[Frontiers in Bioscience 11, 2647-2656, September 1, 2006]

Plasma epigenetic markers for cancer detection and prenatal diagnosis

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1. ABSTRACT

Successful detection of circulating nucleic acids has opened up new possibilities in cancer testing and prenatal diagnosis. Circulating DNA markers are useful in cancer detection, prognostication and monitoring. Cancer-associated molecular changes which can be detected include gene mutations, chromosomal rearrangements, microsatellite alterations, viral sequences, and, to be discussed in more detailed, gene promoter hypermethylation. Methylation analysis is commonly performed by DNA digestion with methylation-sensitive restriction endonucleases followed by polymerase chain reaction (PCR), or bisulfite modification followed by methylation-specific PCR (MSP). The detection of fetal DNA in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis. However, circulating fetal DNA detection has been based on exploiting gender and polymorphic differences between the fetus and mother. The recent discovery of epigenetic differences between the maternal and the fetal DNA detectable in maternal plasma has launched a hunt for fetal-derived epigenetic markers in maternal plasma. It is hoped that this type of universally applicable markers would be made available in a clinical diagnostic setting in the near future.

2. INTRODUCTION

The discovery of cell-free nucleic acids in the circulation by Mandel and Metais in 1948 has opened up exciting possibilities for the molecular detection of human diseases (1). However, the realisation of this goal has taken several decades. In 1989, tumor-derived DNA sequences were detected in the plasma of cancer patients by Stroun and coworkers (2). In the field of prenatal diagnosis and monitoring, Lo and coworkers showed that fetal-derived Y chromosomal sequences were detectable in the plasma of pregnant women (3). From then on, the field has rapidly developed, with new DNA- and RNA-based markers related to different clinical conditions.

Epigenetic markers based on differential DNA methylation of genes are among one type of molecular markers used in cancer detection. Earlier studies had reported on the silencing of tumor suppressor genes by promoter methylation in certain tumors (4). A few years later, advancement in molecular techniques allowed the detection of methylation changes similar to those in the tumor tissues from the plasma of cancer patients (5).

While there are numerous reports on plasma epigenetic markers for cancer detection, the mission to find useful plasma epigenetic markers for noninvasive prenatal diagnosis has progressed relatively more slowly (6, 7). Prior to such developments, circulating fetal DNA detection has generally been carried out by exploiting gender (8-11) and polymorphic (12, 13) differences between the fetus and the mother. It is hoped that some

differentially methylated loci between the fetus and the mother could result in gender- and polymorphism-independent noninvasive prenatal diagnostic tools.

3. CIRCULATING PLASMA DNA AS MOLECULAR MARKERS

3.1. Plasma DNA in cancer patients

In 1989, Stroun and coworkers demonstrated that tumor-associated DNA sequences were detectable in the plasma of cancer patients. Subsequent studies by various groups had identified tumor-derived gene mutations (14, 15), chromosomal rearrangements (16), microsatellite alterations (17, 18), viral sequences (19, 20), and promoter hypermethylation (21, 22) in the plasma of cancer patients. The use of real-time methylation-specific PCR (real-time MSP) to detect these tumor-associated sequences in patients' plasma has further improved the sensitivity of the assays in a quantitative manner (5, 20, 23).

3.2. Plasma DNA in pregnant women

While researchers were exploring ways to make use of fetal cells in maternal circulation (24-26) as a substitute to invasive procedures such as chorionic villus sampling and amniocentesis, the discovery of circulating fetal-derived DNA sequences in maternal plasma by Lo and coworkers has opened up new possibilities in noninvasive prenatal diagnosis and monitoring (3). In contrast to the rarity of circulating fetal cells, cell-free fetal DNA in the maternal circulation is detectable from the first trimester of pregnancy onwards (27), with increasing concentrations as pregnancy progresses (28). Moreover, the circulating fetal DNA species are stable, yet rapidly cleared after delivery (29).

