Review Article

Serum/plasma DNA methylation pattern and early detection of breast cancer

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ABSTRACT

Breast cancer is the most common cancer among women. With its fatality rate reduced significantly if diagnosed early, developing cost-effective, noninvasive methods of early detection is highly investigated. Currently, mammography with magnetic resonance imaging is considered the optimal method of early detection in women who are at a significantly raised risk of developing breast cancer. Due to environmental effects and life-style changes in recent years, elevation of the risk of cancer incidents in lower risk populations is observed and therefore, the development of a relatively easy-performed and low-cost method for early detection of cancer in general and breast cancer in particular is needed. Serum-based analysis techniques have been quite popular subject of research recently as they can be performed with low technical knowledge, become automated and are cheap. In the present article, we have reviewed the literature related to the use of DNA methylation-detection based techniques for diagnosis of early-stage breast cancer using serum or plasma circulating tumor DNA and their power as a future biomarker. A reference to all genes that is reported to be differentially methylated in breast cancer accompanies the article.

Key words: Breast cancer, circulating tumor DNA markers, early detection, methylation pattern

INTRODUCTION

The most frequently diagnosed cancer among women and the second most common cancer overall (except for skin cancers) is breast cancer. According to estimates of the American Cancer Society in 2014, about 200,000 new cases of invasive breast cancer in women will be diagnosed, and about 40,000 of them will die.[1] In the Middle East, according to the estimate of the Iranian Ministry of Health and Medical Education in year 2011, breast cancer ranked first among female cancers, comprising 21.4% of all types of cancer. In developing countries, breast cancer affects women about one decade younger than it does in developed countries; the same situation is reported in Iran.[2,3]

Identifying risk factors for breast cancer could help choosing screening decisions. It has been revealed that two main risk factors for breast cancer are: Extremely dense breast tissue and patient's first-degree relatives with breast cancer, especially if diagnosed at younger age. Each category was associated with more than two-fold increased risk of breast cancer in women age 40–49. Many more risk factors such as race/ethnicity, physical activity, alcohol consumption, cigarette smoking and oral contraceptive pills is investigated, but none of them has been confirmed.[4,5]

It is notable that only 5–12% of patients with breast cancer are in the high risk group carrying inherited factors;[1] thus other risk factors collectively constitute the cause for the great majority of breast cancer emergence. The effects of these factors are not detectable at very early stages by genetic screening.

Breast cancers are broadly categorized into *in situ* carcinoma and invasive (infiltrating) carcinoma. *In situ* breast carcinoma is sub-classified and distinguished on the basis of growth patterns and cytological features as either lobular or ductal carcinoma *in situ* (LCIS and DCIS respectively). DCIS is considerably more common than LCIS.[6] Breast cancer at the gene expression level is categorized into five molecular subtypes: Luminal A, luminal B, HER2+, basal-like, and normal-like each with distinct gene expression patterns. Different subtypes of

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breast cancer tumors have shown a significant difference in overall survival rates.[7-9] Immunohistochemistry and gene expression pattern (Mammaprint and Oncotype DX) are usually the methods of choice to detect these prognostic markers. Accurate classification of breast cancer subtypes coupled with early detection is critical to effective cancer treatments. However, the heterogeneity of different tumors and relatively high prices for these tests can be restrictive, making blood-based techniques appealing clinically.

BREAST CANCER DETECTION METHODS

Disease screening consists of actions taken to find a disease or condition at very early states while the patient has no symptoms or complaints. The recommended available screening methods for early detection of breast cancer are mammography, clinical breast examination (CBE) and breast self-examination (BSE).[2] Currently, mammography with magnetic resonance imaging (MRI) is recommended as the optimal and the most accurate method of early detection of breast cancer in women with significantly raised risk of breast cancer.[10,11] Any woman of 30 years of age or older, with a suspicious breast symptom or sign should undergo further investigations including CBE and diagnostic mammogram.[12]

Mammography screening has a sensitivity (77–95%) and specificity (94–97%), respectively.[13] Sensitivity of mammography in women at increased risk of breast cancer is considerably lower (33–56%). MRI is a highly sensitive substitute screening method to detect breast tumors not seen in mammography.[10,11,14] Since mammography is thought to be more sensitive than MRI in detecting microcalcifications in early stages of breast cancer, they can be considered as complementary. Positive finding on either screening mammography or MRI mandates further diagnostic evaluations.[10,11] There is little evidence to support for using BSE or CBE as useful screening tools.[5,13]

