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Review

Quantitative PCR analysis for methylation level of genome: clinical implications in cancer

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Background: Cancer cells are frequently characterized by hypomethylation of the genome including repetitive sequences. This epigenetic process is believed to be associated with several biological causes and consequences in cancer. Therefore, LINE-1 repetitive sequences demethylation in cancer should result in different clinical outcomes.
Objective: Recently, we have developed an improved quantitative combined bisulfite restriction analysis PCR protocol that efficiently evaluates the methylation status of LINE-1s; the method is referred to as PCR "COBRALINE-1". This article reviewed what have been learned by applying this technique to study methylation level of repetitive sequences from several sources of genomic DNA.
Results: We have found that LINE-1 methylation patterns among normal tissues are distinct. Therefore, this epigenetic event may be continuously altered in adult tissues by the process of cellular differentiation. Moreover, we confirmed that global hypomethylation is an ongoing process that develops during tumor progression, in addition to previous evidence of genomic and LINE-1 hypomethylation occurring as an early event in carcinogenesis. COBRALINE-1 is a highly effective technique for evaluating the genome-wide level of methylation, in particular from tissue samples with minute amounts of low quality DNA. The technique has been applied to study samples from micro-dissected archived paraffin-embedded tissues and sera of several types of cancer.
Conclusion: The COBRALINE-1 technique demonstrated its potential to be a tumor marker and a great tool to explore the biology of global hypomethylation.

Keywords: Cancer, LINE-1, COBRALINE-1, DNA methylation, genomic hypomethylation, global hypomethylation, LINE-1 demethylation, retrotransposon, tumor marker.

Cancer cells are frequently characterized by hypomethylation of the genome [1-5]. This epigenetic process is believed to result in chromosomal instability [6-9], increased mutation events [10], and altered gene expression [11]. Recently, we developed an improved quantitative combined bisulfite restriction analysis (COBRA; [12]) PCR protocol that efficiently evaluates the methylation status of LINE-1 repetitive sequences in genomic DNA derived from micro-dissected tissue and serum samples), the so-called PCR "COBRALINE-1" [3]. We demonstrated that most cancers exhibit significantly increased levels of hypomethylation compared with their normal tissue counterparts [3]. This article reviews the current knowledge of measurement of the LINE-1 level of methylation and the clinical implications in cancer diagnosis.

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Biologic consequence of global and LINE-1 hypomethylation

DNA methylation is one of the main forms of epigenetic control of cells. In cancer, there are two main alterations in epigenetic control [2]: 1) promoter hypermethylation of tumor suppressor genes [13-16] and 2) genome-wide or global hypomethylation [1]. Global hypomethylation has been demonstrated by down-regulation of methylated CpG dinucleotides, which are dispersed throughout the entire genome, both in non-coding repetitive sequences and genes. Global losses of methylation in cancer may lead to

alterations in the expression of proto-oncogenes critical to carcinogenesis [17, 18], and may facilitate chromosomal instability [8] and alteration of host defense from mobile genetic elements, viral DNA and transgene [19]. Moreover, loss of methylation at LINE-1 may have additional consequences. LINE-1s are highly repeated and widely interspersed human retrotransposon sequences [20]. There are up to 600,000 copies of LINE-1s in the human genome. Approximately, 2,000 copies remain full length, some may be transcriptionally active, and less than 50 copies are retrotransposible [21].Therefore, changes in the LINE-1 level of methylation may possess additional consequences regarding global hypomethylation. First, insertion of LINE-1 in the intron may result in down-regulation of the linked gene [22, 23]. Second, methylation in the intron may result in down-regulation of the linked gene [24]. Third, up-regulation of LINE-1 may result to genomic instability by increasing generalized DNA double strand breaks [25] and retrotransposition [26, 27]. Moreover, LINE-1s may have a similar function to oncogenes, whereas LINE-1 up-regulation may promote tumor phenotype [28, 29]. Finally, LINE-1 promoter may function to control transcription in both the upstream antisense sequence [30] and in LINE-1 with alternative splicing, causing LINE-1 chimeric RNA [31]. Consequently, a difference in level of LINE-1 methylation among cancers may reflect several biologic consequences, depending on the location of demethylated LINE-1s. Therefore, LINE-1 demethylation in cancer should result in different clinical outcomes.

