects have turned out to be one of the most common molecular alterations in human neoplasia. Promoter hypermethylation is associated with loss of expression of tumour suppressor genes in cancer. The analysis of aberrant DNA methylation is gaining strength in the fields of cancer risk assessment, diagnosis, and therapy monitoring in different cancer types. These issues are discussed in this review.

Keywords: Methylation, cancer risk assessment, diagnostics, prediction

1. Introduction

Cytosine methylation occurs after DNA synthesis by enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionine to the carbon-5 position of cytosine. Cytosines are methylated in the human genome mostly when located 5' to a guanosine. CpG dinucleotides appear at about 5-fold lower than expected frequency in the overall genome [13]. Small regions with a high G+C content, so-called CpG islands, are an exception to this observation. It has been estimated that half of all human genes contain CpG islands [3]. The generally accepted definition of a CpG island was proposed in 1987 by Gardiner-Garden and Frommer. They defined it as a 200-bp stretch of DNA with a G+C content of 50% and an observed CpG/expected CpG in excess of 0.6 [27]. Takai and Jones used the complete genomic sequences of human chromosomes 21 and 22 to examine the properties of CpG islands in different sequence classes [88]. They found that regions of DNA of greater than 500 bp with a G+C equal to or greater than 55% and observed CpG/expected CpG of 0.65 were more likely to be associated with the 5' regions of genes and this definition excluded most Alu-repetitive elements.

Promoter methylation is generally inversely correlated with gene expression (Fig. 1). The repression is caused on the one hand by changes in chromatin structures due to binding of specific proteins to methylated DNA and on the other hand by decreased affinity of some transcription factors for methylated CpG sites.

In normal tissues de novo methylation of 5' CpG islands rarely occurs. In addition to imprinted genes and inactive X-chromosomal genes, CpG islands associated with non-imprinted autosomal genes can show gradual increases in methylation during ageing [43,44]. It has been increasingly recognized over the past few years that the CpG islands of many genes, which are mostly
unmethylated in normal tissue, are hypermethylated to various degrees in human cancers, thus representing tumour-specific alterations [47,57]. In addition, overall genome hypomethylation is present in most tumours. Hypomethylation of repeated or single-copy DNA sequences has been significantly correlated with disease progression for some tumours [77,79,80].

There are different principles of DNA methylation analysis such as determination of the methylation content, level or pattern. DNA methylation can be detected either by methods which use methylation-sensitive restriction enzymes or by methods which use the so called Sodium Bisulfite modification of genomic DNA [57]. The latter group of methods comprises techniques such as Methylation specific PCR (MSP) [35], MethyLight [21], or Oligo microarrays [1,4], and are mostly used in clinical studies. The basis of Sodium Bisulfite conversion of genomic DNA is the deamination of unmethylated cytosines to uracils, which allows discrimination between methylated and unmethylated cytosines (Fig. 2).

Methylation based markers have an enormous potential in (1) cancer risk assessment, (2) early detection, and (3) therapy monitoring. These applications are discussed below.

2. DNA methylation and cancer risk assessment

The identification of patients who are at high risk of cancer through screening could be very helpful for prophylactic treatment. The use of aberrant methylation as a cancer risk marker seems to be promising as recent publications show. Loss of imprinting (LOI) is an epigenetic alteration of some cancers involving loss of parental origin-specific expression of imprinted genes. Cui et al. found that about one-third of colorectal cancers undergo LOI, and that LOI is also found in the matched normal colon of the same patients [15]. To prove that LOI was not simply a developmental alteration unrelated to cancer, the authors analyzed the normal colon of patients without colorectal cancer, and found that the frequency of LOI was threefold greater in cancer patients. Furthermore it was shown that LOI could also be detected in all blood samples from the analyzed patients whereas only 12% of the control patients without cancer showed LOI in colonic mucosa and 13% in blood samples respectively. In a further study, where the authors investigated the utility of LOI as a marker of colorectal cancer risk, LOI in peripheral lymphocytes was also associated with an increased risk [14]. These data suggest that LOI may identify an important subset of the population with cancer or at an increased risk of developing cancer. The authors suggest creating a DNA methylation based blood test for the detection of individuals who are at high risk for colorectal cancer.

