IDLN-MSP: Idiolocal normalization of real-time methylation-specific PCR for genetic imbalanced DNA specimens

Simeon Santourlidis[1], Foued Ghanjati[1], Agnes Beermann[1], Thomas Hermanns[2], and Cédric Poyet[2]

[1]Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Düsseldorf, Germany and [2]Department of Urology, University Hospital, University of Zurich, Zurich, Switzerland

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Sensitive, accurate, and reliable measurements of tumor cell-specific DNA methylation changes are of fundamental importance in cancer diagnosis, prognosis, and monitoring. Real-time methylation-specific PCR (MSP) using intercalating dyes is an established method of choice for this purpose. Here we present a simple but crucial adaptation of this widely applied method that overcomes a major obstacle: genetic abnormalities in the DNA samples, such as aneuploidy or copy number variations, that could result in inaccurate results due to improper normalization if the copy numbers of the target and reference sequences are not the same. In our idiolocal normalization (IDLN) method, the locus for the normalizing, methylation-independent reference amplification is chosen close to the locus of the methylation-dependent target amplification. This ensures that the copy numbers of both the target and reference sequences will be identical in most cases if they are close enough to each other, resulting in accurate normalization and reliable comparative measurements of DNA methylation in clinical samples when using real-time MSP.

Since its discovery at the beginning of this century (1), many studies have convincingly demonstrated that DNA methylation is of fundamental importance for vertebrate differentiation (2). It is now evident that a complex and diverse, cell-type specific DNA methylome exists that determines cellular identity and functional states. It constitutes a layer of epigenomic information crucial for gene regulation and cellular differentiation, and it is involved in human disease (3).

The reliable detection of cell-type-specific DNA methylation signatures has become increasingly useful for the identification and genome integrity control of various stem cell types (e.g., induced pluripotent stem cells and mesenchymal stem cells), which are broadly used for research and certain clinical applications such as cell replacement therapies. In cancer, many DNA methylation biomarkers with likely diagnostic, prognostic, and predictive power are in clinical trials, particularly because tumor-derived, aberrantly methylated circulating DNA can be detected in the plasma and other body fluids of cancer patients (4). Few of these biomarkers, however, are presently used in clinical settings (5,6).

In this context, methylation-specific PCR (MSP) (7) is generally accepted as an analytically suitable technique for accurately interrogating DNA methylation at single CpG sites. DNA methylation detection can be carried out in a precise, semi-quantitative manner by using intercalating dyes (e.g., SYBR Green) in real-time MSP, which provides high analytical sensitivity and a further improvement in specificity by decreasing the numbers of false-positive and false-negative cases in cancer diagnosis (8). For these reasons this method has already been used in clinical applications for the diagnosis of cancer (9).

In this method the following formula is used:

$\Delta\Delta C_q = \Delta C_q$ (C_q, sample (target locus) - C_q, sample (reference)) - ΔC_q (C_q, calibrator (target locus) - C_q, calibrator (reference)).

For the $\Delta\Delta C_q$ calculation to be valid and to result in reliable measurements, the amplification efficiencies of the methylation-dependent target and of the methylation-independent reference must be approximately equal (10), and it is presumed that the copy numbers

METHOD SUMMARY

In our idiolocal normalization (IDLN) modification of real-time methylation-specific PCR (MSP) assay, the methylation-independent reference sequence used for normalization is selected to be very close to the methylation-dependent target sequence. This guarantees that the copy numbers of the reference and target sequences will be equal in samples such as tumor DNA that may have copy number variations affecting either the reference or target sequence, which could lead to incorrect real-time MSP results.

of these loci are identical in all samples. Unfortunately, this latter prerequisite is in reality compromised by the numerous genetic abnormalities that are prevalent even during early cancer development (11) for almost every cancer type (12) as well as in pluripotent stem cells in culture (13). That means that application of this method to tumor cell DNA harbors the serious danger of false-negative and false-positive detection due to unequal numbers of targeted DNA loci within the compared samples. Unfortunately, it is not feasible to determine copy number variations for each patient DNA sample before applying MSP.

This serious problem was recognized and described previously for MethylLight assays and has been tackled by the significant improvement of using Alu sequences, depleted of CpG dinucleotides, in the control reactions (14). Later on, this kind of normalization has been adopted for SMART-PCR (8) since it minimizes normalization errors caused by aneuploidy and copy number changes often observed in cancer cells (15).

Here we describe a simple technical adaptation of intercalating dye real-time MSP, which we have named idiolocal normalization (IDLN), that overcomes this problem and broadens the application of the method for diagnostic purposes. We chose the locus for reference amplification to be close to the locus of target amplification. Thus, the copy numbers of both the reference and target template sequences for a ΔC_q measurement should be equal in almost every case, guaranteeing that the measurements will be comparable. Genetic changes (e.g., translocations, deletions, etc.) will only interfere with the results in the rare cases where they have affected the short segment between the reference and target template sequences. Therefore, the closer the reference sequence lies to target sequence, the better.

