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Improved results of LINE-1 methylation analysis in formalin-fixed paraffin-embedded tissues with the application of a heating step during the DNA extraction process

포르말린 고정 및 파라핀 포매된 외과병리 검체로부터 DNA를 추출하는 과정에서 가열과정의 도입으로 인한 LINE-1 메틸화 분석결과의 개선

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Improved results of LINE-1 methylation analysis in formalin-fixed paraffin-embedded tissues with the application of a heating step during the DNA extraction process

by
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Abstract

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Formalin-fixed, paraffin-embedded (FFPE) tissues are important resources for profiling DNA methylation changes. However, formalin-fixation introduced inter-strand cross-linking, which might cause incomplete bisulfite conversion of unmethylated cytosines, which might lead to falsely elevated measurements of methylation levels in pyrosequencing assays. To identify whether formalin fixation impact the measured values of methylation in LINE-1 repetitive elements and whether heat-induced denaturation of DNA might reduce the artificial increases in measured values caused by formalin fixation, LINE-1 methylation levels of paired fresh-frozen (FF) and FFPE mouse xenograft tissue samples was measured by pyrosequencing assay. Moreover, to further confirm the effect of additional heating step during the DNA extraction in the measurement of LINE-1 or single gene methylation levels, FFPE gastric cancer and colorectal cancer patient samples were analyzed for the methylation status of LINE-

1 and single genes. Intriguingly, formalin fixation per se led to an increase in the measured values of LINE-1 methylation regardless of the duration of fixation. Application of heating of the DNA at 95° C for 30 min before bisulfite conversion was found 1) to decrease the discrepancy in the measured values between the paired FF and FFPE tissue samples, 2) to decrease the standard deviation of the measured value of LINE-1 methylation levels in FFPE tissue samples of gastric cancer, and 3) to improve the performance in the measurement of single gene methylation levels in FFPE tissue samples of colorectal cancer.

In conclusion, application of heating of DNA samples improves bisulfite conversion-based measurement of LINE-1 or single gene methylation levels in FFPE tissue samples.

Keywords: Archival tissue, CpG island methylator phenotype, DNA methylation, formalin, heat treatment, LINE-1

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FF: Fresh-frozen

FFPE: Formalin-fixed, paraffin-embedded

5mC: 5-methylcytosine

5hmC: 5-hydroxymethylcytosine

5fC: 5-formylcytosine

5caC: 5-carboxylcytosine

DNMT: DNA methyltransferase

LINE-1: Long interspersed nucleotide element-1

PCR: Polymerase chain reaction

PFS: Progression-free survival

OS: Overall survival

GC: Gastric cancer

CRC: Colorectal cancer

CIMP: CpG island methylator phenotype

Introduction

Epigenetics is the study of heritable changes in gene expression or function that do not involve any changes of the DNA sequence [1]. Collectively, essential epigenetic mechanisms include DNA methylation, histone modifications and noncoding RNA regulations [2]. Among these, DNA methylation is the most widely studied epigenetic mechanism due to its broad involvement in normal cellular processes and human malignancies. DNA methylation occurs at the 5’ −position of cytosine (5mC) that precede guanine (CpG) within the dinucleotides of DNA [3]. Establishment and maintenance of DNA methylation are mediated by a family of enzyme named DNA methyltransferases (DNMTs) that transfer a methyl group to the cytosine [4]. Among DNMTs, DNMT3a and DNMT3b act as de novo methyltransferases that can introduce a new methylation pattern to unmethylated DNA, whereas DNMT1 can copy the DNA methylation pattern from the parental DNA strand onto daughter strand to ensure faithful maintenance of 5mC during the replication [5]. Despite its stability, 5mC can return to its unmethylated state through DNA demethylation. One of these mechanisms include active DNA demethylation mediated by

enzymes. The Ten-eleven translocation (TET) family of proteins (TET1, TET2 and TET3) are reported to subsequently oxidize 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) which leads to loss of DNA methylation [6]. The other mechanism is passive DNA demethylation which results from lack of maintenance methylation during DNA replication.

In mammals, DNA methylation is essential for embryonic development and is involved in many genetic events, such as genomic imprinting, X chromosome inactivation, gene repression and transposon silencing [7]. Most often, DNA methylation can result in silencing of corresponding genes, and occurs predominantly in repetitive elements such as transposons in normal somatic cells [8]. However, this genome-wide methylation pattern suffers from a dramatic transformation during carcinogenesis. In normal cells, CpG sites in promoter CpG island loci are protected from methylation and are usually devoid of methylation, whereas CpG sites in genomic sequences other than CpG island loci, particularly on repetitive DNA elements, are usually methylated. In contrast to the situation in normal cells, cancer cells undergo aberrant DNA methylation changes, namely global genomic

hypomethylation and promoter hypermethylation [9]. Indeed, these hypo- and hypermethylation are two of the most common features across human cancers. Promoter CpG island hypermethylation in genes which are actively expressed in normal cells leads to inactivation of the genes, which might contribute to the initiation and progression of tumor cells. Genomic hypomethylation contribute to the progression of tumorigenesis in several ways, including activation of proto-oncogenes, loss of imprinting, activation of transposons and retrotransposons, and induction of chromosomal instability. Activation of transposon and retrotransposon in association with diffuse demethylation of genomic DNA may also provide a selective advantage during tumorigenesis [10].

