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Original Article

Comparison between Absolute and Relative Quantification of *LINE-1* Methylation Level in Breast Cancer

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Abstract: There are two accurate formulas corresponding to absolute and relative methods that were widely used for quantitative DNA methylation level. In the absolute method, DNA methylation is examined by two sets of MSP primers, one is specific for the methylated state and the other for the unmethylated state of the target. In the relative method, DNA methylation is examined by two sets of primers, one is MSP, specific for the methylated state, and the other is MIP, for the measurement of input DNA levels to normalize the signal for each methylation reaction. Both these methods, subjected to quantitative measurement of the *LINE-1* methylation level in breast cancer samples, indicating *LINE-1* hypermethylation in tumour tissue samples as compared with adjacent tissue samples. The methylated *LINE-1* amount is enormous but the unmethylated one is scant, consequently leading to greater measurement variance compared with the comparative quantification.Thus, the relative quantification, may be a more convenient method for quantitatively measuring the *LINE-1* methylation level.

Keywords: DNA methylation, absolute quantification qPCR, relative quantification qPCR.

1. Introduction

Real-time quantitative PCR (qPCR) is a powerful tool to quantify gene expression including absolute and relative expression levels [1]. The absolute quantification requires standard curves to determine the input copy number of the transcript of the target while the relative quantification requires a calibrator to determine the fold change in target expression relative to that in the calibrator.

The DNA methylation at deoxycytidine nucleotides distributed in CpG is well known as an epigenetic regulation mechanism for genomic function. Aberrant DNA methylation, contributing to cancer development, have

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focused extensively on the identification of DNA methylation biomarkers for diagnosing cancer [2]. Actually, DNA methylation at target sequences was routinely analyzed by methods based on the PCR approach [3]. The majority of the PCR-based methods use DNA templates that have been treated with sodium bisulfite. This chemical converts unmethylated cytosine, but not methylated cytosine, to uracil residues that are subsequently converted to thymines after the first round of PCR [4]. Specific primers were designed on the basis of sequences after bisulfite conversion, thus distinguishing the methylated from unmethylated templates [5].

There are two accurate formulas corresponding to absolute and relative methods were widely used for quantitative DNA methylation level. The first formula corresponding to absolute method, is defined as the ratio between methylated molecules (M) and the sum of methylated and unmethylated molecules (M+Un) [5]. The methylated and unmethylated molecules were calculated based on the standard curves performed on serial dilutions with known copy number of the linearised recombinant plasmids containing methylated and unmethylated sequences, respectively [5]. It is worth noting that the unmethylated target is low GC content due to conversion of unmethylated C into T and that poor binding of SYBR to low GC content sequences can result in an undetectable fluorescence signal regardless of the presence of excessive amplicons [6, 7]. The second formula is the classical ΔΔCT approach, the comparative CT method using a calibrator reference [8]. The ΔΔCT method, commonly used for gene expression analysis, shows the relative difference of DNA methylation level between the target sequences and a reference sequence in the sample compared with a calibrator sample [3]. It was first adapted to quantitative methylation-specific PCR (qMSP) using two primer sets: i) The methylation-independent-specific PCR (MIP) primer set, represented as the reference, amplifies the total bisulfite- converted product; and ii) The methylation-dependent specific PCR (MSP) primer set amplifies the methylated target [8]. The ΔΔCT method has been now widely used for evaluating the efficiency of bisulfite conversion of commercial bisulfite conversion kits and validating methylation-based markers used for clinical diagnosis of cancer.

The ΔΔCT method requires a calibrator sample with a known number of the methylated target specifically recognised by the MSP primer set, and a reference sequence specifically recognised by the MIP primer set. The reference that designed from the same or unrelated targets [9]. The calibrator could be achieved by using serial dilutions with known concentrations of a recombinant plasmid containing the methylated and reference sequences [9].

The Long Interspersed Element-1 (*LINE-1*), the only autonomous and active retrotransposon element occupies 17% of the human genome and its altered methylation level is considered a hallmark of cancer [2, 10]. In this study, we quantitatively measured DNA methylation level of the *LINE-1* in breast cancer, using both the absolute and comparative quantification. We found that *LINE-1* methylation level calculated with the absolute methods showed greater measurement variance compared with the comparative quantification. Given its feasibility and popularity, the ΔΔCT method was easily performed to quantification of DNA methylation of repetitive sequences in human genome.