By detecting the circulating Y-chromosomal sequences in maternal plasma, quantitative aberrations of fetal DNA have been reported for different clinical conditions. These include preterm labor (8), certain fetal chromosomal aneuploidies (9), preeclampsia (10, 11), and so on. Some other markers are based on detecting paternally-inherited polymorphisms (30-32). However, a new class of fetal DNA markers which are applicable to all pregnancies is evolving (6, 7). Such markers are identified based on the different epigenetic signatures between maternal and fetal cell populations.

4. DETECTION OF EPIGENETIC CHANGES

4.1. DNA methylation as an epigenetic phenomenon

Epigenetic changes denote alterations in phenotype that are unrelated to DNA sequence changes. Examples of common epigenetic phenomena include methylation of the cytosine residues at CpG dinucleotides, usually in a gene promoter region (33), histone deacetylation, and chromatin remodeling (34). These changes may result in gene silencing. Among these, CpG methylation is a well-described epigenetic phenomenon although the detailed underlying mechanism and the complex interactions between all epigenetic events have yet to be completely elucidated.

4.2. Detection of DNA methylation

It has been known for many years that the cleaving of DNA sequences by certain restriction endonucleases is affected by the methylation status in the targeted DNA sequences (35). Some researchers then started to analyze methylation patterns with restriction enzyme digestion followed by Southern blotting (36), and restriction landmark genomic scanning (RLGS) (37). In a recent paper, Melnikov and coworkers analyzed the methylation status of several cancer cell-lines with methylation-sensitive restriction enzymes and multiplexed PCR (MSRE-PCR) (38).

On the other hand, Frommer and coworkers developed a technique based on bisulfite modification back in 1992, in which cytosine was converted to uracil while 5-methylcytosine remained unchanged. Cloning and sequencing of this bisulfite-converted product revealed the methylation profile of the original template down to a single molecule level (39, 40). Further advancement based on bisulfite modification was described by Herman and coworkers, who introduced methylation-specific PCR (MSP) as a new procedure to detect the methylation status of the CG dinucleotides in a CpG island (41). Later on, the development of fluorescence-based real-time MSP by Lo and coworkers further allows a quantitative dimension in this type of studies (5). Likewise, MethyLight is another example of a fluorescence-based real-time PCR technology that is capable of quantitating DNA methylation (42). Moreover, genomewide screening of hypermethylation in cancer cells can be achieved with an array-based method, termed differential methylation hybridization (DMH) (43).

Besides the bisulfite-based approach, Rauch and coworkers described a method termed methylated-CpG island recovery assay (MIRA). Such an approach relied on the specific binding of methyl-CpG-binding domain proteins to methylated DNA sequences. PCR was performed on the specifically bound DNA for the detection of DNA methylation (44).

5. ABERRANT DNA METHYLATION IN THE PLASMA OF CANCER PATIENTS

5.1. DNA methylation in tumors of cancer patients

Aberrant DNA methylation in tumor tissues has been well-documented for different cancers. It is believed that hypermethylation of the promoter CpG island of tumor suppressor or related genes is associated with gene silencing (45, 46). Recent literature has documented that promoter hypermethylation triggers local histone code modifications. Gene promoters are sequestered away from transcription factors, hence resulting in stable gene silencing. Some workers believe that this process may affect virtually every pathway important to cancer formation (47). Methylation profiling of the human genome reveals tissue-specific epigenetic signatures (48), while tumor-specific signatures are observed in different cancers (47). Examples of tumor suppressor genes hypermethylation in various types of cancer are described as follows.