Retrotransposons are DNA elements that can mobilize throughout the genome via an RNA intermediate. Long interspersed nucleotide element-1 (LINE-1), a type of non-long terminal repeat (non-LTR) retrotransposon, is repeated half a million-times in the human genome and comprises approximately 17% of the human genome [11]. It has a high density of CpG dinucleotides in its 5' untranslated region, and these CpG sites are usually heavily methylated in normal cells. Because of both the extremely high frequency of LINE-1 and the heavy methylation in its 5' CpG

sites, the level of LINE-1 methylation has been thought to be closely associated with genomic DNA methylation levels. In 2005, Weisenberger et al. demonstrated a strong relationship between the levels of LINE-1 methylation and genomic DNA methylation [12]. In that study, the LINE-1 methylation level was assessed using the MethyLight assay, a sodium bisulfite-dependent, probe-based real-time PCR assay [13]. Sodium bisulfite treatment of genomic DNA converts unmethylated cytosine into uracil which becomes thymine in subsequent PCR amplification, whereas methylated cytosine are resistant to bisulfite modification and remains unaffected. Bisulfite modification is widely accepted as a gold standard method for evaluating DNA methylation [14]. This modification creates methylation-dependent distinct CpG sites, and thus these CpG sites can be quantified by real-time PCR with primer pairs and fluorescence-labeled probes. The high sensitivity and specificity of MethyLight make it suitable for assessment of low-frequency methylation events [15]. More recently, a pyrosequencing-based LINE-1 methylation assay has also become widely used. Pyrosequencing methylation assay is also bisulfite conversion-dependent, and a sequence-by-synthesis method that detects incorporation of nucleotides complementary to the base of template strand bioluminometrically [16]. Following an initial PCR

amplification to introduce biotin-tagged single strand DNA, the streptavidin-coated sepharose beads are used to bind to the biotin-tagged single strand DNA, which is the template for the pyrosequencing reaction [17]. Then this template will be incubated with a reaction cascade including enzymes and substrates. Typically, a deoxribonucleotide triphosphate (dNTP) is involved in this reaction one by one, and incorporated by DNA polymerase with pyrophosphate (PPi) when it found complementary to the base of template strand [18]. Ultimately, the released PPi is converted into ATP to generate visible light that is detected and recorded as a peak in the pyrosequencing data. Of note, pyrosequencing assay can identify differentially methylated positions in close proximity [19], and also has been validated for its precision and reliability in formalin-fixed, paraffin-embedded (FFPE) tissue samples. [20].

LINE-1 hypomethylation in tumors has been demonstrated in virtually all tissue types of human cancer except for thyroid cancer and renal cell carcinoma [21-28]. However, prior to the report of Tournier et al. in 2012, there had been no study which compared the results of the PCR-based LINE-1 methylation assay between paired fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) tissue samples [29]. Tournier and colleagues analyzed

paired FF and FFPE tissue samples for their LINE-1 methylation levels using a pyrosequencing assay to identify whether formalin fixation induced deviations in the measured value of methylation levels in individual genes or in LINE-1. Tournier et al. found that a significant discrepancy existed in the measured LINE-1 methylation levels between paired FF and FFPE tissue samples. This discrepancy raised doubts regarding the utility of the pyrosequencing LINE-1 methylation assay in FFPE tissue samples.

In formalin-fixed tissue samples, formaldehyde induces several types of DNA damage on either double strand or single strand, including formaldehyde-induced crosslinks, DNA fragmentation, abasic sites, and deamination of cytosine bases [30]. Of various formaldehyde-induced crosslinks, interstrand DNA crosslinks and protein-DNA crosslinks are thought to affect the efficacy of bisulfite modification which is an essential step for genomic DNA methylation analysis. Because the reaction of bisulfite with cytosine residues is highly single strand-specific and cannot occur on double-stranded DNA [31], interstrand DNA crosslinks are thought to cause some resistance against heat- and alkaline denaturation. Incomplete denaturation of DNA leads to incomplete bisulfite conversion, which might cause the discrepancy in the measured

values of LINE-1 methylation between paired FF and FFPE tissue samples. Because formaldehyde-induced crosslinks are known to be reversible by heat treatment [32, 33], the application of heat treatment during DNA preparation process might increase the performance of bisulfite modification in DNA samples obtained from FFPE tissues [29]. The present study aimed to identify whether formalin fixation is related to the increased values of LINE-1 methylation detected in FFPE tissues and whether the addition of a heating step during the DNA extraction process helps to decrease the discrepancy in the measured values of LINE-1 methylation between paired FF and FFPE tissue samples. Mouse xenograft tissue samples were used for identifying the effects of formalin-fixation and heating in the pyrosequencing-based assay of LINE-1 methylation. Finally, the human FFPE tissue samples were used in confirm the effect of heating in the methylation analysis of LINE-1 or of single genes.

Materials and Methods

Cell culture

The human gastric cancer cell lines, MKN-45 and SNU-638 and colorectal cancer cell lines, SW620, SNU-C5, and LoVo, were obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in a 37℃ incubator with 5% CO_2. Cells were grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 100 U / ml penicillin and 100 μg / ml streptomycin. Culture medium was replaced approximately every 48 h.

In vivo xenograft experiment

A total of ten 6-week-old normal BALB/c-nu mice were used for the tumor xenograft experiment. 2×10^6 cells in phosphate-buffered saline were bilaterally injected subcutaneously into the flanks of the mice to generate two tumor masses per mouse. The mice were maintained in a pathogen-free barrier facility and fed a standard diet. All mice were euthanized 8 to 10 weeks after subcutaneous injection. The xenograft experimental plan and protocol were approved by the Biomedical Research Institute of Seoul National University Hospital (15-0111-C1A0).