2. Experimental

2.1. Sample Collection

Tumour tissue samples and their corresponding pair-matched adjacent normal tissue samples from 35 primary breast cancer patients were collected at the 175 Hospital (Ho Chi Minh City) during 2023 year. Informed consent was obtained from healthy participants and patients in written form, and all collection methods were performed in accordance with the relevant guidelines and regulations by the Ethics

Committee of the Vietnam Academy of Science and Technology (01-2023/NCHG-HDDD).

2.2. DNA Isolation and Bisulfite Conversion

DNAs were extracted from tissues using the DNeasy Blood & Tissue Kit (Qiagen). After DNA quantification using the Qubit4 Fluorometer (Thermo Fisher Scientific), a concentration of 0.5 ng of genomic DNA was subjected to bisulfite conversion using EZ DNA Methylation-Gold TM kit (Zymo Research). The bisulfite converts unmethylated cytosine, but not methylated cytosine, to uracil residues [4].

2.3. Construction of a Calibrator

A calibrator sample with the *LINE-1* methylation level is defined by using serial dilutions concentrations of a recombinant plasmid containing the methylated *LINE-1* sequences specifically recognised by the MSP primer set and the bisulfite-converted *LINE-1* reference specifically recognised by the MIP primer set [11, 12]. A serial dilution of the linearized recombinant plasmids pLINE-Ref and pLINE-Me respectively containing the bisulfite-converted *LINE-1* and the methylated *LINE-1* sequences were mixed with used as a calibrator for measurement of the *LINE-1* methylation level [11, 12].

2.4. Quantitative Real-Time Methylation Specific PCR Reaction (qMSP)

qMSP is carried out in 20 µl per reaction using one µl of bisulfite converted DNA as template and SsoAdvanced Universal SYBR Green Supermix (Biorad). Real-time PCR assays were duplexed for each of the 3 reactions: (1) and (2) using the MSP primers to quantify methylated *LINE-1* (LINE-Me) and unmethylated *LINE-1* (LINE-Un), and (3) using the MIP primers to quantify bisulfite converted *LINE-1* (LINE-Ref), respectively. Water with no DNA template was included in each PCR reaction as a control for contamination. The primer sequences have been described previously [11, 12]. All qPCR reactions were performed using the 7500 Real-time PCR instrument (Applied Biosystems, CA).

2.5. Methylation Level Calculation

The alsolute quantification using qPCR with the primers specific to methylated-CpG and unmethylated-CpG in the *LINE-1* sequences The *LINE-1* methylation level was defined as the ratio between the methylated *LINE-1* molecules and the sum of the methylated *LINE-1* and unmethylated *LINE-1* molecules [3]. These molecules were calculated based on standard curve performed on serial dilutions from 10^6 to 10 copies of the linearized recombinant plasmid pLINE-Me and pLINE-Un. To calculate the *LINE-1* methylation level with the ΔΔCT approach as relative quantification, a calibrator sample with a defined methylation level of 10% was obtained by mixing linearized plasmids pLINE-Me and pLINE-Ref with 10 ng of genomic DNA extracted from lymphocytes of healthy donors. The relative amount of the methylated *LINE-1* level was calculated for each sample as following [6]:

ΔΔCTSample = ΔCTSample−ΔCTCalibrator, ΔCTSample = CTSample (Methylated *LINE-1* – CTSample/Ref-*LINE-1;*

ΔCTCalibrator = CTCalibrator/Methylated*LINE-1*– CTCalibrator/Ref- *LINE-1*.

ΔΔCTs were measured in duplicate. Methylation Sample = Methylation Calibrator (10%) x $2^{\Delta\Delta^{CTSample}}$. Samples were excluded from the study when two replicates of the quantification methylation assay showed CT value > 34.

2.6. Statistical Analysis

In all box plots, methylated and unmethylated *LINE-1* were expressed as medians with interquartile values. Comparisons between 2 groups on a quantitative value were assessed using the student t-test when normality was met, otherwise by the Wilcoxon test. For all statistical analyses, a p-value < 0.05 was considered as significant.

All analyses were performed with the STATA program version 12 (https://www.stata.com/) and GraphpadPrism program version 9 (https://www.graphpad.com/ scientific-software/prism/).