Another study where the role of the methylation of MLH1, a DNA mismatch repair gene, in the normal colonic mucosa of patients with colorectal cancer was analysed, showed that methylation of this gene in-
increases with age, and correlates with the occurrence of tumours which are positive for microsatellite instability [72]. DNA-Methylation in normal colon might pre-dispose the tissue directly to cancer. Thus DNA methylation has also been proposed as a candidate mediator of field defects, leading to cancer.

Germline epimutations are thought to be a basis for epigenetic disease in humans. Suter et al. hypothesized that some individuals are predisposed to develop cancer because they carry germline epimutations of tumor suppressor genes [86]. They report two patients, who fit the criteria for hereditary nonpolyposis colorectal carcinoma (HNPCC), with soma-wide, allele-specific loss of MLH1 and mosaic hypermethylation of carcinomas (HNPCC), with soma-wide, allele-specific fit the criteria for hereditary nonpolyposis colorectal suppressor genes [86]. They report two patients, who dispose the tissue directly to cancer. Thus DNA methylation increases with age, and correlates with the occurrence of tumours which are positive for microsatellite instability [72]. DNA-Methylation in normal colon might predispose the tissue directly to cancer. Thus DNA methylation has also been proposed as a candidate mediator of field defects, leading to cancer.

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These facts show the possible power of aberrant DNA-methylation for the development of screening tests to predict the individual risk for different types of cancer.

3. DNA methylation and diagnostics

DNA methylation has several advantages over protein markers, RNA or mutations. Firstly, the methylation signal can be amplified and therefore DNA methylation analysis by PCR is very sensitive. Secondly, DNA is a very stable molecule in comparison to protein or RNA. In addition, DNA hypermethylation takes place in a defined area of the gene - the CpG island in the promoter region, whereas mutations can take place in various regions of a gene. For these reasons methylation analysis seems to be a promising tool in molecular diagnostics.

For most types of cancer, early detection of the disease is associated with an improved clinical outcome. Because DNA methylation changes have been reported to occur early in carcinogenesis [56], the identification of aberrant DNA methylation offers the exciting possibility of developing diagnostic tests.

The early detection of cancer by methylation markers is based on the premise that tumour-derived DNA is released into body fluids or other remote samples and is detected by the abnormal DNA methylation patterns specific for malignant cells [58]. It is thought that tumour DNA in luminal samples (sputum, urine, stool, vaginal fluid and lavage) is derived from cells which are sloughed off from the tumour. There are several hypotheses regarding how free circulating DNA can enter the blood system. One possibility could be that free circulating DNA derives from circulating tumour cells. It is assumed that DNA enters the plasma following lysis of cells on the interface between the primary tumour and the circulation, breakdown of circulating cancer cells or destruction of tumour micrometastases. Stroun et al. showed that 10.000 tumour cells per millilitre blood would be necessary to account for the amount of free DNA circulating in blood of cancer patients [84]. This indicates that circulating tumour cells would be a poor explanation for free circulating DNA. In addition to cell lysis, apoptosis has been proposed as the origin of circulating DNA [28]. However, the phenomenon...
of spontaneous active release of DNA from cells might also be expected.

Many studies have used serum or plasma to detect aberrant DNA methylation in breast [19,41,71,81,82], colorectal [33,45,59,63,73,100], lung [2,5,6,22,26,55,65,90], head and neck [78,87,98,99], esophageal [36], liver [12,67,95–97], gastric [10,42,49,51,52,60,62,66], prostate [34,74], bladder cancer [18,91] and melanoma [39,68]. In recent years other sources of DNA have been analysed. Vaginal secretions on tampons for the detection of endometrial cancer [23] and cervical cancer [94], nipple aspirate fluid [54], breast fine needle washings [46] for breast cancer, sputum [6,38,53,75,93], bronchial brush samples [83] and bronchioalveolar lavage [8,17,50,89] for lung cancer, prostatic fluid [31], ejaculate [30,85], urine [32] and urine sediment [40] for prostate cancer, urine [9,20] or urine sediments [25] for bladder cancer, peritoneal fluid [16,69] for ovarian cancer and stool for the detection of colon cancer [7,11,61,64,70,76].