DNA extraction from xenograft tissue samples

FFPE tissue Mouse xenograft tissue slides were made according to one of five formalin-fixation conditions: no fixation (fresh-frozen), fixation for 1 day, 3 days, or 5 days, or delayed fixation (room temperature for 2 days prior to fixation for 1 day). Ten micrometer-thick sections were cut from the FFPE tissue blocks or from the FF tissue blocks. Deparaffinization was accomplished by first heating the glass slides mounted with the paraffin section to no more than 60°C. The paraffin was dissolved in xylene. Deparaffined and rehydrated sections of the FFPE tissue blocks or sections of the FF tissue blocks were stained with hematoxylin and eosin. The FFPE or FF tissue slides were examined under a microscope, and the areas with the highest tumor cell density were selectively dissected using knife blade. The scraped tissues were collected in microcentrifuge tubes containing 50 μL of tissue lysis buffer (0.5% Tween 20 [Sigma, St Louis, MO, USA], 100 mM Tris HCl buffer [pH 7.6], 1 mM EDTA, and 20 μg of proteinase K [Sigma]). After incubation at 55℃ for 2 days to ensure complete lysis, the microcentrifuge tubes were centrifuged at 10,000 x g for one minute to remove insoluble debris. The supernatant was transferred to a newly labeled microcentrifuge tube. DNA samples were prepared from the human FFPE tissue blocks using the same

protocol for the xenograft FFPE tissue blocks.

Patient specimens

Retrospectively analysis of the clinicopathologic data of 476 patients who underwent surgery and extended lymph node dissection (D2) for advanced gastric cancer in the Seoul National University Hospital, Seoul, Korea, from January 2007 to December 2008. Patients who had a history of other primary malignancies within 5 years or were treated with neoadjuvant chemotherapy were excluded. The following pathological parameters were evaluated by gross and microscopic examination: tumor location, tumor differentiation, histological type, lymphatic invasion, perineural invasion, venous invasion, and TNM stage (American Joint Committee on Cancer, 7th edition). A total of 497 colorectal cancer patients who received curative surgery and adjuvant chemotherapy in the Seoul National University Hospital between June 2005 and November 2011 were included. Because each FFPE tissue blocks were made soon after the surgery, tissue blocks of gastric cancer and colorectal cancer ranged in age from 6-7 and 4-10 years, respectively, at the time of DNA extraction. Microscopically, tumor areas with high tumor density and representative histology were marked for each case, were manually

dissected, and were collected into microcentrifuge tubes containing tissue lysis buffer and proteinase K. The tissue solution was kept at 55℃ for 2 days.

The study protocol was reviewed and approved by the institutional review board of Seoul National University Hospital (1312-051-542) and was performed in accordance with the recommendations of the *Declaration of Helsinki* (2013) for biomedical research involving human subjects. Patient records/information was anonymized and de-identified prior to analysis.

Bisulfite conversion and Alu-based MethyLight control reaction

20 μL of the supernatant was used for the bisulflte modification which was performed using the EZ DNA methylation kit according to the manufacturer's protocol (Zymo Research, Irvine, CA, USA). In order to measure input DNA (bisulfite-modified DNA), Alu-based MethyLight control reaction which is a CpG-independent, bisulfite specific control reaction was performed [12]. Determined threshold cycle [C(t) value] of this reaction in which the Alu reaction fluorescence was detected. To keep the C(t) value of bisulfite-modified DNA samples in the range from 18 to 20, distilled water was added to dilute bisulfite-modified DNA samples

with C(t) values lower than 18. MethyLight PCR was performed in a 25 μL reaction volume with 200 μM dNTPs, 0.3 μM forward and reverse PCR primers, 0.1 μM probe, 3.5 mM $MgCl_2$, 0.01% Tween-20, 0.05% gelatin and 0.2 units of Taq polymerase on a 96-well plate (BioRad) using the following PCR program: 95℃ for 10 min, then 50 cycles of 95℃ for 15 s followed by 60ºC for 1 min.

Pyrosequencing methylation assay

The converted DNA samples were PCR-amplified with oligonucleotide primers that were designed against a consensus LINE-1 sequence by the Issa group for pyrosequencing [13]; the forward primer was 5'-TTTTGAGTTAGGTGTGGGATATA, and the reverse biotinylated primer was 5'-biotin-AAAATCAAAAAATTCCCTTTC. The PCR reaction was carried out in a 25 μL final volume comprised of 2 μL of bisulfite-treated DNA (input DNA was approximately 33ng), 2.5 μL Coral Load PCR Buffer, 1.5 μL of 25 mM $MgCl_2$, 1 μL of the forward and biotinylated reverse primers (0.4 μM final concentration), and 0.75 U of HotStarTaq Plus DNA polymerase (Qiagen, Valencia, CA, USA). The PCR cycling conditions were as follows: initial denaturing at 95℃ for 10 min, 50 cycles of 94℃ for 30 s, 57℃ for 40 s, and

72℃ for 40 s followed by a final extension at 72℃ for 5 min. The PCR products were added to the binding buffer (Qiagen) and the Streptavidin Sepharose High Performance beads (GE Healthcare Bio-Sciences Corp., Uppsala, Sweden). The biotinylated DNA-bound beads were collected and retained using the PyroMark Vacuum Prep WorkStation (Qiagen). The purified single-stranded PCR product was added to the annealing buffer (Qiagen) with 0.3 μM of sequencing primer (5' -AGTTAGGTGTGGGATATAGT), and the pyrosequencing reaction was performed using the PyroMark Q24 platform (Qiagen). The level of methylation at each of the four analyzed CpG sites (GenBank accession number X58075 sites 1-4: nucleotide positions 328, 321, 318, and 306) was determined by the percentage of methylated cytosines. The pyrosequencing assay was repeated in triplicate, and the median value of the three replicates was reported as the representative value of LINE-1 methylation.

Measurement of single gene methylation level

For evaluation of the DNA methylation status in individual genes, the MethyLight assay was performed as previously described [43]. The converted DNA samples were analyzed for methylation status in 8 individual genes (*CACNA1G*, *CDKN2A* (*p16*), *CRABP1*, *IGF2*,

MLH1, *NEUROG1*, *RUNX3*, and *SOCS1*). A complete list of MethyLight reaction probes and primers has been previously reported [44]. The MethyLight assay was repeated in triplicate, and the median methylation level (determined by the percentage of methylated reference [PMR]) was obtained.

Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 21.0) (International Business Machines Corp., Armonk, NY, USA). Two-sided P-values < 0.05 were considered significant. The clinical database for gastric cancer patients was updated in January 2014. Progression-free survival (PFS) was calculated from the date of resection of advanced gastric cancer to the first date of documented recurrence or the date of death from any cause. Overall survival (OS) was measured from the date of resection to the date of death or the date of the last clinical follow-up before January 2014. Kaplan-Meier survival analysis was performed to compare OS and PFS using the log-rank test. Because the data on the level of LINE-1 methylation in CpG sites 1 to 4 did not follow the normal distribution, mean values across two or more groups were compared using both parametric and non-parametric tests. The Mann-Whitney U test and ANOVA test were used for the

comparison of mean values across two groups, while the Kruskal–Wallis test and Student's t–test were used for the comparison across three or more groups. Pearson's correlation test was used to assess the correlation between the LINE–1 methylation levels in paired FF and FFPE xenograft tissue samples. The Wilcoxon signed–rank test and paired Student's t–test were used to analyze the paired differences.

Results

Each xenograft cancer tissues (n=10) were cut into five slices, which were treated with five different durations of formalin fixation (no fixation, 1 day−fixation, 3 day−fixation, 5 day−fixation, or a 2 day−delay prior to 1 day−fixation). Ten μm sections cut from the FFPE tissue blocks or from the FF sections were stained with hematoxylin and eosin, and examined under a microscope. Tumor areas with the highest tumor cell density were scraped into microtubes containing lysis buffer solution. The lysed tissue solution was divided into two halves, one of which was treated with heating (95°C for 30 min) and the second with no heating step. The overall experimental design was indicated in Fig. 1.

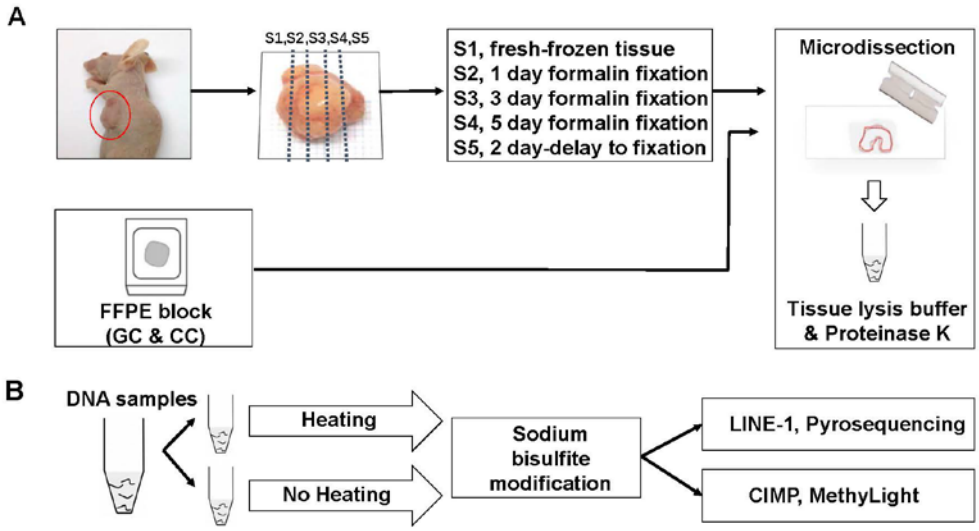

Fig. 1. The overall experimental design

(A) DNA preparation of mouse xenograft tissue samples (n=10) and human archival tissue samples (gastric cancer (GC), n=476, colorectal cancer (CRC), n=497). Each xenograft tumor tissue was cut into five slices. Each slice was treated using five different formalin fixation protocol. (B) Both heat-treated and -untreated DNA samples were subjected to bisulfite conversion and subsequent pyrosequencing methylation assay or MethyLight assay.

Effect of formalin fixation in the assessment of LINE-1 methylation

When the LINE-1 methylation level was compared between paired fresh and formalin-fixed tissues from xenograft tissue slices, FFPE tissue samples showed increased levels of LINE-1 methylation compared with paired FF tissue samples. Regardless of the duration of formalin fixation, FFPE tissue samples exhibited significantly higher values of methylation than those of FF tissue samples (56.6% vs. 53.7%) (Fig.2). No difference was noted in the values of LINE-1 methylation level among FFPE tissue samples across the various fixation parameters.

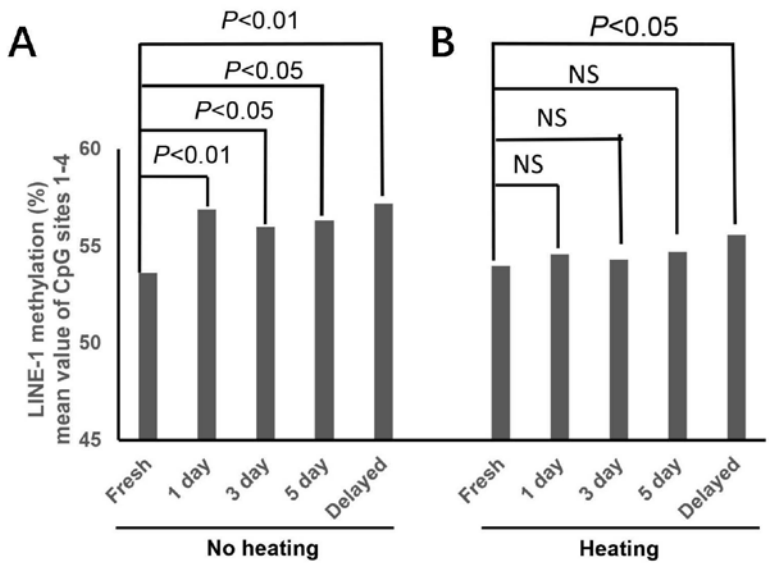

Fig. 2. LINE-1 methylation level in mouse xenograft tissue samples with or without heating

Mean methylation levels of the four LINE-1 CpG sites for xenograft tissue samples (n=10) with five different durations of formalin fixation. DNA samples were heat-untreated (A) and heat-treated (B). Both the paired Student's t-test and the paired Wilcoxon signed rank test were performed to compare the mean methylation values of the four CpG sites between paired fresh frozen and formalin-fixed, paraffin-embedded tissue samples. P-values in the bar graph represent the values of both the parametric and the non-parametric tests.