3. Results and Discussion

3.1. Standard Curves used for the Absolute and Relative Quantifications

A serial dilution of the linearized recombinant plasmids pLINE-Me, pLINE-Un and pLINE-Ref, respectively containing the methylated *LINE-1* sequence, the unmethylated *LINE-1* sequence, and the reference *LINE-1* sequence, from 10^6 to 10 copies, were used as as templates in qPCR with the MIP and MSP primer sets (Figure 1). The CT values have a strong linear relationship with the DNA input for both primer sets (R^2>0.99). There was a statistically insignificant difference in amplification efficiency (E value) with 96.07% for the *LINE-1* reference amplicons and 101.3% for the methylated *LINE-1* amplicons (Figure 1A, B). The difference in CT values of both the *LINE-1* reference and the methylated *LINE-1* sequences was then plotted against the logarithm of the template input amount, with the slope of the fitted line to be -0.1113, thus demonstrating the Livak formula suitable for the relative quantification of the methylated *LINE-1* (Figure 1C). samples were identical to the input Taken together, these results indicate the designed MIP and MSP primer sets ensure accurate measurement of the *LINE-1* methylation levels using the Livak formula.

Standard curves with the CT values plotted against the known concentrations of recombinant plasmids containing the methylated *LINE-1* (LINE-Me) (A), the unmethylated *LINE-1* sequences (LINE-Un) (B), and the reference Ref (LINE-Ref) (C). The amplification efficiency (E value) and the coefficient of correlation (R^2 value) were shown in each standard curve. The data were fitted using least-squares linear regression analysis (N = 6-16). Validation of the relative method for the quantification of *LINE-1* methylation. The ΔΔCT method can be used for the MIP primers since the amplification efficiencies of both the methylated *LINE-1* (101.307 %) and Reference (96.607%) amplicons are similar with the slope of the fitted line to be -0.1113 (D). The data were fitted using least-squares linear regression analysis (N = 3-16).

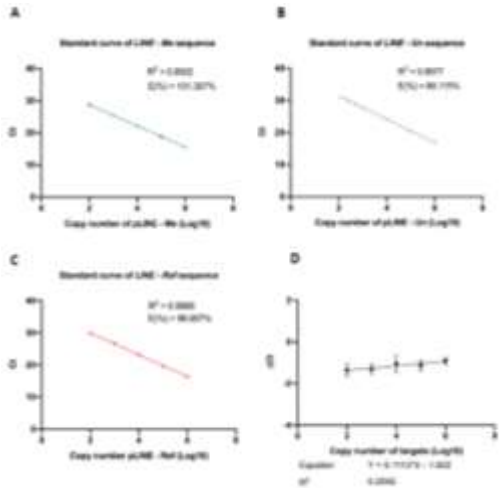

Figure 1. Generating a standard curve to assess absolute and relative quantification of the *LINE-1* methylation.

3.2. Absolute and Relative Quantifications of the LINE-1 Methylation Level

Based on the standard curves (Figure 1A, B), the methylated and unmethylated *LINE-1* molecules in breast tumour and adjacent tissue samples were absolutely quantified as the ratio between the methylated *LINE-1* molecules and the sum of the methylated *LINE-1* and unmethylated *LINE-1* molecules. Results showed that *LINE-1* is hypermethylated in tumour tissue samples when compared with adjacent tissue samples (Figure 2A). On the other hand, based on the relative Livak formula, the relatively calculated *LINE-1* methylation level in tumour and adjacent tissue samples was presented in Figure 2B.

Based on the standard curves (Figure 1A, B), the methylated and unmethylated *LINE-1* molecules in breast tumour (BC) and adjacent tissue (AD) samples were quantified using the

absolute formula. Results showed that *LINE-1* is hypermethylated in breast tumour tissue samples when compared with adjacent tissue samples (A). Based on the validation of amplification efficiency (Figure 1D), the *LINE-1* methylation level in breast tumour (BC) and adjacent tissue (AD) samples was calculated based on the Livak formula (B). The result showed that *LINE-1* is hypermethylated in breast tumour tissue samples when compared with adjacent tissue samples. The Wilcoxon matched-pairs signed rank test was used in statistical analysis.

