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DNA Hypermethylation in Breast Cancer

Le Huyen Ai Thuy, Lao Duc Thuan and Truong Kim Phuong

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Abstract

Cancer development is a complex process with multiple steps. Many factors, including radiation, chemicals, viruses, genetic and epigenetic changes, lead to abnormal proliferation of a single cell, which results in the outgrowth of a population of clonal-derived tumour cells. It has established that DNA hypermethylation, an epigenetic mechanism that occurred by the addition of a methyl group at 5′ position of the pyrimidine ring of cytosine residues at CpG islands through the action of DNA methyltransferase enzymes, has been considered as the cause of human tumorigenesis, including breast cancer development. Moreover, DNA hypermethylation holds a promising application as a potential biomarker for the early detection, prognosis and prediction of drug sensitivity in cancer. Therefore, this chapter focuses on the description and exemplification of the DNA hypermethylation changes, particularly, highlight the DNA hypermethylation as a potential biomarker applied in predictive, diagnostic, prognostic and therapeutic monitoring of breast cancer.

Keywords: breast cancer, epigenetics, hypermethylation, tumour suppressor gene

1. Introduction

Epigenetics, which was first coined by Waddington in 1942, literally means as ‘outside conventional genetics’, refers to the heritable, reversible changes in gene expression that occur without alteration DNA sequence [1]. Epigenetic modifications are natural processes and essential for mammalian development and cell proliferation. These epigenetic changes could also be affected by many random factors or environmental influences. Disruption of epigenetic modification resulting in regulating patterns of gene expression is the feature of a number of severe human diseases, including malignant cellular transformation [2–4]. Three main epigenetic modification systems, including DNA methylation, histone covalent modification, and non-coding RNA modification, leading to associated-gene silencing, have been observed [5, 6]. This chapter aims
to introduce the reader to the concept of DNA methylation, especially DNA hypermethylation, with examples of its involvement in human breast cancer.

2. DNA hypermethylation: a kind of epigenetic modification that plays a key role in silencing tumour suppressor genes

DNA methylation is one of the epigenetic mechanisms that is closely associated with normal cell development and a number of key processes including imprinting, X-chromosome inactivation, repression of repetitive element transcription, chromatin organization, etc. [7–9]. Aberrant methylation patterns are known to be presented in the genomes of cancer cells. Two patterns of aberrant methylation have been observed, including global hypomethylation along the genome and hypermethylation at the specific sites, namely the CpG islands (CGIs) within the promoter regions, according to the decreased and increased the level of methyl group modification, respectively [4, 8, 10–12]. Disordered DNA methylation contributes to a number of human diseases, including breast cancer. Increased level of genome-wide hypomethylation results in increased chromosomal instability and activation of regulatory DNA sequences, including transcription of oncogenes, retrotransposons as well as genes encoding proteins involved in malignant cell development. DNA methylation refers to a covalent modification of cytosine ring at the 5' position of a CpG dinucleotide by adding a methyl group in the 5th carbon of the ring using S-adenosyl methionine as a methyl donor (Figure 1) [8, 12].

This methylation process is catalysed by DNA (cytosine-5) methyltransferases (DNMTs). In mammalian, DNMTs are a highly conserved family protein encompassing DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L, which could be distinguished by their function [13–15] (Figure 2). DNMT1 was the first methyltransferase to be discovered [1], then DNMT3 was discovered and characterized. Regarding to DNMTs function, DNMT3A and DNMT3B perform de novo methylation by adding the methyl groups to unmethylated CpG, which is responsible for the establishment of new methylation pattern in genomic DNA, whereas DNMT1, which has a high preference for hemi-methylated DNA, maintains the existence of methylation patterns following DNA replication on the newly synthesized strand [3, 4, 13, 14, 16, 17]. DNMT3L (DNA (cytosine-5)-methyltransferase 3-Like) has no catalytic activity, DNMT3L has been shown to act as a general stimulatory factor for de novo methylation and facilitate methylation of DNMT3A and DNMT3B [2, 18].