Heating decreases the formalin-associated elevation of LINE-1 methylation levels

The discrepancy of LINE-1 methylation values between paired FF and FFPE tissue samples was found to decrease with the application of a heating step (95℃, 30min) during the FFPE DNA extraction process. With the addition of a heating step during DNA preparation, the differences in the measured levels of LINE-1 methylation between paired FF and FFPE tissue samples became insignificant (Fig. 2). However, FFPE tissue samples with delayed fixation showed significantly increased measured values of LINE-1 methylation level despite the application of a heating step. The correlation of the measured values became stronger between paired FF and FFPE tissue samples with the application of a heating step. Although another condition of heating was also tested, heating at 95°C for 1hr, no difference was found in LINE-1 methylation levels between samples treated with heating at 95℃ for 30 min and those with heating at 95℃ for 1hr (Fig. 3).

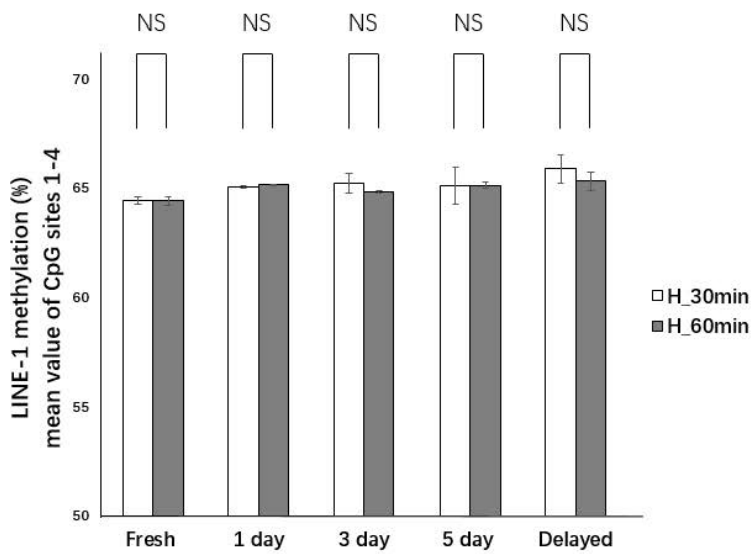

Fig. 3. LINE-1 methylation level in mouse xenograft tissue samples with heating at 95℃ for 30min and 60min

Methylation levels of the four LINE-1 CpG sites for 4 xenograft DNA samples (MKN-45, SNU-638, SW620, and LoVo xenograft tumors) with heating at 95℃ for 30min or 60min. Both the paired Student's t-test and the paired Wilcoxon signed rank test were performed to compare the mean methylation values of the four CpG sites between paired fresh frozen and formalin-fixed, paraffin-embedded tissue samples. P-values in the bar graph represent the values of both the parametric and the non-parametric tests.

Effect of heating on LINE-1 methylation analysis of archival tissue samples and survival analysis

Methylation assays for 4 LINE-1 CpG sites were performed on both heat-treated and on untreated DNAs from 476 cases of advanced gastric cancer. When the methylation levels of four individual LINE-1 CpG sites were compared between advanced gastric carcinoma DNA samples with and without heat, all but CpG site 2 showed decreased values of methylation with concomitant decrease in the standard deviation of the measured value in all four CpG sites (Fig. 4). When advanced gastric cancer cases were split into 4 groups according to their tumor LINE-1 methylation levels, different survival curves were observed for each group after the application of a heating step during DNA extraction compared to unheated extractions. In the survival analysis of heat-treated DNA samples, lower LINE-1 methylation level correlated with decreased PFS and OS (Fig. 5).

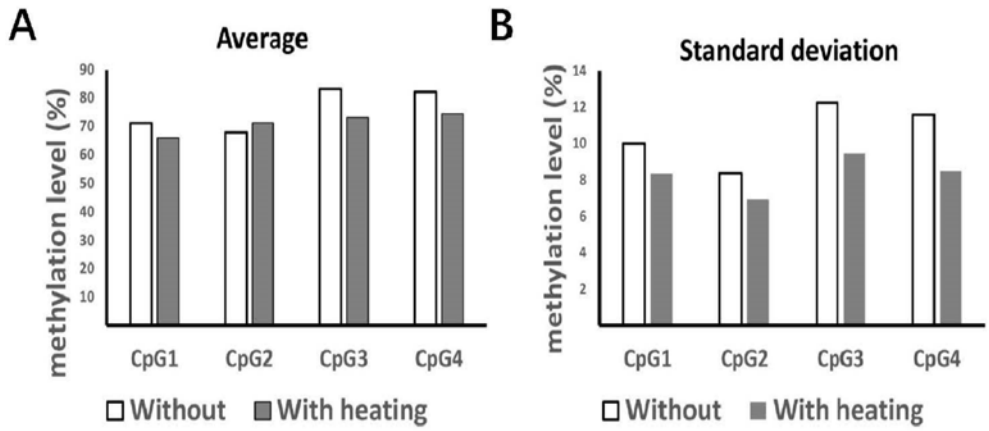

Fig. 4. LINE-1 methylation level in gastric cancer tissue samples

Comparison of the methylation levels (A) and the standard deviations (B) in the four LINE-1 CpG sites of gastric cancer tissue DNA samples (n=476) with and without heat treatment.