In the past decade, a very active area of cancer research has been the development of diagnostic methods based on readily accessible body fluids (urine, nipple discharges, blood, and saliva).[15] Here, we discuss the most promising investigations, which are based on blood molecular markers. One method to detect cancer is an investigation of the patterns of proteins in serum and other bodily fluids. It seems that SELDI-TOF's specificity and sensitivity in the serum proteome profiling are greater than some older methods, despite its technical difficulties, some studies showed that protein markers are valuable complimentary markers to diagnose cancer.[16-19]

CIRCULATING TUMOR DNA

Scientists for more than 50 years have been able to detect small quantities of DNA in the plasma of normal individuals. In patients with conditions such as cancer and chronic autoimmune disorders, increased quantities of circulating DNA are detected. Cell-free nucleic acids in plasma were reported in 1948 by Mandel *et al*. for the 1st time but recognized in 1977 and reported by Leon *et al*. In this study, the elevated circulating tumor DNA (ctDNA) concentration in serum of cancer patients was compared to normal controls.[20-22] Tumor DNA has been identified in the serum and plasma of different cancers patients[23] as reported in breast cancer patients and 3–4 times more ctDNA has been reported to be present in the serum of these patients when compared with that of healthy controls.[24]

The source of this cell-free DNA remains unclear. Different hypotheses are proposed for the origin of this circulating DNA. A tiny amount may originate from blood lymphocytes. Other possible sources of ctDNA include released DNA from necrotic or apoptotic tumor cells of primary and metastatic tumors or tumor DNA secretion and a partly contamination of serum by DNA released from leukocytes.[20,25-28] The most common hypothesis for ctDNA origin in the serum of cancer patients is the lyses of circulating tumor cells (CTCs).[26,27] There is also some evidence that DNA is released from the tumor cells as a glyconucleoprotein complex, which may protect it from nucleases.[25]

Ashworth reported existence of CTC in the peripheral blood of patients with cancer for the 1st time in 1869;[29] from then existence of CTC of many different tumors in the peripheral blood of patients has been revealed and been thought to be a source of ctDNA. Among all the cancers in women, presence of CTC in the blood has been most investigated in breast cancer.[30]

Different studies have searched for genetic and epigenetic alterations in plasma ctDNA in different types of cancers. ctDNA can be used for the development of noninvasive tests for cancer patients in screening, prognosis, prediction, monitoring of therapies and drug resistance.[24,25,31-33] In recent years, many studies have shown the usefulness of ctDNA as a surrogate marker for breast cancer detection.[26,34,35] Several studies have shown that circulating DNA of cancer patients contains most of the DNA alterations specific to cancer.[20,21,25] The sensitivity of polymerase chain reaction (PCR) techniques in the detection of these alterations in plasma DNA may be as high as 86%; and the specificity shown in these studies range from about 28% to 100%.[36] In cancer patients, the same mutations such as K-ras, N-ras, p53, have been found in tumor tissue and

ctDNA.[20,33] Divella *et al*. quantified the hTERT gene in the plasma of patients with primary breast cancer and tested its correlation with the clinical parameters of disease. They used reverse transcription-PCR and it was revealed that circulating hTERT DNA has a better diagnostic value than glycoprotein cancer antigen 15.3 biomarker in early breast cancer disease and hTERT could be a possible candidate as a tumor marker in patients with histology of infiltrating ductal carcinoma.[37] Chen *et al*. isolated DNA from the plasma or serum of patients with a suspected diagnosis of breast cancer to analyze this DNA for the presence of microsatellite instability and loss of heterozygosity and compared it with corresponding tumor DNA.[38]

Changes in the status of DNA methylation represent one of the most common molecular alterations in human neoplasia including breast cancer. These epigenetic alterations induce neoplastic process by transcriptional silencing of tumor suppressor gene expression and are responsible for initial steps of induction of tumor cell proliferation. Therefore, analysis of gene methylation patterns in tissues has the potential to play a profound significance in the early detection of cancer.[39] The most efficient method of early diagnosis would be the analysis of the methylation pattern in body fluids like serum, urine or milk in early cancer development. It has been shown that these methylation pattern changes originate from cancer cells. In Tables 1 and 2 in this review, we have provided reported examples of many genes in hypermethylated or hypomethylated states in breast cancer using a variety of techniques for detection of methylation in DNA sequence.