The term CpG refers to the base cytosine (C) linked by a phosphate bond to the base Guanine (G) in the DNA nucleotide sequence, which usually cluster together in 'CpG islands (CGIs)' and typically locate at or near the promoters and transcription sites of genes. The molecular mechanisms underlying CpG island hypermethylation in many human cancers, including breast cancer, have been explored. The hypermethylation of CGIs located at tumour suppressor genes can result in transcriptional silencing of genes through a number of mechanisms, including (i) DNA hypermethylation directly affects the RNA polymerase II and DNA interactions by inhibiting the binding of transcriptional factors on specific sequences, such as AP-2, c-Myc/Myn, E2F, NF-κB, etc. and (ii) hypermethylated DNA recruits methyl-CpG binding proteins (MeCP1 and MeCP2), and methyl-CpG binding domain protein (MBD1, MBD2, MBD3 and MBD4) [4].
Figure 1. (A) The DNMTs catalyse the methyl cytosine modification. (B) The structure of SAM and SAH.

Figure 2. The roles of DNMTs.
Tumour suppressor genes (TSGs) normally suppress or negatively regulate cell proliferation by encoding proteins that block the action of growth-promoting proteins. A hallmark of cancer involves the loss of function of TSGs through the silencing genetic information. The silencing of TSGs by the high levels of 5-methylcytosine in their CpG island promoter regions, considered as the ‘first and second hit’, is equivalent to mutations and translocations, in Knudson’s two-hit model of tumorigenesis [19, 20]. Here, the methyl groups become chemically bonded to the cytosine in CGIs, leading to disruption of the normally controlled cell proliferation and drive it to malignancy (Figure 3). Thus, the presence of m5CpG dinucleotide in tumour suppressor gene promoters is recognized as an important event in many human tumour types.

Figure 3. The typical CpG island of a tumour suppressor gene is represented in a normal and a tumour cell. White dots: unmethylated CpG; black dots: methylated CpG.

3. DNA methylation in circulation as a cancer biomarker

The high presence of cell-free circulating tumour DNA (ctDNA), which is derived from primary tumour cells, can be found in blood and non-invasive samples of patients with cancer, such as urine, bronchoalveolar lavage, mammary aspiration fluids, saliva, sputum, etc. makes an ideal candidate biomarker for prognosis and early diagnosis of breast cancer. ctDNA can be distinguished from circulating DNA derived from healthy cells by the presence of genomic aberrant modifications. For example, upon the tumour development, ctDNA carries tumour specific epigenetic modifications, i.e. DNA hypermethylation, is released due to the lysis of circulating cancer cells or micro-metastases. Therefore, the detection of genetic and epigenetic alterations in ctDNA offers a potential source of development of prognostic and predictive biomarkers for cancer. Quantitative evaluation of ctDNA can reflect tumour burden relevant to provide information on genetic and epigenetic profiles associated with human cancer development. The concentration of methylated ctDNA is presented in an even smaller portion of this amount, thus, presenting a challenging substrate to work with. Fortunately, even in the low concentration, ongoing technical developments and much of the progress in...
molecular biological techniques have provided a chance that they can be directly applied in ctDNA collection and validation even smaller amounts of ctDNA [10, 21, 22].

4. Hypermethylation of TSGs in breast cancer: a prognostic and early diagnostic indicator

DNA aberrant methylation patterns, like hypermethylation of TSGs, global hypomethylation, etc. have been observed in human breast cancer. Silencing of TSGs expression by DNA hypermethylation provides a molecular mechanism by which DNA hypermethylation could trigger tumour development by interfering with the binding of transcription factors located at TSG gene’s promoter. Thus, numerous studies have been attempted to focus on the role of hypermethylation of the TSG genes’ promoter in breast cancer as well as the correlation between methylation of specific CGIs in TSGs and many breast cancer clinical states. Table 1 shows the most relevant hypermethylated genes involve in various functions in breast cancer reported so far. Methylation of these TSG promoters is associated with the complete loss of TSG protein products in cancer cells and development of malignant phenotype.