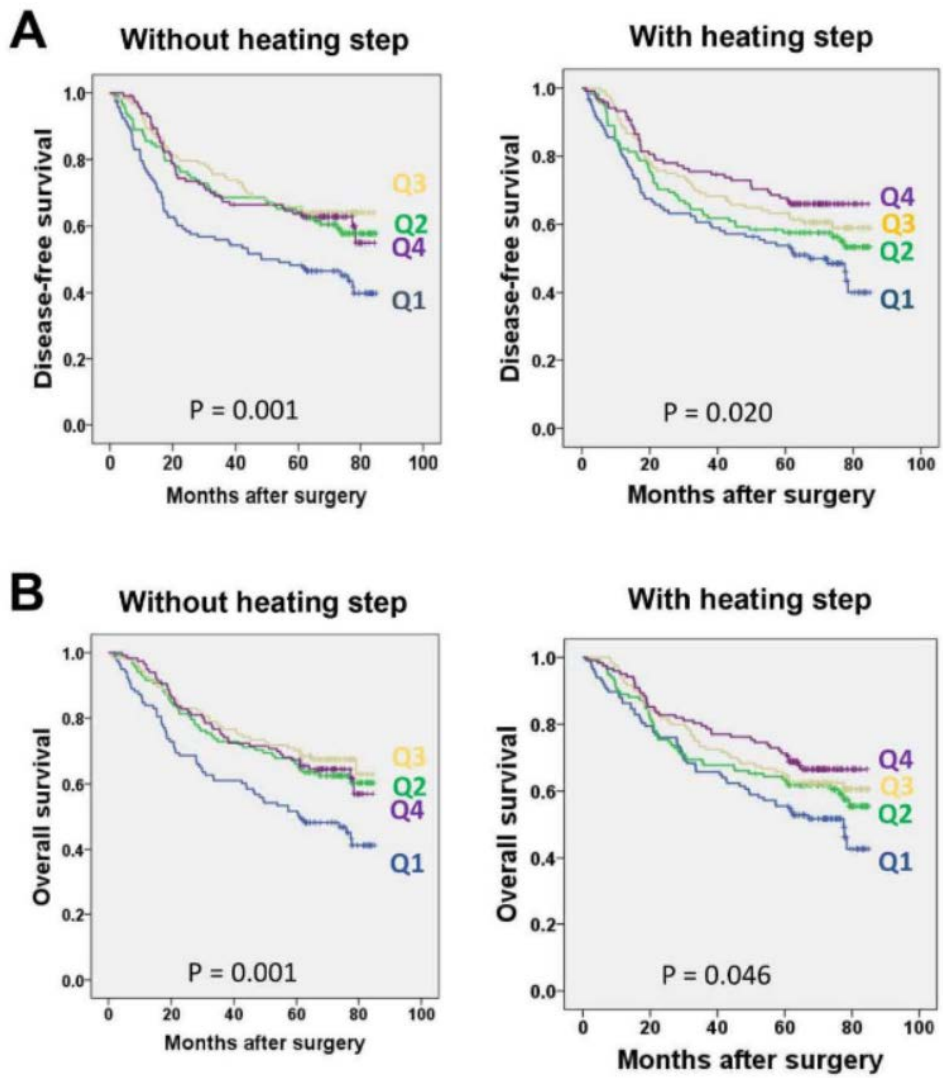

Fig. 5. Kaplan-Meier Survival curves in gastric cancer patient set

Gastric cancer patients (n=476) were divided into four groups according to their tumoral LINE-1 methylation levels and its association with dissected-free survival (A) and overall survival (B) was observed. Q1, Q2, Q3 and Q4 are in the order of increasing LINE-1 methylation levels.

Effect of heating on the methylation analysis of single genes in archival tissue samples

To identify whether the application of heat during DNA extraction might affect the results of single gene methylation analysis, the methylation levels and frequencies of eight CpG island methylator phenotype (CIMP) panel markers between DNA samples with and without heat treatment. The samples from 497 colorectal cancer cases were split into two, and CIMP analysis was performed on paired heat-treated and untreated DNA samples using the MethyLight assay. Of the eight markers, all but MLH1 showed increased methylation frequencies and levels in the DNA samples subjected to heat treatment during extraction compared with DNA samples without heat treatment (Fig. 6). The application of heat during DNA extraction thus resulted in enhanced detection of CIMP-high CRC (Fig. 7).

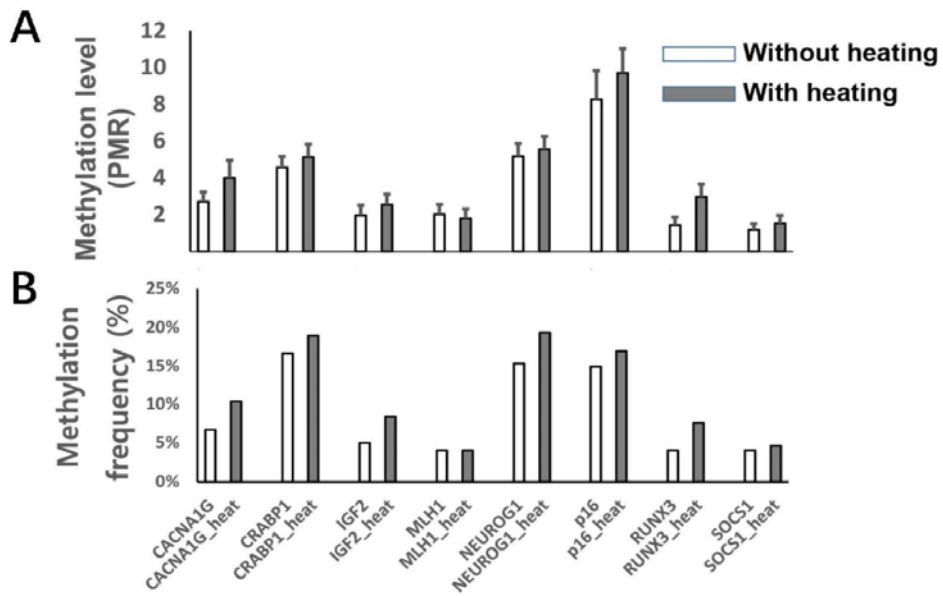

Fig. 6. Methylation level and frequency of single genes in colorectal cancer tissue samples

Comparison of the methylation levels (A) and frequencies (B) in the eight individual CpG island methylator phenotype panel markers in colorectal cancer tissue (n=497) DNA samples with and without heat treatment.