Theoretical models easily demonstrate how aneuploidy at the involved target loci that changes the C_q values inserted into the $2^{-\Delta\Delta C_q}$ formula inevitably results in a misleading DNA methylation value. For instance, the occurrence of a triploid target locus for the methylation-dependent target amplification would lead to an overestimate of methylation.

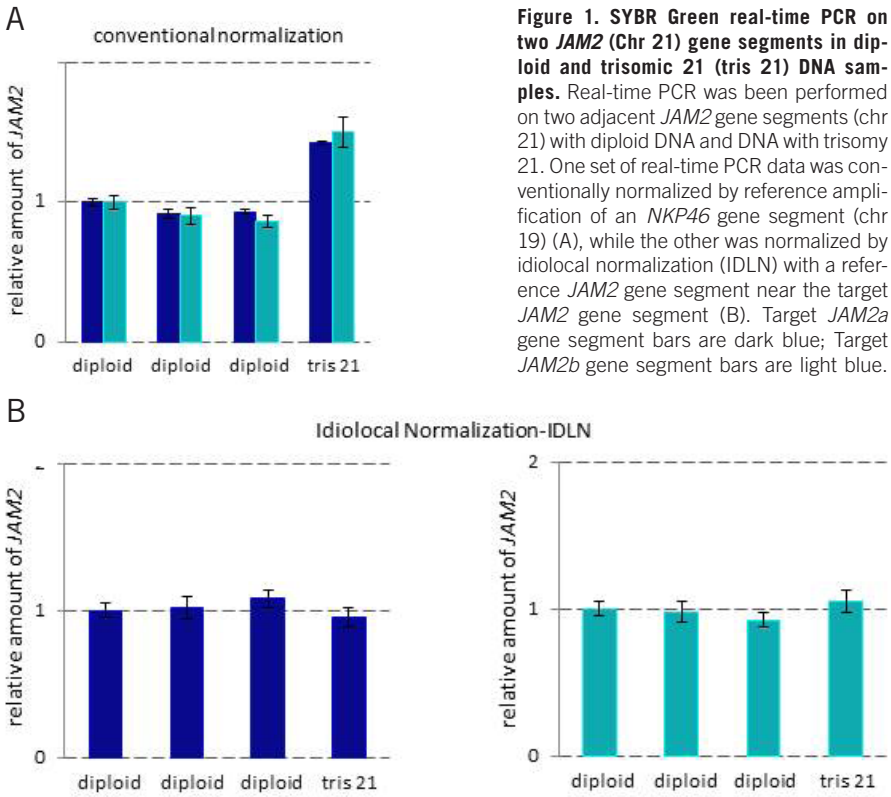

Figure 1. SYBR Green real-time PCR on two *JAM2* (Chr 21) gene segments in diploid and trisomic 21 (tris 21) DNA samples. Real-time PCR was been performed on two adjacent *JAM2* gene segments (chr 21) with diploid DNA and DNA with trisomy 21. One set of real-time PCR data was conventionally normalized by reference amplification of an *NKP46* gene segment (chr 19) (A), while the other was normalized by idiolocal normalization (IDLN) with a reference *JAM2* gene segment near the target *JAM2* gene segment (B). Target *JAM2a* gene segment bars are dark blue; Target *JAM2b* gene segment bars are light blue.

To test if this is really valid in practice, we first chose an experimental set-up mimicking a triploid situation (Figure 1). In this example, we measured by SYBR Green real-time PCR the amount of 2 *JAM2* gene segments (JAM2a and JAM2b) that are 136 bp apart from each other on chromosome 21 in equal amounts of DNA samples from diploid individuals and probands with trisomy 21. For each *JAM2* gene segment, we either normalized conventionally with the unlinked diploid gene locus *NKp46* on chromosome 19 or by our IDLN method using the other *JAM2* gene segment as the reference for normalization (see protocol in the Supplementary Material for details). With IDLN, the same relative amount of each *JAM2* target segment on chromosome 21 was repeatedly measured for the trisomic as well as the disomic DNA samples (Figure 1B). In contrast, the conventionally normalized MSP approach using an unlinked reference locus that was disomic in all samples clearly indicated an enrichment of the *JAM2* gene segments within the trisomic DNA sample (Figure 1A).

This result suggests that methylation of an unaltered, fully methylated target locus would be overestimated by SYBR Green MSP in a trisomic situation if normalization was conventionally conducted using an arbitrarily chosen disomic normalization locus. On the other hand, correct levels of DNA methylation would be measured in a trisomic situation by IDLN since it compensates for underlying genetic imbalances within samples so that methylation-dependent amplifications remain solely dependent on the methylation status.