4. DNA Methylation and prediction of response to therapy

Some papers show that the methylation status of individual genes or patterns of multiple genes are associated with disease prognosis and response to therapeutics. Methylation markers which can identify subtypes of cancer could be useful in the choice of treatment. A recently published work describes methylation patterns specific for glioma grade and glioma subtypes [90].

Adjuvant systemic therapy (a strategy that targets potential disseminated tumour cells after complete removal of the tumour) has clearly improved survival of cancer patients. To date no tool is available to monitor efficacy of these therapies, unless distant metastases arise, a situation that eventually leads to death. A study where the CpG island methylator phenotype (CIMP) status, characterized by concurrent methylation of multiple CpG islands in tumour DNA, was evaluated in 103 stage III colorectal cancer samples from patients treated with surgery alone and in an additional 103 cases from patients treated with surgery and adjuvant 5-fluorouracil-based chemotherapy. This showed that CIMP has independent predictive significance for the survival benefit from adjuvant 5-fluorouracil (5-FU) chemotherapy in colorectal cancer [92]. The authors concluded that a positive CIMP has independent predictive significance for the survival benefit from 5-FU chemotherapy in colorectal cancer and that this molecular marker should be incorporated into prospective clinical trials of fluorouracil-based therapies to confirm its clinical value. Recently in a study of 148 patients with breast cancer receiving adjuvant therapy the measurement of serum DNA methylation of RASSF1A was used to monitor treatment efficacy [24]: Disappearance of RASSF1A DNA methylation in serum throughout treatment with tamoxifen indicates a response, while persistence or new appearance means resistance to adjuvant tamoxifen treatment.

In a study of 47 melanoma patients who received biochemotherapy it was shown that detection of circulating methylated RASSF1A DNA in serum can predict response to the biochemotherapy and disease outcome [68].

DNA methylation is potentially an important driving force for drug resistance. There is growing evidence that DNA methylation of genes, which are directly involved in drug responses, has a potential role in predicting disease outcome following chemotherapy. The analysis of MLH1 methylation in plasma samples of ovarian cancer patients before chemotherapy and at relapse showed that 25% of patients acquired MLH1 methylation during chemotherapy, which was independently associated with poor overall survival [29].

It remains to be seen whether modifications made in adjuvant therapeutic strategies based on detection of circulating nucleic acids will improve survival as well as quality of life.

5. Conclusion

Over the past few years the field of epigenetics in cancer has moved centre stage. It has become apparent that cancer is as much a disease of aberrant epigenetics as it is a disease of genetic mutations. Epigenetic silencing is now recognized as a “Third pathway” in Knudson’s model of tumour-suppressor gene interaction in cancer. Knudson’s hypothesis that two hits are required for the full inactivation of a tumour-suppressor gene has been shown to be fundamentally correct in almost all cases of human cancer. So the suggested pathways for the inactivation of tumour suppressor genes are intragenic mutations, loss of chromosomal material (loss of heterozygosity (LOH) or homozygous deletion) and DNA methylation [48].

The ubiquity of DNA methylation changes has opened the way to risk assessment, early detection, molecular diagnostic of resected specimens, chemoprevention and monitoring for disease recurrence. How-
ever, if we are to use these changes in cancer diagnostics or even to modify epigenetic information with demethylating agents, we must gain more insight into the mechanisms that underlie the DNA methylation changes in cancer cells.

The different methods for detecting methylation markers are not as yet validated and standardized. But a successful clinical diagnostic assay requires careful consideration of several issues. Firstly optimal sample quality is a prerequisite to generate valid quantitative data. Hence sample collection, transport and preparation are all critical parameters in test performance and must be optimized and standardized. Secondly analytical sensitivity and specificity are critical parameters of any diagnostic assay. Analytical sensitivity refers to the smallest number of molecules that can be detected and distinguished from a zero result. Analytical specificity is determined by the degree to which analytes other than that in question react in an assay; the higher the level of cross-reactions, the lower the analytical specificity. Finally there must be agreement between replicates within and between runs of the assay, as this provides important information about the reliability of the assay. Repetitability is measured as the amount of agreement between replicates tested in different analysis. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents and controls). It is important to maintain the internal quality control by monitoring the assay for both parameters. Therefore the protocols for sample preparation and analysis must meet strict requirements for consistency and performance.

Epigenetic markers are now under clinical trials. Based on the published studies it appears that methylation markers will play an exciting role in cancer in the future.

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