<table>
<thead>
<tr>
<th>TSGs</th>
<th>Function</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Inhibitor of β-catenin, cell proliferation, migration and adhesion</td>
<td>5q21</td>
</tr>
<tr>
<td>BRCA1</td>
<td>DNA damage repair</td>
<td>17q21</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>Regulators of CDK kinases</td>
<td>12p13</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Conjugation to Glutathione, prevention of oxidative DNA damage</td>
<td>11q13</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>Cyclin-dependent kinase inhibitor</td>
<td>9p21</td>
</tr>
<tr>
<td>PTEN</td>
<td>Negatively regulating AKT/PKB signalling pathway</td>
<td>10q23</td>
</tr>
<tr>
<td>RARβ</td>
<td>Retinoic acid receptor</td>
<td>3p24</td>
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<tr>
<td>RASSF1A</td>
<td>Ras effector homologue, cell cycle arrest</td>
<td>3p21</td>
</tr>
<tr>
<td>ZMYND10</td>
<td>Inhibitor of colony formation of cancer cells</td>
<td>3p21.3</td>
</tr>
</tbody>
</table>

Table 1. Examples of TSGs that undergo CpG island hypermethylation in breast cancer.

This DNA hypermethylation is a reversible signal, maybe due to the activity of Demethylase, which performs the reverse reaction to DNA methyltransferase and is an excellent candidate to be one of its important partners in shaping the methylation pattern of genomes [23, 24]. Thus, nowadays, many studies have been focused on an innovative approach in cancer treatments in which aimed to inhibit DNA hypermethylation and/or re-expression of silenced TSGs.

Therein, the hypermethylation of the CGIs promoter of BRCA1 gene is now recognized as one of the most common molecular abnormalities associated with breast cancer development and is quoted as a significant example. BRCA1 (Breast cancer 1) gene (HGNC: 1100; Entrez Gene: 672; OMIM: 113705; UniProtKB: P38398), which locates at 17q12-21, also known by many other names such as IRIS, PSCP, BRCA1, BRCC1, RNF53, BROVCA1, etc. is a tumour
suppressor gene that conferred genetic pre-disposition to early onset of human breast and ovarian cancer [25–27]. This gene encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability. The encoded protein combines with many other tumour suppressor, DNA damage sensors, and signal transducers to form a large multi-subunit protein complex that is called as BRCA1-associated genome surveillance complex (BASC). Therefore, the BRCA1 protein is involved in multifunction, such as repairing damaged DNA of double-stranded break, transcriptional regulation, ubiquitinylation, recombination and controlling the cell cycle check points as well as other functions. The hypermethylation of the BRCA1 promoter has been considered as an inactivating mechanism of BRCA1 expression, leading to breast tumourigenesis. In addition, some evidences have shown the significant association between the inactivation or low expression of BRCA1 protein expression and the aberrant methylation status of CGIs in the BRCA1 promoter in breast cancer tumorigenesis.

It is well known that breast cancer constitutes a heterogeneous complex of diseases characterized by different distinct morphologies, biological behaviours and clinical outcomes. The classification and diagnosis of breast cancer have been based on the expression of different proteins, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [28, 29]. An example of such a target molecular therapy is Trastuzumab (Herceptin®), which has been approved to directly against HER2-expressing tumours. Among the variety of breast cancer types, a subtype called triple-negative breast cancer (TNBC), which is clinically defined by the lack of expression of ER, PR and HER2, presents a challenge for effective clinical management [28]. Therefore, it is essential to find a reliable biomarker, which are not only useful for the screening, early diagnosis and prognosis prediction for breast cancer, but also provide insight into the mechanisms driving tumourigenesis as well as an innovative approach in breast cancer treatments.

Over the past few years, a considerable amount of studies has been conducted to evaluate the association between BRCA1 promoter methylation and many clinicopathological characteristics of breast cancer. Therefore, tentatively, a meta-analysis was carried out, a total of 44 studies including 25 case-control studies and 19 cohort studies were eligible, enrolled into the meta-analysis research. According to our research, the prevalence of the hypermethylated BRCA1 promoter has been reported to fall in the range from 9.1 to 59.2%, which was statistically significant higher in breast cancers than non-cancerous controls (OR = 4.00, 95% CI = 2.336–6.878, \(P < 0.001\), \textbf{Figure 4}). Because of large heterogeneity (\(P_H \leq 0.0001, I^2 = 73.82\%\)), we continued to clarify the potential source of heterogeneity via stratified analysis based on sample materials, methods for identifying methylation and ethnicity; with the detailed results were summarized in \textbf{Table 2}.