It would also be expected that an arbitrarily chosen normalization locus that is present in higher or lower copy numbers, such as commonly occurs in tumor DNA samples, would also result in false DNA methylation values. To test this, we first precisely determined the representative methylation status of LINE-1 elements in urinary cell DNA of one bladder cancer patient and one healthy proband. LINE-1 hypomethylation in bladder cancer has been described as a common, early, and, for diagnostic purposes, very promising DNA methylation alteration (16). The results of our bisulfite genomic sequencing reveal the relative DNA methylation of every single CpG dinucleotide within the relevant, distinct

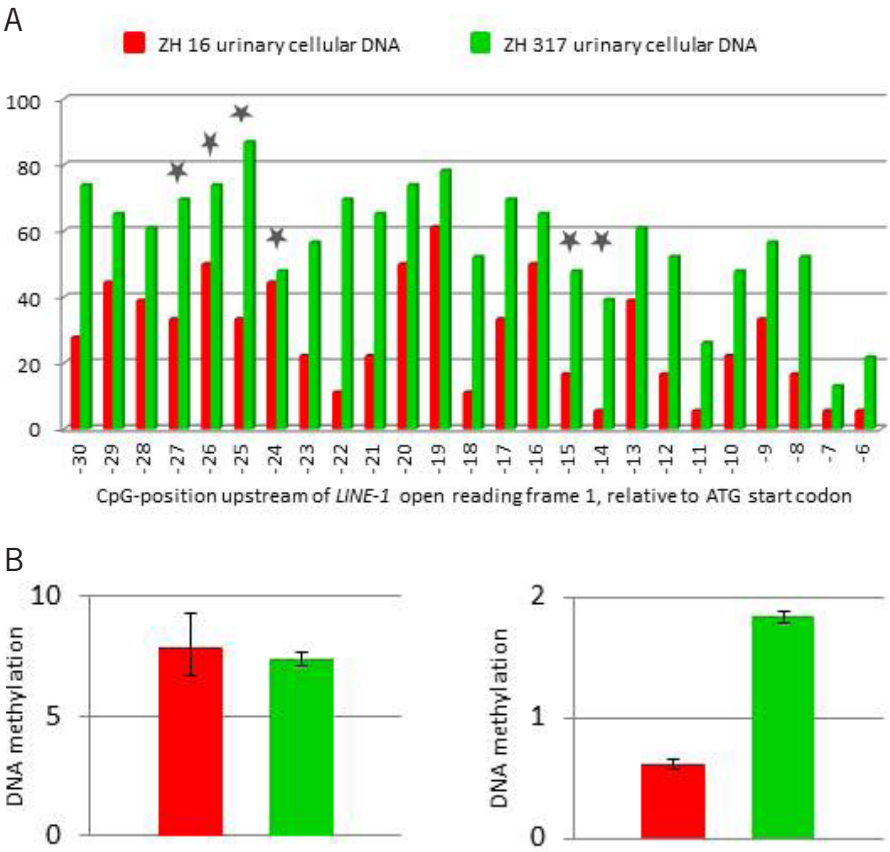

Figure 2. Bisulfite sequencing, conventional normalized real-time methylation-specific PCR (MSP), and idiolocal normalization (IDLN)-MSP of urinary cellular DNA from one bladder cancer patient and one healthy proband. DNA methylation profiles of 436-bp long, CpG-rich, *LINE-1* promoter regions from urinary cellular DNA of one bladder cancer patient (ZH 16, red) and one healthy proband (ZH 317, green) were analyzed by bisulfite genomic sequencing as shown in (A). Each bar shows the frequency of methylated CpGs at the CpG position indicated by number below the bar. CpG positions are relative to the ATG start codon of first *LINE-1* open reading frame; for example, -6 means the sixth CpG upstream of this ATG. Stars above the bars indicate the positions of the primers used in the real-time MSP assays on the same samples. This result is shown in (B), where the left panel shows the *GAPDH*-normalized result and the right panel shows the idiolocal normalization result.

promoter segment of LINE-1 DNA elements (Figure 2A). Only real-time MSP using IDLN with a reference sequence 185 bp downstream of the target sequence in the LINE-1 promoter (see protocol in the Supplementary Material for details) reproduced the results from bisulfite sequencing showing hypomethylation of bladder cancer patient DNA, whereas the real-time MSP result normalized conventionally with an unlinked gene locus (GAPDH) yields a misleading result (Figure 2B) due to the aneuploidy found in transitional cell carcinoma of the bladder (17). The reliability of IDLN real-time MSP has been validated for this experimental set-up in additional patient samples. In addition, we have also validated the procedure by direct comparison to conventionally normalized measurements in more than 100 samples of cell-free and cellular urinary DNA (4).

Therefore, for all measurements using relative quantifying intercalating dye real-time MSP in DNA samples with likely genetic imbalances, idiolocal normalization of real-time MSP is a useful option. Samples of this sort include not only cancer samples but also cells after prolonged cultivation, (e.g., stem cells).

Author contributions

S.S. contributed to the conception, development, and writing of the manuscript. F.G. contributed to the execution and analysis of experiments. A.B. contributed to the execution and analysis of experiments. T.H. and C.P. defined and prepared clinical samples.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Simeon Santourlidis, Schürkesweg 3, 40670 Meerbusch, Germany. E-mail: simeon.santourlidis@med.uni-duesseldorf.de