Gene silencing by 5' CpG island hypermethylation was identified in the tumor suppressor gene (TSG), the *von Hippel-Lindau* (*VHL*) gene, in renal carcinoma (4). In addition, methylation of the promoter CpG island of the TSG *p16 (INK4A)* was found in prostate cancer (49), lung cancer (50), colon cancer (51), ovarian cancer (52), and other cancers. Another TSG, *retinoic acid receptor-beta2* (*RARbeta2*), is silenced by its promoter methylation in lung carcinomas (53, 54), breast cancer (55), esophageal squamous cell carcinoma (56), and so on. Another example of such genes is *breast cancer 1* (*BRCA1*) (57). Transcriptional inactivation by its promoter hypermethylation has been reported in sporadic breast cancer (58, 59) and ovarian tumors (60, 61). Aberrant methylation of the *adenomatous polyposis coli* (*APC*) gene promoter is also reported in breast and lung carcinomas (62), colorectal cancer (63), and adult T-cell leukemia/lymphoma (64). Furthermore, promoter hypermethylation of *RAS association domain family protein 1A* (*RASSF1A*) is detected in lung, breast and ovarian tumors (65), bladder carcinomas (66), colorectal cancer (67, 68), Wilms' tumor (68), uterine cervix adenocarcinoma (69), and esophageal squamous cell carcinoma (70). As the last example, promoter hypermethylation of the *RUNX3* gene was extensively studied by Homma and coworkers in gastric cancer and was found to be useful in cancer diagnosis and risk assessment (71). *RUNX3* promoter hypermethylation was also found to be an important molecular event in lung adenomcarcinoma (72).

5.2. DNA methylation changes detected in plasma of cancer patients correspond to those identified in tumors

Aberrations in DNA methylation patterns have been reported in the tumor tissues and cancer cells in a wide range of malignant diseases, as discussed previously. In addition, corresponding DNA methylation changes have also been found to be detectable in the patients' plasma samples. The identification of such markers would be of great value to noninvasive cancer detection and monitoring.

In 1999, Wong and coworkers discovered an identical p16(INK4a) methylation change between the tumors and the plasma in a group of liver cancer patients (21) and later concluded that real-time quantitative MSP would be useful in HCC detection and monitoring (73). In addition, Silva and coworkers published comparable findings in the plasma of breast cancer patients (22). This *p16* hypermethylation was then found to have potential diagnostic and prognostic value for breast cancer (74). Moreover, similar *p16* hypermethylation in tumor tissues and plasma samples was also detected from non-small cell lung cancer (NSCLC) patients (75-77), head and neck squamous cell carcinoma (HNSCC) patients (78), and nasopharyngeal carcinoma (NPC) patients (79). In addition, concurrent *p16* and *p15* hypermethylation was detected in the plasma of patients with acute leukemias (80) and hepatocellular carcinoma (HCC) (81).

It is known that hypermethylation of the promoter of the *glutathione S-transferase P1* (*GSTP1*) gene is found in a proportion of prostatic carcinoma. By using MSP, Goessl and coworkers were able to detect *GSTP1* promoter hypermethylation in a proportion of tumors, plasma and other bodily fluids from prostate cancer patients (82, 83). Their findings have been confirmed by other groups, showing a strong correlation between detectable aberrant methylation of the *GSTP1* promoter in prostate cancer patients' tumor tissues and their plasma samples (84-86).

In a study by Kawakami and coworkers, promoter hypermethylation of the *APC* gene was identified in both esophageal adenocarcinoma and esophageal squamous cell carcinoma. The same methylation aberration was detected in the plasma of some patients (87). They found that high levels of methylated *APC* in plasma were associated with reduced patient survival. In another study, promoter methylation of *APC* was also described in the plasma and/or serum in a cohort of lung cancer patients (88).

In a study on TSG inactivation by epigenetic changes in cutaneous melanoma, a profile of methylated regions were identified in the tumors and plasma of the patients (89). Among those genes, methylated *RASSF1A*, together with the *O-6-methylguanine DNA methyltransferase* (*MGMT*) gene and *RAR-beta2*, were present in the plasma of a proportion of the patients. In a group of pediatric cancer patients, hypermethylated *RASSF1A* was found in a variety of tumors. *RASSF1A* methylation was also found in the patients' plasma during chemotherapy. This is associated with cell death and good response to treatment (90). In addition, methylated *RASSF1A* in HCC patients' plasma was found to be associated with the size of the tumor (91).