History of global hypomethylation

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells and was reported at a symposium at Johns Hopkins in 1982 on tumour-cell heterogeneity by Feinberg and Vogelstein [1]. Since then, genome-wide hypomethylation has been reported in several malignancies [2, 3]. In addition, the extent of global hypomethylation appears to correlate with tumor progression and invasiveness in several cancer types, thus indicative of its role in tumor development and progression [32, 33].

What do we learn from COBRALINE-1?

We developed a PCR-based analysis for quantifying the genome-wide level of methylation to improve upon the conventional Southern blot and hybridization approach [3]. Southern blot requires large amounts of DNA and consequently it is difficult to analyze tumor DNA derived from micro-dissection. We applied a modified COBRA PCR protocol to efficiently and quantitatively evaluate LINE-1 methylation status in the entire genome of isolated cell populations. This new protocol targets shorter amplicon sizes of the widely distributed LINE-1 sequences, which greatly improves the yield when amplifying genetic material derived from micro-dissected paraffin-embedded tissue. Interestingly, based on our experience, DNA from paraffin-embedded tissue is difficult to be amplified by conventional PCR methods [34, 35] because DNA is usually degraded by formalin [34]. Interestingly, we experienced a very limited failure rate of PCR

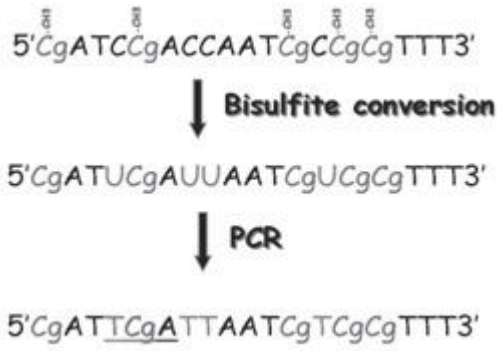

Fig. 1 Schematic representation of DNA sequences. From above to below, the sequences were methylated DNA, bisulfited DNA and bisulfited DNA after PCR, respectively. Bisulfite reaction converted unmethylated cytosines to uracils but methylated cytosines were not changed. After PCR, uracils were amplified as thymine. The underline nucleotide represents sequence that was digestible by *Taq*I.

by COBRALINE-1, which may be due to the large number of repetitive sequence in the genome [20].

Before PCR, to distinguish between methylated and unmethylated sequences, the DNA is treated with bisulfite [14]. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR (**Fig. 1**). This generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified LINE-1 sequences. The modified DNA was amplified by 5'UTR LINE-1 bisulfited sequence primers and then digested with *Taq*I and *Tas*I restriction enzymes, which recognize methylated and unmethylated sequences, respectively. The level of LINE-1 hypomethylation in each sample was calculated by dividing the measured intensity of *Tas*I digestible amplicons with the sum of the *Tas*I and *Taq*I products [3] (**Fig. 2**).