Many studies have demonstrated alterations in methylation patterns of ctDNA in various malignancies.[23,24,26,27,31,34,40-42] Mirza found a significant positive correlation between promoter hypermethylation of estrogen receptor β (ERβ) and retinoic acid receptor β2 (RAR-β2) in matched tissue DNA and serum DNA. They identified no hypermethylation in serum DNA without presence in the primary tumor.[43] However, the frequency of detected methylated markers is two-fold lower in plasma samples compared with their frequency in the tissues of cancer patients.[39]

To find out whether changes observed in ctDNA is identical to those found in primary tumor, Silva *et al*. investigated one of the inactivation mechanisms of p16INK4a (i.e. hypermethylation) in their patients with breast carcinomas and searched for the possibility of finding this phenomenon in plasma DNA of these patients. They used a PCR-based methylation assay, based on the inability of some restriction enzymes to cut methylated DNA sequences, they found out aberrant DNA hypermethylation in exon 1 of p16INK4a in plasma DNA of these patients is identical to the alteration present in the corresponding carcinomas.[36] They also demonstrated the same microsatellite alterations in ctDNA tumor of 61% of breast cancer patients.[44] Also, Yazici *et al*. examined RASSF1A promoter methylation and found that RASSF1A promoter hypermethylation was more frequent in affected cancer patients, and their unaffected siblings compared with population-based controls.[45]

Based on the fact that extracellular nucleic acids circulate in blood and not only in plasma and these molecules are also bound to the surface of blood cells, Skvortsova *et al*., evaluated the cell surface-bound DNA as a source of material for methylation specific PCR (MSP) diagnostics along with ctDNA isolated from the plasma. Aberrant hypermethylation of RASSF1A, RAR-β2 and HIC-1 gene promoters was determined by MSP. The hypermethylation of all three genes was detected considerably more frequently in the cell bound ctDNA than in the plasma ctDNA of the tumor-bearing patients.[39]

The potential to diagnose different cancers including breast cancer[23] based on abnormal hypermethylation or hypomethylation in ctDNA has been demonstrated.[46] Yamamoto *et al*. analyzed methylation status in serum DNA before and after surgery and their results demonstrated that the origin of the aberrantly methylated genes was the tumor tissue.[47] Also, benign, inflammatory and malignant diseases could be differentially identified through methylation analysis of ctDNA[46] Fiegl *et al*. demonstrated that RASSF1A DNA methylation disappears in serum throughout treatment with tamoxifen.[32]

Tumor suppressor genes such as APC, RASSF1A, TMS-1, DKK3 and DAPK, cell cycle-related genes (14-3-3-sigma, GSTP1, p16 and RAR-β), metastasis-related genes (ITIH5 and E-cadherin), and others (ESR1, SLC19A3 and HIN-1) have also been studied as potent biomarkers.[28] Skvortsova *et al*. demonstrated the prevalence of RAR-β2 and RASSF1A promoter hypermethylation in serum of 95% of the cancer patients and 60% of the patients with fibroadenoma and was not found in healthy women.[39] Another study showed significant promoter hypermethylation in HIN-1, RAR-β, RASSF1A and Twist genes in serum DNA of patients with DCIS or invasive ductal carcinoma (IDC). In this study detection of hypermethylation of at least one of the RAR-β and RASSF1A genes from ctDNA provided a sensitivity and specificity of 94.1% and 88.8%, respectively for the detection of DCIS/IDC.[23]

Significantly higher methylation frequencies of two genes (APC and RASSF1A) have been shown in the serum DNA of breast cancer patients. Also, in this study, it was shown that detection of a methylated gene in serum was significantly associated with the detection of CTC in