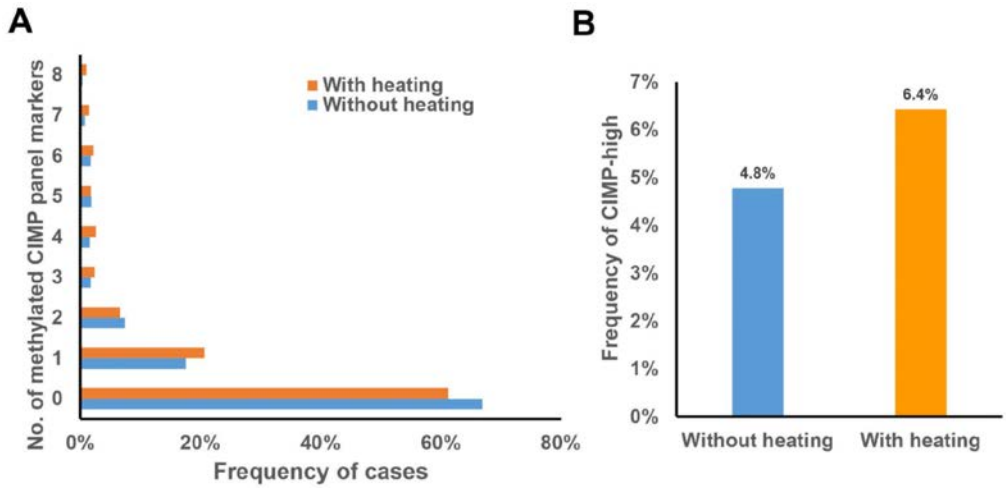

Fig. 7. Application of additional heating step in methylation analysis of single genes

(A) Application of a heating step during DNA extraction increased the mean number of methylated CIMP panel markers from 0.8 to 0.9. Cases with no methylation of CIMP panel markers decreased from 66.9% to 61.2%. (B) Application of a heating step allowed identification of 1.8% more CIMP-high colorectal cancer cases.

Discussion

FFPE tissue is an invaluable resource for oncologic epigenetic studies because large collections of FFPE tissue samples with clinical annotations are managed in hospitals. Histologic examination and delineation of areas with the highest tumor cell content is important for the quantitative methylation analysis of cancers to prevent dilution of the neoplastic cell signal by contributions from non-neoplastic cells in the cancer tissue samples. FFPE tissue samples are more suitable for manual microdissection than FF tissue samples. Minimal amounts of tissue, even a 10-micrometer thick section from an endoscopic biopsy specimen, are required for the PCR-based quantitative methylation analysis of single genes or repetitive DNA elements. Tissue samples dissected from the mounted tissue slices are subjected to incubation in lysis buffer containing proteinase K at 55°C for 2 days. Then, the lysed tissue solutions are subjected to bisulfite conversion using commercially available kits, including the EpiTect or EZ DNA methylation kit, which generate bisulfite-modified DNA samples. In this study, the addition of a heating step, 95°C for 30min, between the 2-day incubation step and the bisulfite conversion procedure was found to

decrease the discrepancy between the measured values of LINE-1 methylation of paired fresh and fixed tissue samples.

In the present study, formalin fixation was found to increase the measured value of LINE-1 methylation levels compared to the paired FF xenograft tissue samples. But, the measured value of LINE-1 methylation levels was not different among FFPE xenograft tissue samples of varying fixation lengths from 1 day to 5 days. These findings suggest that formalin fixation per se, rather than duration of fixation, plays an important role in the causation of deviation in the measured value of LINE-1 methylation level. However, because of the effect of fixation duration > 7 days or < 1 day on the performance of pyrosequencing-based LINE-1 methylation assay was not examined, it is not enough to demonstrated that the duration of fixation does not affect the pyrosequencing assay of LINE-1 methylation. However, of note, there has been no study which investigated how duration of formalin fixation affects bisulfite conversion of genomic DNA and its performance in downstream methylation analysis. Formaldehyde reacts with amino groups in nucleobases, leading to the formation of methylene bridges between complementary strands of DNA. Because the generation of methylene crosslinks is a time-

dependent process [34], longer fixation time is expected to form more inter-strand crosslinks. However, no difference was found in the measured value of LINE-1 methylation among fixation time from 1 day to 5 days.

As a surrogate marker for genomic DNA methylation content, methylation of LINE-1 has been measured by various assays, including the combined bisulfite restriction assay [35], the MethyLight assay [12], pyrosequencing, and absolute quantitative analysis of methylated alleles [36], which target CpG sites located in the 5' untranslated region of LINE-1. However, 85% of LINE-1 elements are truncated in their 5' sequences [37], and thus PCR-based assays only assess 15% of the LINE-1 elements in the human genome. Weisenberger et al. demonstrated that the MethyLight assay-based measurement of LINE-1 methylation level correlates with genomic DNA methylation content as measured by high performance liquid chromatography (HPLC). However, before the study of Lisanti et al. [38], there had been no study that directly analyzed the correlation between the pyrosequencing-based measurement of LINE-1 methylation and genomic DNA methylation content. Lisanti et al. showed a strong correlation between the methylation levels in LINE-1 measured via

a pyrosequencing assay versus a high performance liquid chromatography.