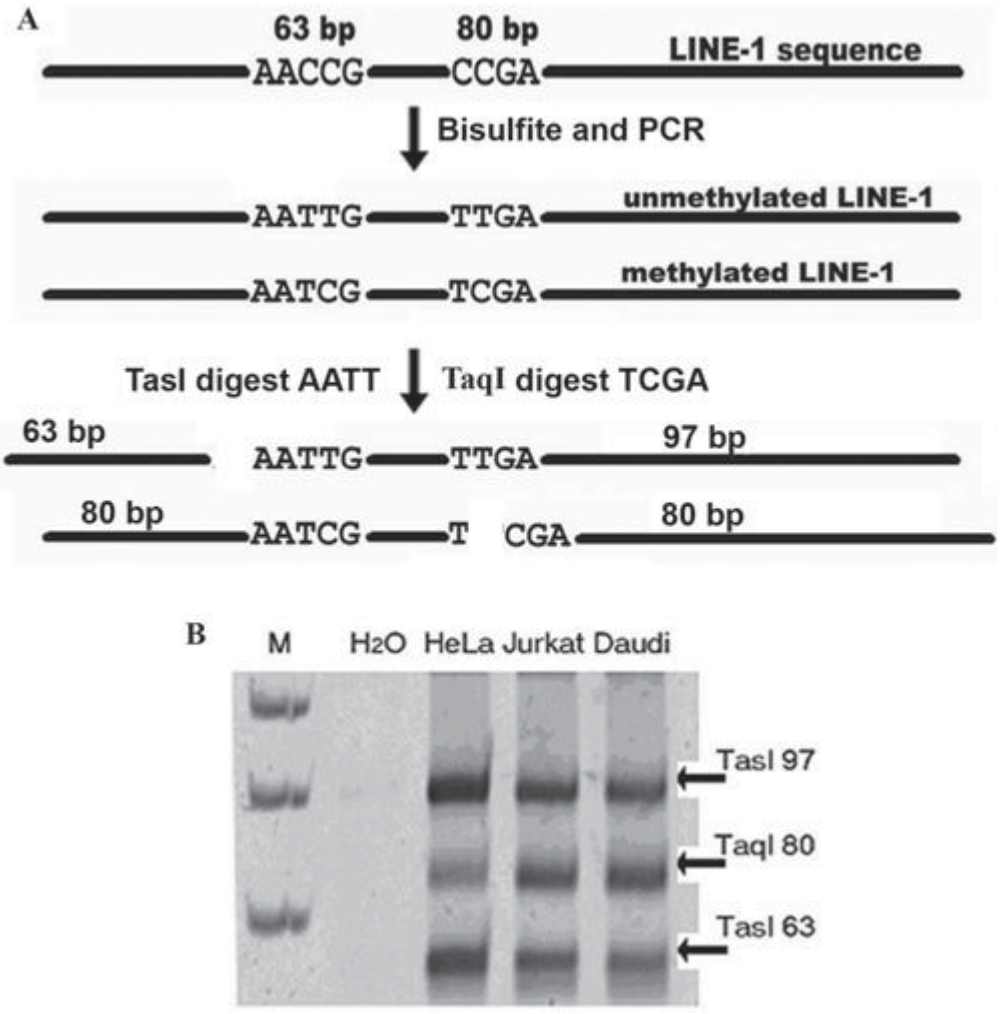

Fig. 2 Combined bisulfite restriction analysis of LINE-1 (COBRA LINE-1) [3]. LINE-1 methylation level was calculated as a percentage of intensity of *Taq*I divided by the sum of *Taq*I-and *Tas*I-positive amplicons. (**A**) Schematic illustration of COBRA LINE-1. The LINE-1 amplicon size is 160 bp. Methylated amplicons, *Taq*I positive, yielded two 80 bp DNA fragments, whereas unmethylated amplicons, *Tas*I positive, yielded 63 and 97 bp fragments. (**B**) An example of COBRA LINE-1. M, 10-bp DNA size marker; numbers under each sample, methylation levels; TasI 63 and 97, unmethylated amplicons; TaqI 80, methylated amplicons. H_2O is water, HeLa, Jurkat and Daudi are cell lines. HeLa LINE-1s are the most hypomethylated among the three.

Interestingly, there are distinctive LINE-1 methylation patterns among normal tissues [3]. In most cases, the distribution of detectable LINE-1 hypomethylation within normal tissue of the same type was consistently clustered, whereas the levels of methylation in thyroid and esophageal tissues were widely distributed. Strikingly, the levels of LINE-1 methylation were significantly different among different tissue types. Our data suggest that this epigenetic event may be continuously altered in adult tissues by the processes of cellular differentiation (**Fig. 3A**). Moreover, this finding implies the physiologic function of LINE-1 level of methylation, which would be new knowledge regarding how human genes are controlled.