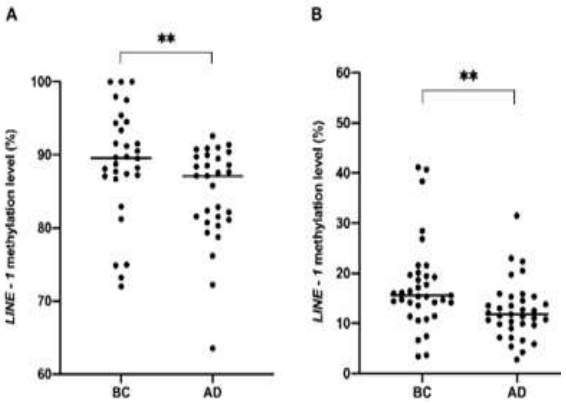

Figure 2. Absolute (A) and relative (B) quantifications of the *LINE-1* methylation.

Both absolute and relative methods can be used to quantify DNA methylation level. In the absolute method, DNA methylation is examined by two sets of MSP primers, one is specific for the methylated state and the other for the unmethylated state of the target. The *LINE-1* sequences were heavily methylated but scantily unmethylated, consequently leading to greater measurement variance of the low GC content due to conversion of unmethylated C into T (Figure 2A). In the relative method, DNA methylation is examined by two sets of primers, one is MSP, specific for the methylated state, and the other is MIP, for the measurement of input DNA levels to normalize the signal for each methylation reaction. The MIP primers can be used not only for a specific target but also for different targets. For example, the MIP primers derived from either the single copy gene *ACTB* or the repetitive *Alu* have been used for the analysis of the methylation status of different specific genes as well as the repetitive *Alu*, *LINE-1* and *Sat2* elements [13-15]. A further advantage is that the MIP primers could be derived from the same or unrelated locus with the MSP primers while both the methylated and unmethylated MSP primer sets should be designed from the same region [5]. Moreover, it is worth noting that the low GC content of the unmethylated target, due to conversion of unmethylated C into T, and that poor binding of SYBR to low GC content sequences can result in an undetectable fluorescence signal regardless of the presence of excessive amplicons [6, 7]. In addition, the inefficient amplification of the unmethylated sequences has been reported by Wojdacz et al., [15], Ji et al., [16], Šestáková et al., [17], and Taryma-Lesniak et al., [18]. In our study, the *LINE-1* methylation level calculated with the absolute method showed greater measurement variance compared with the comparative quantification. Our result revealed that *LINE-1* was hypermethylated in breast cancer; consistent with previous reports on an increase in *LINE-1* methylation level in patients with age-related macular degeneration (AMD), multiple sclerosis (MS), and high-grade cervical intraepithelial neoplasia (CIN2+) when compared with healthy controls [19, 20]; thus, supporting to its repressor role in carcinogenesis [21].

4. Conclusion

Regarding all the reasonable evidence presented above, the relative quantification, using the MIP and the MSP primers, may be a more convenient method for quantitatively measuring the *LINE-1* methylation level.

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References

[1] T. D. Schmittgen, K. J. Livak, Analyzing Real-time PCR Data by the Comparative C(T) Method, Nature Protocols, Vol. 3, 2008, pp. 1101-1108.

[2] J. Ibrahim, M. Peeters, G. Van Camp, K. Op de Beeck, Methylation Biomarkers for Early Cancer Detection and Diagnosis: Current and Future Perspectives, European Journal of Cancer, Vol. 178, 2023, pp. 91-113.

[3] L. S. Kristensen, L. L. Hansen, PCR-based Methods for Detecting Single-locus DNA Methylation Biomarkers in Cancer Diagnostics, Prognostics, and Response to Treatment, Clinical Chemistry, Vol. 55, 2009, pp. 1471-1483.

[4] S. J. Clark, J. Harrison, C. L. Paul, M. Frommer, High Sensitivity Mapping of Methylated Cytosines, Nucleic Acids Research, Vol. 22, 1994, pp. 2990-2997.

[5] J. G. Herman, J. R. Graff, S. Myöhänen, B. D. Nelkin, S. B. Baylin, Methylation-specific PCR: A Novel PCR Assay for Methylation Status of CpG Islands, Proceedings of the National Academy Sciences of the USA, Vol. 93, 1996, pp. 9821-9826.