Studies have shown that hypomethylation of LINE-1 in tumors is closely associated with poor patient prognosis for many types of human cancers, including gastric carcinoma [23, 39], colorectal carcinoma [26], esophageal squamous cell carcinoma [40], and lung adenocarcinomas [41, 42]. In particular, the association between LINE-1 hypomethylation in gastric carcinoma and poor prognosis has been reported by three studies [23, 28, 39]. However, all three of these studies, including Shigaki et al. [23], used FFPE tissue samples. In the Shigaki's study, DNA was extracted from FFPE tissue samples using the QIAamp DNA FFPE tissue kit, which includes a heating step for 60 min at 90°C in its protocol. However, the previous studies did not use commercial DNA purification kits and the extraction protocol did not include any heating step. In the present study, the effect of heat during DNA extraction from FFPE tissue samples was analyzed by comparing the survival curves of four groups for both heat-treated DNAs and untreated DNA. With heat-treated DNA samples, PFS and OS decreased as tumoral LINE-1 methylation decreased. This trend was not observed with unheated DNA samples. Regardless of whether the heating step was

included during DNA extraction, tumoral LINE-1 hypomethylation was found to be an independent parameter for identifying gastric cancer patients with a poor prognosis.

In the examination of the methylation frequencies and levels of eight CIMP panel markers between DNA samples with and without heat treatment, all of the genes except for MLH1 showed increased methylation frequencies and levels in the heat-treated DNA samples relative to the unheated DNA samples. Consequently, heat treatment enabled identification of more cases of CIMP-high CRCs. At present, it is unclear why assessment of methylation at MLH1 CpG island locus was not affected by heat treatment. Interestingly, the increased methylation level of individual genes in association with heat treatment is in contrast to the decreased methylation levels of LINE-1 under the same conditions. The question arises why heat treatment led to a decrease in methylation levels in repetitive DNA elements but an increase in individual genes. The reason of this apparent discrepancy is unclear, but it might be attributable to the difference in the methylation assays employed: LINE-1 methylation was analyzed using a pyrosequencing assay, which measures the mean methylation level of all the DNA alleles at individual CpG sites [12], whereas the methylation levels of

individual genes were assessed by the MethyLight assay, which evaluates the relative amount of specific DNA alleles with concurrent methylation of serial CpG sites [13]. When the region of interest is incompletely converted and thus contains non-converted CpG and non-CpG cytosines, MethyLight probe cannot anneal to the incompletely converted region of interest and does not generate fluorescent signal. Thus, on the condition that all CpG sites located in the region of interest are methylated, complete conversion leads to an increase in the measured value of methylation levels compared to an incomplete conversion. In contrast with the MethyLight assay, enzymatic cascade of the pyrosequencing reaction cannot discriminate incompletely converted template sequence (region of interest) and generates luciferase light signals not only from methylated CpG cytosines but also nonconverted unmethylated CpG cytosines, which causes an increase in the measured value of methylation levels compared to complete conversion.

Conclusions

The present study demonstrated that formalin fixation can result in elevated values of the LINE-1 methylation level, irrespective of the duration of fixation, but the heating of the DNA solution prior to bisulfite conversion helps decrease the discrepancy between paired FF and FFPE tissue samples. However, heating did not offset the discrepancy in FFPE tissue samples prepared using delayed fixation. Moreover, the study results indicate that application of heating of DNA samples obtained from FFPE tissues is necessary for proper evaluation of DNA methylation levels, regardless whether single genes or repetitive DNA elements are assayed.

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국문 초록

포르말린 고정 및 파라핀 포매 (FFPE) 된 조직은 DNA 메틸화연구에 쓰이는 매우 중요한 자원이다. 그러나 포르말린 고정이 초래하는 DNA inter-strand 교차결합 (crosslinking)은 메틸화 되지 않은 싸이토신에서 불완전한 산성아황산염 전환 (bisulfite conversion)을 야기 시킴으로써 파이로시퀀싱을 이용한 메틸화분석에서 메틸화수준의 과도한 향상을 일으킬 수 있다. 포르말린 고정이 LINE-1의 메틸화수준 측정에 영향을 미치는지, 추가적인 가열 처리를 이용한 DNA 변성과정이 포르말린 고정에 의한 메틸화수준의 거짓된 향상을 감소시키는 지 여부를 확인하기 위해, 본 연구에서는 마우스의 종양이식조직을 이용하여 신선동결조직 (FF)과 포르말린고정조직에서의 LINE-1의 메틸화레벨을 파이로시퀀싱으로 측정하여 비교하였다. 또한, 가열 과정이 LINE-1 및 개별 유전자의 메틸화측정에 미치는 효과를 검증하기 위해 위암 (n=476)과 대장암 (n=497) 환자의 FFPE 조직샘플에서 LINE-1과 8개 개별 유전자의 메틸화정도를 각각 분석하였다.

결과, 포르말린의 고정은 그 고정 시간에 상관없이 LINE-1 메틸화정도를 보다 향상시킴을 발견하였다. 그밖에, 산성아황산염 전환과정 전에 추가되는 DNA 샘플에 대한 가열 처리 (95° C, 30분)는 1) FF와 FFPE조직 샘플 간에 생긴 메틸화수준의 차이를 해소시키는 데 도움이

되고, 2) FFPE 조직 샘플에서 메틸화 측정값의 표준편차를 감소시키는 데 효과적이며, 3) 개별 유전자에 대한 메틸화정도 측정에도 개선효과가 있음을 발견하였다.

결론적으로, DNA 샘플에 대한 추가적인 가열처리 과정은 FFPE조직 샘플을 이용한 산성아황산염을 기반으로 하는 LINE-1 및 개별 유전자의 메틸화측정에서 보다 나은 결과 도출에 도움이 된다.

주요어: 저장 조직, CpG 메틸화 표현형, DNA 메틸화, 포르말린, 가열처리, LINE-1

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