For cancer, our finding is consistent with the previous notion that global hypomethylation is a common epigenetic event in cancer. Carcinomas, including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, reveal a greater percentage of hypomethylation than their normal tissue counterparts [3] (**Fig. 3B**). Only lymphomas and carcinomas of the kidney and thyroid do not exhibit significant LINE-1 methylation alterations (**Fig. 3B**). Collectively, with findings from other approaches, global hypomethylation has been found in almost all types of cancers, including colon [1], lung [3], breast [36, 37], stomach [38], prostate [3], ovary [32], urinary bladder [39], and head and neck [3]. It is interesting to note that global hypomethylation is detectable in those cancer types known to have premalignant lesions (Shuangshoti S, *personal communication*). Therefore, global hypomethylation evolves through a multistep process during carcinogenesis. This hypothesis is consistent with our finding that LINE-1 hypomethylation increases in direct relation with tumor progression. Therefore, global hypomethylation is an ongoing process developing during tumor progression, in addition to previous evidence of genomic and LINE-1 hypomethylation occurring as an early event in carcinogenesis [3].

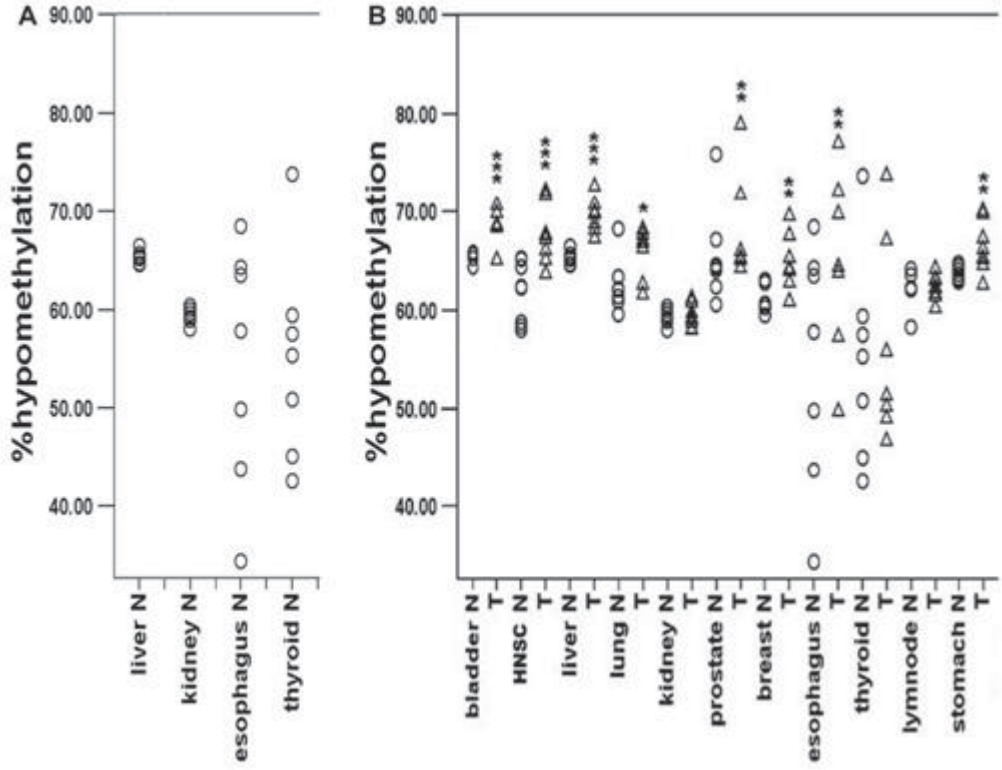

Fig. 3 LINE-1 hypomethylation levels in several tissue types [3]. Circles and triangles are levels of COBRA LINE-1 from normal and malignant, respectively. The vertical axis displays percentage levels of LINE-1 hypomethylation. Sample types are labeled. **(A)** is the hypomethylation levels of adjacent normal tissues and **(B)** is normal and cancers. Single, double, and triple asterisks indicate significant differences in hypomethylation levels between normal tissues and the tested samples at $P<0.05$, <0.01, and <0.001, respectively. N and T are normal and malignant tissues, respectively. The figure was modified from Chalitchagorn K, *et al*. [3].