Table 1: Hypermethylated genes in breast cancer

Genes	EntrezGene IDs	Reference (s)
14-3-3σ (14-3-3 Sigma)	10971	66
ABCB1 (ATP-Binding Cassette B1)	5243	67
ACADL (Acyl-CoA Dehydrogenase, Long Chain)	33	68
APC (Adenomatous polyposis coli)	324	69
RhoGDI2 (Rho GDP-dissociation inhibitor 2)	397	70
BPHL (Biphenyl Hydrolase-Like)	670	71
BRCA1 (Breast cancer 1, early onset)	672	66,69,72
BRCA2 (Breast cancer 2, early onset)	675	73
C1S (Complement Component 1, S Subcomponent)	716	71
CAMK2N1 (Calcium/Calmodulin-Dependent Protein Kinase II Inhibitor 1)	55450	71
CDH1 (Cadherin-1)	999	66,69
CDKL2 (cyclin-dependent kinase-like 2)	8999	68
COX7A1 (cytochrome c oxidase subunit VIIa polypeptide 1)	1346	68
CCND2 (CyclinD2)	894	67,69
DKK3 (Dickkopf WNT signaling pathway inhibitor 3)	27122	40
DOK7 (Docking protein 7)	285489	72
ERBB3 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3)	2065	71
ERα (estrogen receptor alpha)	2099	66
FOXC1 (forkhead box C1)	2296	67
GSTM2 (glutathione S-transferase mu 2)	2946	71
GSTP1 (glutathione S-transferase pi 1)	2950	66,67
HIC-1 (Hypermethylated In Cancer 1)	3090	69
HIN-1 (High in normal 1)	92304	67,69
HLA-DPA1 (major histocompatibility complex, class II, DP alpha 1)	3113	70
HLA-DRA (major histocompatibility complex, class II, DR alpha)	3122	70
IL8 (interleukin 8)	3576	70
ITIH5 (inter-alpha-trypsin inhibitor heavy chain family, member5)	80760	40
ITR (inverted terminal repeats)	160897	68
LAMB2 (laminin, beta 2)	3913	71
LPXN (leupaxin)	9404	71
MAPK12 (mitogen-activated protein kinase 12)	6300	70
MCT1 (Monocarboxylate transporter 1)	6566	69
MGAT1 (mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase)	4245	71
MGMT (O-6-methylguanine-DNA methyltransferase)	4255	67
MLH1 (mutL homolog 1)	4292	67
p16INK4A/CDKN2A (cyclin-dependent kinase inhibitor 2A)	1029	66,67,69
PER1 (period circadian clock 1)	5187	74
PER2 (period circadian clock 2)	8864	74
PER3 (period circadian clock 3)	8863	74
PPP2R2B (protein phosphatase 2, regulatory subunit B, beta)	5521	67
PR (Progesterone receptor)	2099	66
PTEN (phosphatase and tensin homolog)	5728	67
PTPRO (protein tyrosine phosphatase, receptor type, O)	5800	68
RARB2 (retinoic acid receptor, beta2)	5915	66,67
RASSF1A (Ras association domain family member 1A)	11186	67,69
RECK (reversion-inducing-cysteine-rich protein with kazal motifs)	8434	68
SFRP1 (secreted frizzled-related protein 1)	6422	40
SFRP2 (secreted frizzled-related protein 2)	6423	40,68
SFRP5 (secreted frizzled-related protein 5)	6425	40
SST (somatostatin)	6750	68
TIMP-3 (TIMP Metallopeptidase Inhibitor 3)	7078	66,69
TWIST (Twist Family BHLH Transcription Factor 1)	7291	73,75
UAP1L1 (UDP-N-acteylglucosamine pyrophosphorylase 1-like 1)	91373	68
UGT3A1 (UDP glycosyltransferase 3 family, polypeptide A1)	133688	68
WIF1 (WNT inhibitory factor 1)	11197	40
WT-1 (Wilms Tumor 1)	7490	69
ZNF154 (zinc finger protein 154)	7710	68

peripheral blood. CTCs were detected in 45% of patients with at least one gene methylated.[26] Chimonidou *et al.* demonstrated CST6 promoter hypermethylation in serum DNA of 39.8% of the patients, while it was not found in healthy individuals.[31] Detection of methylated SLC19A3 gene promoter in serum DNA showed that hypermethylation of this gene differentiated between breast cancer patients and healthy controls with a sensitivity of 87% and a specificity of 85%.[35]

Recently, an analysis published by Chimonidou *et al.*,[48] evaluated connection between the presence of CTCs and ctDNA in patients with breast cancer, after surgical removal of the primary tumor. They demonstrated concordance