As shown in \textbf{Table 2}, the pooled OR for BRCA1 promoter hypermethylation in breast cancer tissues was 4.312 (95% CI = 2.395–7.765, \(P < 0.001\)) compared with normal or benign tissues, and was higher than the pooled OR in peripheral blood of breast cancer patients (OR = 2.485, 95% CI = 1.433–4.310, \(P = 0.001\)) compared with non-cancer controls. In addition, the pooled OR for BRCA1 promoter hypermethylation detected by MSP was 5.059 (95% CI = 2.214–11.561, \(P < 0.001\)), significant higher than other methods (OR = 2.506; 95% CI = 1.409–4.457, \(P = 0.002\)). Meanwhile, the frequency of BRCA1 promoter hypermethylation in Asians (OR = 4.006, 95% CI = 2.122–7.560; \(P < 0.001\)) was higher than in Caucasians (OR = 2.291, 95% CI = 1.147–4.576, \(P = 0.006\)). Furthermore, our studies also demonstrated that the BRCA1 promoter hypermethylation was significant
correlated with the clinicopathological characteristics which included ages, meant that the prevalence of hypermethylation status was higher in the group of age under 55 (OR = 1.227, 95% CI = 1.604–1.414, P = 0.05) (Figure 5); histological grade, meant that the hypermethylated BRCA1 in the case of histological grade 3 and 4 was higher than in the histological grade 1 and 2 (OR = 1.858, 95% CI = 1.499–2.301, P < 0.001) (Figure 6); disease stages, meant that the prevalence of the hypermethylation of BRCA1 gene in the case of late stages was higher than in early stages (OR = 1.339, 95% CI = 1.023–1.752, P = 0.033) (Figure 7). Additionally, the hypermethylation status of BRCA1 gene’s promoter was correlated with the ER(−) (OR = 2.02, 95% CI = 1.525–2.675, P < 0.001), PR(−) (OR = 1.823, 95% CI = 1.374–2.41, P < 0.001) and especially with triple-negative phenotype (OR = 2.814, 95% CI = 1.811–4.371, P < 0.001) under fixed or random effect mode (Figure 8). Thus, those meta-analysis results confirmed that the BRCA1 promoter hypermethylation was significant correlated with the increased risk of breast cancer, associated with several specific clinicopathological characteristics of breast cancer, which indicated that BRCA1 promoter hypermethylation could be utilized as an effective biomarker in predictive and diagnostic breast cancer.

Study or Subgroup | Breast cancer | Non-cancer | Weight (%) | Odds Ratio M-H, Random, 95% CI | Year |
<table>
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<td>Li 2015</td>
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<td>49</td>
<td>6.87</td>
<td>3.316 (1.344-7.948)</td>
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<td>7</td>
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Total events: 944
Total events: 525

Heterogeneity: Tau^2=0.024, Chi^2=91.18, df=24 (P<0.0001), I^2=73.82%
Test for overall effect: Z=5.84 (P<0.001)

Figure 4. Forest plot for evaluating the association between BRCA1 promoter methylation and breast cancer under fixed or random effect mode.