Current clinical application

Applying COBRALINE-1 in analyzing the genome-wide level of methylation has the advantage over conventional Southern blot and hybridization techniques in that minute quantities of low quality of DNA can be analyzed. Therefore, several sources of DNA can be analyzed, in particular DNA sources from body fluids. Moreover, the analysis can be performed in a quantitative fashion. Consequently, the correlation among large sample sizes can be effectively determined.

Cancer DNA is released into a patient's circulation by an unknown mechanism [40]. The amount of tumor DNA depends on the type and severity of the malignancy. For example, whereas Epstein Barr viral DNA is found in serum/plasma from the majority of patients with nasopharyngeal carcinoma, few patients with cervical cancer have human papilloma viral DNA in their plasma [41]. Moreover, patients with cervical cancer in whom the plasma is HPV-positive are strongly associated with metastasis [41]. Because LINE-1 hypomethylation is a common event in cancer and the level may directly correlate with clinical severity, we evaluated the use of COBRALINE-1 in the serum of cancer patients. Our first systematic approach was to evaluate the sera of patients with hepatocellular carcinoma (HCC) [42]. Serum genome hypomethylation, the percentage of unmethylated LINE-1, was significantly increased in patients with HCC. The levels of serum LINE-1 hypomethylation on initial presentation correlated significantly with the presence of HBsAg, large tumor sizes, and advanced tumor stages, as classified by the CLIP score. Multivariate analyses showed that serum LINE-1 hypomethylation was a significant and independent prognostic factor of overall survival. Therefore, serum LINE-1 hypomethylation may serve as a prognostic marker for patients with HCC [42].

Our ongoing approach was to evaluate whether LINE-1 hypomethylation is a potential prognostic factor for epithelial ovarian cancer (EOC). We found that LINE-1 levels of methylation in EOC were lower than in representative normal ovarian tissues. The mean percentage of the level of methylation in the cancer group was lower than in the control group. In addition, the LINE-1 levels of methylation among specific histologic subgroups were different. The mean LINE-1 levels of methylation ranked higher-to-lower were as follows: mucinous, serous, endometrioid, and clear cell carcinomas. An increase in the level of hypomethylation was correlated with a higher FIGO stage, advanced tumor grade, elevated CA 125 level, and tumor recurrence. Patients with greater hypomethylation had poorer mean overall survival and a lower mean progression-free interval. Therefore, LINE-1 hypomethylation is a common and important epigenetic process in ovarian carcinogenesis. Moreover, the COBRALINE-1 method has the potential to be used as a tumor marker for EOC.

Cho et al. [43] studied hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathologic features. They found that prostate adenocarcinoma with hypermethylation of ASC, COX2, RARB, TNFRSF10C, MDR1, TIG1, RBP1, NEUROG1, RASSF1A, and GSTP1 showed a significantly lower level of methylation of Alu or LINE-1 than prostate adenocarcinoma without hypermethylation. In addition, hypomethylation of Alu or LINE-1 was closely associated with one or more of the above prognostic parameters. These data suggest that with respect to tumor progression, a close relationship exists between CpG island hypermethylation and the hypomethylation of repetitive elements, and that CpG island hypermethylation and DNA hypomethylation contribute to cancer progression [43].