The followings are further examples on the detection of identical methylation changes in tumors and plasma of cancer patients. *Death-associated protein (DAP)-kinase* is a gene frequently found inactivated in cancer by promoter methylation. This methylation pattern was also observed in the plasma in NPC patients (79, 92). Another gene is the DNA mismatch repair gene *hMLH1*, which is found hypermethylated in both the tumor and plasma of a proportion of ovarian cancer patients (93). It is known that *E-cadherin* (*CDH1*) plays a role in controlling cell proliferation, invasion and metastasis. Hypermethylated *CDH1* was found detectable in patients with breast cancer (74) and NPC (79).

Table 1 shows a selection of hypermethylated genes and their corresponding cancers, with detection rates in the tumors and the plasma samples of patients.

6. EPIGENETIC MARKERS FOR PRENATAL DIAGNOSIS

6.1. Detection of aberrant methylation or genomic imprinting disorder for prenatal diagnosis

In some earlier studies, molecular analysis on fragile X syndrome, characterized by amplification of a CGG repeat and aberrant DNA methylation, was reported. Such aberrant methylation was observed in full fragile X

Table 1. A selection of hypermethylated genes in their corresponding cancers

Gene	Cancer	Detection in tissue DNA, %	Detection in plasma DNA, %	Reference
p16(INK4A)	HCC[6]	16/22, 73%	13/16, 81%	21
	Breast cancer	8/35, 23%	5/8, 63%	22
	NSCLC[7]	73/92, 79.3%	64/73, 87.7%	75
	NSCLC	22/35, 63%	12/22, 55%	76
	NSCLC	14/33, 42%	2/33, 6%	77
	NPC[8]	~	17/41, 42%	79
GSTP1[1]	Prostate cancer	16/17, 94%	23/32, 72%	82
	Prostate cancer	18/20, 90%	23/32, 72%	83
	Prostate cancer	63/69, 91.3%	6/69, 13.0%	84
APC[2]	Esophageal adenocarcinoma	48/52,92%	13/52, 25%	87
	Esophageal SCC[9]	16/32, 50%	2/32, 6.3%	87
	Lung cancer	95/99, 96%	42/89, 47%	88
RASSF1A[3]	Cutaneous melanoma	49/86, 57%	6/31, 19%	89
	HCC	37/40, 92.5%	17/40, 42.5%	90
DAP-kinase[4]	NPC	~	8/41, 20%	79
	NPC	12/12, 100%	6/12, 50%	92
CDH1[5]	Breast cancer	9/36, 25%	7/36, 20%	74
	NPC	~	19/41, 46%	79

Detection rates in the tumor tissues and in plasma are listed. Abbreviations: [1] *GSTP1*, *glutathione S-transferase P1*; [2] *APC*, *adenomatous polyposis coli*; [3] *RASSF1A*, *RAS association domain family protein 1A*; [4] *DAP-kinase*, *Death-associated protein kinase*; [5] *CDH1*, *E-cadherin*; [6] HCC, hepatocellular carcinoma; [7] NSCLC, non-small cell lung cancer; [8] NPC, nasopharyngeal carcinoma; [9] Esophageal SCC, esophageal squamous cell carcinoma.

mutations in chorionic villi and other fetal tissues (94, 95). However, DNA methylation of the *FMR-1* gene was found to be inconsistent in chorionic villi of the affected individuals in some later studies (96, 97).

Some of the fetal abnormalities are related to genomic imprinting. Genomic imprinting is a natural phenomenon in which only the maternally-inherited allele or the paternally-inherited allele is expressed in certain loci in our genome. Under normal circumstances, the silencing of the opposite allele is usually achieved through CpG methylation of the promoter of the gene. However, imprinting disorders have been reported. For example, the loss of maternally or paternally imprinted loci within the chromosome 15q11-q13 region would result in Angelman syndrome (AS) or Prader-Willi syndromes (PWS). Methylation analysis on specimens obtained from amniocentesis and chorionic villus sampling (CVS) correctly diagnosed the major molecular classes (deletion, uniparental disomy and imprinting mutation) of PWS and AS (98). However, the consistency of the methylation analysis is tissue-dependent, with fetal blood as the most reliable sampling material. Accurate diagnosis of the diseases is usually made in a combination of morphological, cytogenetic and other molecular analyses (99, 100).