Up to now, a significant proportion of breast cancer patients who have poor prognosis will develop recurrence. This needs to find a more sensitive and specific biomarker, which can be a powerful prognostic indicator and help make therapeutic decisions to prolong the survival time of patients. Then, we included 10 articles provide disease-free survival (DFS) and/or overall survival (OS) to evaluate the role of the BRCA1 promoter hypermethylation in the prognosis of breast cancer. Overall survival (OS), which was defined as the length of time from either the date of diagnosis or the start of treatment for breast cancer, that patients diagnosed with the disease are still alive, and disease-free survival (DFS), which was defined that the length of time after primary treatment for a cancer ends that the patient survives...
without any signs or symptoms of that cancer. In detail, in the Asian population, the OS and DFS were 2.163 (95% CI = 1.212–3.858, \(P < 0.001\)) and 2.47 (95% CI = 1.331–4.584, \(P = 0.004\)), respectively, using single variable analysis. In the case of using multiple variables analysis, the OS and DFS were 1.611 (95% CI = 1.116–2.324, \(P = 0.011\)), and 2.872 (95% CI = 1.389–5.937, \(P = 0.004\)), respectively. Those analytic results indicated that hypermethylated \textit{BRCA1} gene’s promoter was significant associated with OS, DFS, meant that it was poor prognosis to breast cancer patients, in both single and multiple variables analysis. Hence, \textit{BRCA1} promoter hypermethylation is suggested to be a potential biomarker for prognostic assessment.

<table>
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<tr>
<th>Test of association</th>
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<td>Africa</td>
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\(P\)-value of \(Q\) test for heterogeneity among studies; F: fixed-effects model; R: random-effects model; NA: non-analysis.

Table 2. Overall and subgroups analyses of \textit{BRCA1} methylation and breast cancer risk in 25 cases control studies.

![Figure 5](image_url)  
Figure 5. Forest plot for evaluating the association between \textit{BRCA1} promoter methylation and ages under fixed or random effect mode.
Figure 6. Forest plot for evaluating the association between BRCA1 promoter methylation and histological tumour grades under fixed or random effect mode.

Figure 7. Forest plot for evaluating the association between BRCA1 promoter methylation and disease stages under fixed or random effect mode.

Figure 8. Forest plot for evaluating the association between BRCA1 promoter methylation and triple negative phenotype under fixed or random effect mode.
5. DNA hypermethylation-targeted drug in cancer therapy

The process of DNA methylation is catalysed by DNMTs which typically occurs at CpG dinucleotides. As mentioned earlier, it is also a reversible process. Removal of a methyl group from DNA must involve a cleavage of a carbon-carbon bond, which is carried out by DNA demethylase (dMTase). In addition, the methylation reaction can be blocked by the inhibitors of DNA methylation drugs, such as 5-azacytidine, 5-aza-2′-deoxycytidine, etc. which contains a nitrogen in the place of carbon at 5’ position of cytosine ring (Figure 9) [30]. This drug is cooperated into DNA, then, replaces the natural base cytosine and acts as a potent inhibitor of the DNMTs, inducing the DNA demethylation [31]. Since DNA methylation is reversible, an aberrant hypermethylation of tumour suppression genes can be reverted. This consequently supports DNA methyltransferases (DNMTs) as attractive therapeutic targets. Indeed, epigenetic drugs (epi-drug)—methylation inhibitors through DNMT inactivation, used alone or in combination with other biomarkers, including by dietary agents, for targeted preventive and therapeutic interventions, have attracted attention recently.

Figure 9. Inhibition of DNMTs by 5-azacytidine.

DNMT inhibitors (DNMTi), such as 5-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine) (Figure 10), are epi-drugs which are first announced and currently marketed as hypomethylation therapeutics. They are nucleoside analogues, derivatives of cytidine that work by incorporating into the DNA sequence at cytosine positions during DNA replication to be active and then form a suicidal covalent complex with the DNMTs. These drugs have been approved by Food and Drug Administration (FDA) for clinical tests on the myelodysplastic syndrome, malignant mesothelioma, pre-leukemic disease, breast cancer, nasopharyngeal carcinoma and some other diseases.
Zebularine is another cytidine analog that has a mechanism similar to 5-azacytidine, integrating into DNA and forming a covalent bond with DNMT1, resulting in inhibition of methylation reaction. Moreover, Zebularine is reported to be a DNMT1 inhibitor with low toxicity and has a high sensitivity in selective cancer cells. Particularly, this drug showed the reactivated functions on some important tumour suppressor genes that were disrupted in breast cancer cell lines, even at low concentrations. Although the drug is not yet FDA approved, a preclinical study on mouse models showed that Zebularine can inhibit DNA methylation and induce re-expression-silenced gene, even given orally.