Similarly, the PCR-based method has been used to evaluate the significance of methylation of repetitive sequences in particular Alu and LINE-1 in association with chromosomal instability, and microsatellite instability (MSI) remains unclear in colorectal cancer. Matsuzaki et al. [44] investigated the relationship between global methylation status, loss of heterozygosity (LOH), and MSI in sporadic colorectal cancer. They found LOH was observed more frequently in microsatellite stable (MSS) cancers than in MSI cancers at all loci. MSS cancers showed significantly lower global methylation levels when compared with MSI cancers. Tumors with higher global hypomethylation had a significantly increased number of chromosomal loci with LOH than did tumors without global hypomethylation. This suggests that global hypomethylation plays an important role in inducing genomic instability in colorectal carcinogenesis [44].

Interestingly, the level of global hypomethylation from white blood cells may reflect head and neck

cancer risk. Hsiung et al. [45] determined whether global methylation in DNA derived from whole blood was associated with head and neck cancer. They found that hypomethylation lead to a significant 1.6-fold increased risk for disease in models controlled for other head and neck squamous cell cancer (HNSCC) risk factors. Smoking showed a significant differential effect on blood levels of methylation between cases and controls. Therefore, DNA hypomethylation in non-target tissues was independently associated with HNSCC and had a complex relationship with the known risk factors associated with the genesis of HNSCC [45]. The mechanism to account for this finding is not known. However, it is interesting to speculate that changes in the genome-wide level of methylation are systemic rather than local. Chronic feeding of a methyl-donor (methionine, choline, folic acid, and vitamin B12) deficient diet induces hepatocellular carcinoma in rats. Asada et al. [46] determined cytosine methylation status in the LINE-1 repetitive sequences of rats fed a choline-deficient (CD) diet of various durations and compared the cytosine methylation status with rats fed a choline-sufficient diet. Progressive hypomethylation was observed in LINE-1 liver of rats fed a CD diet as a function of feeding time [46].

Other applications

LINE-1 methylation may be continuously altered in adult tissues by the processes of cellular differentiation [3]. Therefore, it is interesting to explore the role of LINE-1 methylation during the human developmental process. Perrin et al [47] studied global DNA methylation of trophoblastic tissues. Partial hydatidiform mole and normal placenta have identical global levels of DNA methylation. Surprisingly, LINE-1 sequences are hypermethylated in partial hydatidiform mole tissues. This confirmed the physiologic roles of LINE-1 methylation.

Several causes and consequences of global hypomethylation appear to occur in cis that is linked to nearby unmethylated DNA. For example, studies in ICF syndrome (immunodeficiency, chromosomal instability, and facial anomalies) with loss-of-function mutations in the cytosine DNA methyltransferase *DNMT3B* [48] and Wilm's tumor [49] demonstrated the direct association between loss of DNA methylation and rearrangements in the pericentromeric heterochromatin. Therefore, hypomethylation could lead to spontaneous mutations in cis. Moreover, LINE-1 methylation may influence the linked gene expression [22, 30, 31]. Consequently, LINE-1 demethylation may lead to an alteration of linked gene expression. Therefore, COBRALINE-1 should be a great tool for evaluating the biology of these phenomena. Finally, alteration of DNA methylation should not only affect tumor phenotype, but a potential role of changes in human DNA methylation patterns in other conditions, such as atherosclerosis and autoimmune diseases (e.g., multiple sclerosis, psoriasis, and lupus) has been recognized [50-52]. Therefore, in addition to cancer, COBRALINE-1 should be a great tool to study fundamental biologic processes and may be a diagnostic tool in the future.

Further improvement

The potential clinical application of the COBRALINE-1 approach is illustrated by a significantly higher serum LINE-1 hypomethylation levels in HCC patients when compared to normal individuals [42]. However, the two levels are, as expected, considerably overlapped. Further improvements of the current method in order to show a more distinct LINE-1 hypomethylation pattern for tumor versus normal tissues, if possible, are crucial. Our previous study [3] revealed that there are differences of LINE-1 methylation levels not only among normal tissues but also cancer types and stages. Our ongoing researches indicate that the differences may be due to the distinctive patterns of methylation among LINE-1 loci. Therefore, LINE-1 hypomethylation from several loci may serve as potential prognostic markers for certain cancers.

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