6.2. Differential methylation between mother and fetus

Regardless of the inconsistency in methylation analysis on amniotic fluid and CVS for the detection of imprinting disorders, genomic imprinting, as a phenomenon, was shown to be useful in detecting fetal-derived DNA in maternal plasma.

In 2002, Poon and coworkers first outlined the strategy for using fetal epigenetic markers for the purpose of plasma-based noninvasive prenatal diagnosis. Poon *et al* made use of a differentially methylated region and a single-nucleotide polymorphism (SNP) to prove that the maternally-inherited fetal allele was distinguishable from the background maternal DNA in maternal plasma (6). The target was within the *IGF2-H19* locus, in which the paternally-inherited allele is always methylated and the maternally-inherited allele is always unmethylated. By using MSP and primer extension, the fetal-derived unmethylated allele, which was inherited from the mother's methylated allele, was detectable in maternal plasma.

At the moment, detection of the existing circulating fetal DNA markers largely depends on using genetic traits which the fetus has inherited from the father, and which are distinguishing from the maternally-derived DNA in the plasma of the pregnant woman. While some researchers are trying to explore the possibilities of using gender- and polymorphism-independent fetal RNA markers for noninvasive prenatal diagnosis and monitoring (101, 102), Poon's work has highlighted the possibilities to identify universal fetal DNA markers by means of an epigenetic approach.

6.3. Methylation pattern of the placenta

Following the demonstration of the successful detection of a fetal-derived differentially methylated locus in maternal plasma, researchers started to search for additional differentially methylated loci in the maternal circulation. Based on the characteristic of the trophoblasts in early pregnancy (103), a direction for investigation is outlined.

It is known that gene silencing by promoter CpG methylation is a mechanism contributing to neoplastic development. During early pregnancy, the invasiveness of the trophoblastic cells resembles that of the malignant cells in cancer patients. It is possible that DNA methylation and demethylation may play an important role during embryonic development in controlling gene expression. Furthermore, trophoblasts release placental-specific DNA into the maternal circulation while cancer cells release tumor-specific DNA into the patient's circulation. Based on the similarities between pregnancy and cancer, Muller and

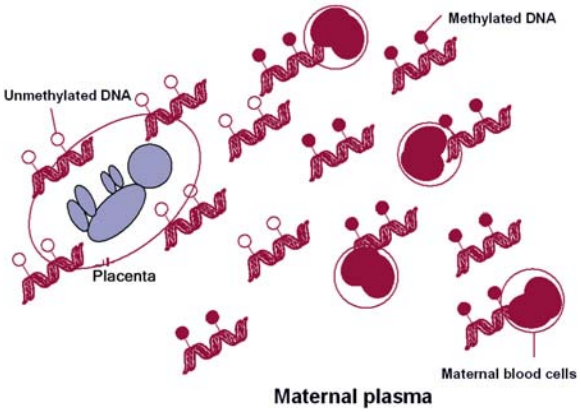

Figure 1. Hypomethylated DNA sequences in placenta versus hypermethylated DNA sequences in maternal blood cells. Bisulfite sequencing results confirm that the *maspin* promoter sequences are hypomethylated in placental tissues and hypermethylated in maternal blood cells. Both placenta and maternal blood cells release DNA into the maternal plasma. By using methylation-specific PCR, the hypomethylated DNA sequences derived from placental tissues can be specifically detected in maternal plasma.

coworkers were able to identify similar DNA methylation changes in the circulation of early gestation pregnant women and advanced breast cancer patients (104). However, this observation does not apply to all the genes that are found methylated in various forms of cancer. Hypermethylation of some genes are only documented in certain gestational trophoblastic diseases.