Other trends related to DNA methylation including the inhibition of DNMTs through siRNA, ribozymes, antisense oligonucleotides have also been considered. Some drugs have proven effective impact on cell cultures, animal models and clinical trials as well such as MG98, a 20 bp anti-sense oligonucleotide that directly prevents the translation of DNMT1 or RG108—a new small molecule that can act on active site of DNMT1. Unlike the nucleoside analogs, RG108 did not demonstrate cytotoxic or genotoxic effects on cells even at high concentrations.

The combination of the histone deacetylase inhibitors (HDACi), such as Trichostatin A (TSA) and phenylbutyrate, with DNMTi is a new trend giving promising efficacy in the treatment of cancer. In breast cancer, triple negative metastatic patients that do not express estrogen receptor (ER), progesterone receptor (PR) and HER, do not respond to agents such as trastuzumab (Herceptin) and tamoxifen. Particularly, the loss of ER in some triple negative breast cancers is epigenetically silenced by abnormal methylation and histone modifications. Consequently, the patients express the resistance of anti-estrogen. Triple negative metastatic breast cancer patients were pre-treated with decitabine—a DNMTi and LBH 589—an HDACi, to restore the ER and then treated with tamoxifen. This combination can remove the epigenetic modifications including DNA methylation and histone deacetylation and reactivate ER. Thus, this reactivated ER cells become sensitive to agents like tamoxifen. Similarly, the combination of azacitidine with TSA also induces the re-expression of ER function to increase the sensitivity of breast cancer cell lines that previously show negative expression with ER in tamoxifen therapy or the combination of HDACi and trastuzumab has taken to effectively suppression of the development and apoptosis induction into breast cancer cell lines.

In addition, the combination of epi-drugs with chemotherapeutic agents or natural dietary ingredients also increases the effectiveness of treatment. A pre-clinical study has shown that the combination of decitabine and docetaxel (an anti-mitotic drug) can increase treat-
ment outcomes against cancer cells in experiments conducted on breast cancer cell lines [32, 33]. Decitabine in combination with another substance, amsacrine or idarubicin, also shows therapeutic effect. Green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), may cause re-modelling of chromatin structure and the ERα promoter by histone acetylation and DNA methylation mechanisms, and consequently reactivating ERα. The combination of TSA and EGCG leads to reactivation of numerous tumour suppressor genes by inhibiting directly or indirectly DNMTs. Dietary sulforaphane—an inhibitor of histone acetylation also shows very effective activity in the inhibition of proliferation and survival of breast cancer cells without affecting normal cells.

Therefore, methylation combined therapy is very promising in the treatment of breast cancer. Clinical trials in the combination of trastuzumab with HDACi for the treatment of breast cancer, and a phase II trial in breast cancer—valproic acid combined with FEC100 (5-fluorouracil, epirubicin and cyclophosphamide) also are being investigated. Up to date, several other classes of epi-drug have been studied, developed with new drugs, which based on the DNMT inhibitors, HDAC inhibitors, HMT inhibitors, etc. in early preclinical trial development.

6. Conclusion

DNA hypermethylation has become established in recent years as being one of the important causes of breast tumorigenesis and potential biomarkers in clinical applications, prognosis and early diagnosis of breast cancer. As the release of tumour-associated DNA into body fluids, thus the screening of plasma or serum DNA may provide information on epigenetic profiles which are tightly associated with breast cancer development, progression and response to therapies. This is the real advantage of an aberrant DNA methylation property as a great versatility, promising biomarker for the molecular monitoring of cancer patients, and applied in early detection, prognosis and predicting drug sensitivity in cancer.

Abbreviations

- APC: Adenomatous Polyposis Coli
- BASC: BRCA1-associated genome surveillance complex
- BRCA1: Breast cancer 1
- CGIs: CpG islands
- CI: Confidence interval
- ctDNA: Cell-free circulating tumour DNA
- DFS: Disease free survival
- DNMTi: DNA methyltransferase inhibitors
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References