[6] H. Gudnason H, D. Martin, B. B. Bang, A. Wolff, Comparison of Multiple DNA Dyes for Real-time PCR: Effects of Dye Concentration and Sequence Composition on DNA Amplification and Melting Temperature, Nucleic Acids Research, Vol. 35, 2007, pp. e127.

[7] S. Giglio, P. Monis, C. Saint, Demonstration of Preferential Binding of SYBR Green I to Specific DNA Fragments in Real-time Multiplex PCR, Nucleic Acids Res, Vol. 31, 2003, pp. e136.

[8] K. J. Livak, T. D. Schmittgen, Analysis of Relative Gene Expression Data using Real-time Quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods. Vol. 25, 2001, pp. 402-408.

[9] H. Thomassin, C. Kress, T. Grange, MethylQuant: A Sensitive Method for Quantifying Methylation of Specific Cytosines within the Genome, Nucleic Acids Research, Vol. 32, 2004, pp. e168.

[10] E. S. Lander et al., International Human Genome Sequencing Consortium, Initial Sequencing and Analysis of the Human Genome, Nature, Vol. 409, 2001, pp. 860-921.

[11] D. A. T. Pham, S. D. Le, T. M. Doan, P. T. Luu, U. Q. Nguyen, S. V. Ho, L. T. T. Vo, Standardization of DNA Amount for Bisulfite Conversion for Analyzing the Methylation Status of LINE-1 in Lung Cancer, PloS one, Vol. 16, 2021, pp. e0256254.

[12] T. T. Q. Trang, P. T. Tung, N. T. Than, D. H Trang, L. T. T. Phuong, N. Q. Uyen, V. D. Linh, N. N. Quang, H. V. Son, D. V. Hang, H. V. Tong, V. T. T Lan, An Appropriate DNA Input for Bisulfite Conversion Reveals *LINE-1* and *Alu* Hypermethylation in Tissues and Circulating Cell-free DNA from Cancers, PLoS One, Vol. 19, 2924, pp. e0316394.

[13] C. A. Eads, K. D. Danenberg, K. Kawakami, L. B. Saltz, C. Blake, D. Shibata, P. V. Danenberg, P. W. Laird, MethyLight: A High-throughput Assay to Measure DNA Methylation, Nucleic Acids Research, Vol. 28, 2000, pp. e32.

[14] S. Jin et al., Efficient Detection and Post-surgical Monitoring of Colon Cancer with a Multi-marker DNA Methylation Liquid Biopsy, Proceedings of the National Academy Sciences of the USA. Vol. 118, 2021, pp. e20174-21118.

[15] D. J. Weisenberger, M. Campan, T. I. Long, M. Kim, C. Woods, E. Fiala, M. Ehrlich, P. W. Laird, Analysis of Repetitive Element DNA Methylation by MethyLight, Nucleic Acids Research, Vol. 33, 2005, pp. 6823-6836.

[16] T. K. Wojdacz, L. L. Hansen, Reversal of PCR Bias for Improved Sensitivity of the DNA Methylation Melting Curve Assay, BioTechniques, Vol. 41, 2006, pp. 274-278.

[17] L. Ji, T. Sasaki, X. Sun, P. Ma, Z. A. Lewis, R. J. Schmitz, Methylated DNA is Over-represented in Whole-genome Bisulfite Sequencing Data, Frontiers in Genetics, Vol. 5, 2014, pp. 341.

[18] Š. Šestáková, C. Šálek, H. Remešová, DNA Methylation Validation Methods: A Coherent Review with Practical Comparison, Biological Procedures Online, Vol. 21, 2019, pp. 19.

[19] M. Barchitta, A. Quattrocchi, A. Maugeri, LINE-1 Hypermethylation in White Blood Cell DNA is Associated with High-grade Cervical Intraepithelial Neoplasia, BMC Cancer, Vol. 17, 2017, pp. 601.

[20] K. Y. Neven, M. Piola, L. Angelici, Repetitive Element Hypermethylation in Multiple Sclerosis Patients, BMC Genet, Vol. 17, 2016, pp. 84.

[21] M. M. Kelsey, Reconsidering LINE-1's Role in Cancer: Does LINE-1 Function as a Reporter Detecting Early Cancer-associated Epigenetic Signatures? Evolution, Medicine, and Public Health, Vol. 9, 2021, pp. 78.