Table 2: Hypomethylated genes in breast cancer

Genes	EntrezGene IDs	Reference (s)
BIN2 (bridging integrator 2)	51411	71
CCR7 (chemokine (C-C motif) receptor 7)	1236	71
CCR8 (chemokine (C-C motif) receptor 8)	1237	71
CD160 (CD160 molecule)	11126	71
CD27 (CD27 molecule)	939	71
CD37 (CD37 molecule)	951	71
CD3D (CD3d molecule, delta)	915	71
CD5 (CD5 molecule)	921	71
CTGF (connective tissue growth factor)	1490	70
DDR2 (discoidin domain receptor tyrosine kinase 2)	4921	70
DOCK8 (dedicator of cytokinesis 8)	81704	71
DSG1 (desmoglein 1)	1828	70
FAM113B (Family With Sequence Similarity 113, Member B)	91523	71
IL2RB (interleukin 2 receptor, beta)	3560	71
MAGE (Melanoma Antigen Family A, 1)	57692	69
MGC29506 (marginal zone B and B1 cell-specific protein)	51237	71
NCKAP1L (NCK-associated protein 1-like)	3071	71
RTN4IP1 (reticulon 4 interacting protein 1)	84816	71
S100A2 (S100 calcium binding protein A2)	6273	70
SP140 (SP140 nuclear body protein)	11262	71
SPDEF (SAM pointed domain containing ETS transcription factor)	25803	71
SNCG (Synuclein, Gamma)	6623	69
TFF1 (trefoil factor 1)	7031	71
TNFRSF10A (Tumor Necrosis Factor Receptor Superfamily, Member 10a)	8797	71
uPA (plasminogen activator, urokinase)	5328	69

between serum level of SOX17 hypermethylation and CTC for 70.9% of the patients. Hypermethylation of ITIH5, DKK3 and RASSF1A in ctDNA of breast cancer patients achieved 67% sensitivity with a specificity of 69%.[40] Silva *et al*. investigated microsatellite alterations (in D17S855, D17S654, D16S421, TH2, D10S197, and D9S161 polymorphic markers) as well as mutations in the p53 gene and aberrant hypermethylation at the first exon of p16INK4a in tumor and plasma DNA. They identified 56 cases (90%) in which there were at least one alteration in tumor DNA and 41 cases (66%) with a similar alteration in ctDNA.[44] One-step MSP assay of methylated GSTP1, RASSF1A, and RAR-β2 gene promoters showed that hypermethylation of these three genes differentiated between breast cancer patients and healthy controls with a sensitivity of 78%. In this study in which samples exhibited hypermethylation of GSTP1, RASSF1A, and RAR-β2 genes in serum DNA, hypermethylation of the same genes in tumor tissues were reported.[47] In another study Bae *et al*. demonstrated almost all of the primary breast tumors (97%) were abnormally methylated in at least one of cyclin D2, RAR-β, twist and HIN-1 genes. Hypermethylation in the ctDNA was detected in 67% of the patients with confirmed hypermethylation of the genes in the tumor.[41] RAR-β2 and ERβ promoters were reported to be hypermethylated in 26% and 61% of tumors and in 20% and 50% of ctDNA of breast cancer patients, respectively. Thus, concordance between tumor and serum hypermethylation status was observed.[43] Papadopoulou *et al*. analyzed ctDNA and methylation patterns of GSTP1, RASSF1A and ATM genes in plasma of breast and prostate cancer patients. They demonstrated that the combination of DNA increase and promoter hypermethylation of GSTP1, RASSF1A, and ATM genes can identify 54% of breast cancer and 88% of prostate cancer patients.[49]

Branham *et al*. reported hypermethylation for 110 CpG islan (CPGI) within more than 60 cancer-related genes in tumors with triple-negative (TN) features. Breast cancers identified as TN are defined as tumors that lack the expressions of estrogen and progesterone receptors and epidermal growth factor receptor 2. Their results revealed that 18 of CpGI (located within 16 different cancer-involved genes) were associated with TN tumors, indicative of a specific methylation profile. This panel includes: Five hypermethylated genes (CDKN2B, CD44, MGMT, RB, p73) and 11 hypomethylated genes (GSTP1, PMS2, MSH2, MLH1, MSH3, MSH6, DLC1, CACNA1A, CACNA1G, TWIST1, ID4).[50]

Martínez-Galán *et al*. investigated ESR1, APC, RAR-β, 14-3-3-σ and E-cadherin promoter hypermethylation in serum of 106 women with breast cancer, 34 with benign breast disease and 74 with no evidence of breast disease. Results indicated that hypermethylation of ESR1 and 14-3-3-σ genes differentiated between breast cancer patients and healthy individuals with a sensitivity of 81% and a specificity of 88%.[51] In 2003, Ramirez *et al*. reported hypermethylation of MGMT, p16, DAPK and RASSF1A genes in serum DNA among glioblastoma patients. The methylation profile of ctDNA has also been used to identify other cancers.[24] Forshew *et al*. identified mutations throughout the tumor suppressor gene TP53 in ctDNA from

46 to 69 plasma samples (67%) of advanced ovarian cancer patients.[52] Methylation pattern changes in cancer genes in blood could be a prime target for early detection of cancers in the future. Currently, technologies are being developed to detect minor changes in the blood DNA of cancer patients.