Promoter hypermethylation of the *tissue inhibitor of metalloproteinase 3* (*TIMP3*) gene in choriocarcinoma was described (105). A more comprehensive study on the analysis of hydatidiform mole and choriocarcinoma revealed a significant difference in the methylation pattern of the promoter regions of a number of genes, when compared with normal trophoblasts. These genes include *E-cadherin*, *hypermethylated in cancer 1* (*HIC-1*), *p16*, *TIMP3* for choriocarcinoma, and *E-cadherin*, *HIC-1*, *p16* for hydatidiform mole. Aberrant methylation of these genes was observed in the abnormal trophoblasts while none of the normal placenta showed hypermethylation (106). In addition, aberrant methylation of *PTEN* in molar pregnancy was also reported by another group (107).

6.4. Fetal-derived methylation pattern in maternal plasma

Since only a proportion of genes found to be methylated in cancer appear to be methylated in normal placenta, additional approaches should be used in the exploration of fetal epigenetic markers. In fact, any genes that show a differentially methylated pattern between placenta and maternal blood cells would be useful in the development of universal DNA markers. Given that plasma DNA is of hematopoietic origin (108), the identification of genes that are hypomethylated in placenta and hypermethylated in the maternal blood cells would also be of practical use.

Poon and coworkers' work has initiated a search for fetal epigenetic markers which can be used in all pregnancies. Recently the epigenetic signature of the *maspin* gene has been shown to be different between the placenta and maternal blood cells (7). The unmethylated form of the *maspin* gene is found in the placenta, whereas the maternal blood cells are densely methylated in this region (Figure 1). By using real-time quantitative MSP, the placental-derived unmethylated *maspin* has been detected in maternal plasma and has been found to be cleared 24 hours after delivery. Moreover, the level of this unmethylated form of the gene has been found to be elevated in preeclamptic pregnancies when compared with normal pregnancies. The results were comparable to those using a Y chromosomal marker, except that the *maspin* epigenetic marker can be applied in all pregnancies, regardless of the gender of the fetus.

7. PERSPECTIVE

Studies on circulating nucleic acids in cancer patients and pregnant women have grown rapidly over the last few years. The circulating nucleic acid approach is potentially beneficial to patients due to its lack of invasiveness. In the search of the ideal plasma nucleic acid marker, different types of markers have a different profile of advantages and disadvantages. For epigenetic DNA markers, the main advantages include the following: first, DNA molecules are stable. Second, gene silencing by promoter methylation appears to be a common event in many cancers. The main disadvantage concerns the methodology that is currently used for methylation analysis, as bisulfite-based methods are relatively difficult to be standardised (109-111) and such methods tend to degrade most of the target DNA in a particular sample (112). Furthermore, for the fetal DNA field, there is also a need to increase the number of epigenetic markers which can be used to distinguish the mother from the fetus. With the launching of the Human Epigenome Project (HEP) (113-115) and the development of new, high throughput methods for methylation analysis, such as mass spectrometry (116, 117), it is believed that more and more plasma epigenetic markers would be developed in the near future.

For the eventual clinical application, standardization of protocols between laboratories is very important. This would mean that a standard protocol is needed from sample collection (118, 119), to sample processing, including centrifugation (120), DNA extraction (3, 121) and DNA analysis (122, 123). If and when all these are achieved, it is expected that many new epigenetic markers will be able to impact clinical decision making.

8. ACKNOWLEDGEMENT

Supported by the Hong Kong Research Grants Council Earmarked Research Grant CUHK 4279/04M.

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Key Words: Plasma, Epigenetic markers, Cancer detection, Prenatal diagnosis and monitoring, Methylation, Methylation-sensitive restriction endonucleases, Polymerase chain reaction, Bisulfite modification, Methylation-specific PCR, Tumor suppressor genes, Genomic imprinting, Differential methylation, Review

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