METHODS USED IN DNA METHYLATION PATTERN ANALYSIS

A wide range of methods is used to discriminate methylated and umethylated cytosine in DNA sequence. These methods are based on the chemical and physical differences of cytosine and 5 m-cytosine that can combine into various techniques. Three of the methods are briefly described below, and the remaining are listed in Table 3.

Bisulfite modification

This powerful chemical reaction can change the indistinguishable cytosine to uracil and leaves 5-methylcytosine residue intact. This technique allows specific changes in the DNA sequence depending on the DNA methylation status. The use of this technique allows downstream processes like PCR and sequencing of DNA and the use of sequence specific restriction enzymes for identification of methylation patterns.[53]

Allele-specific bisulfite sequencing

It is very much like regular sequencing of single alleles. Primers not overlapping CpG sites are used to amplify the desired sequence, the design of such primers have been much easier after the initiation of ENCODE and CANCER methylome projects, these databases allow the rapid and accurate detection of differentially methylated regions in cancer and normal cells and they help locating optimal sequence. After bisulfite conversion and PCR, products are ligated to a cloning vector to amplify individually. Each vector is then sequenced by any sequencing method suitable for the study. If a sufficient number of clones are sequenced, this method can be quantitative, as each clone represents a single allele.[54,55]

Methylation specific quantum dot fluorescence resonance energy transfer

Another technique to detect methylation of different genes is methylation specific quantum dot fluorescence resonance energy transfer (MS-qFRET). It is a nanotechnology assay that enables the detection of methylation and its changes in an ultrasensitive manner. MS-qFRET is a combination of the high specificity of MSP and the high sensitivity of quantum dot fluorescence resonance energy transfer technology. The procedure of this method composed of treating DNA template with sodium bisulfite that converts unmethylated cytosines to uracil. Amplification of converted template is done using biotinylated methylation-specific primers (biotinylated forward primer and the reverse primer is labeled with an organic fluorophore). Quantum dots, conjugated with streptavidin, serve as a scaffold to capture amplicons and as a donor for transferring energy to the Cy5 acceptor. Cy5 acceptor has been incorporated into the amplicons during PCR. Thus, the status of DNA methylation can be determined according to the level of fluorescence resonance energy transfer. This technique causes reduction in a number of PCR cycles, and allows for multiplexed analyses.[56,57]

These techniques offer different costs of the utilization, specificity, output range and speed and ease. In Table 3, we have listed the most popular methods used for detection of DNA methylation patterns.

PERSPECTIVE

The cancer phenotype is a combination of genetic and environmental effects like most other traits in living organisms. The promise of DNA methylation analysis as a cancer biomarker partially comes from the fact that sequencing methods utilized in the process of methylation pattern analysis can investigate the effects of genetic and environmentally induced epigenetic changes at the same time.

Many studies have shown that ctDNA methylation analysis can be a highly selective method for screening early breast cancer. Regarding the variety of methods used to evaluate

Table 3: Popular methods used for detection of DNA methylation patterns

Methods	Distinguishing protocol	Detection protocol	Sensitivity	Relative cost	References
Methylation specific PCR	Bisulfite conversion and specific primers	Gel electrophoresis	Low	Very low	58-60
Combined bisulfite restriction analysis	Restriction enzyme	Gel electrophoresis	Low	Low	61
Bisulfite-PCR followed by MALDI-TOF MS	Bisulfite modification	Mass spectrometry	High	High	62
Restriction landmark Genome scanning	Methylation sensitive enzymes	2D-gel	Low	low	63
Live cell imaging	Fluorescent probes	Microscopy	Low	low	64
Methylation specific digital karyotyping	Met-specific enzyme	sequencing	Low	high	65

methylation patterns, it is not unimaginable for robust methods to be developed for assessing cancer progression by methylation pattern analysis specifically, the lower costs of DNA sequencing in recent years make sequence-based methods more widely accessible by many laboratories and eventually patients. This method is particularly applicable in cases where the bodily fluids are easily obtained and may be routinely used in diagnostic laboratories in the near future.

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