BioFeedback

[Letter to the editor] Whole-genome amplification of sodium bisulfite-converted DNA can substantially impact quantitative methylation analysis using pyrosequencing

Epigenetic gene silencing due to DNA hypermethylation plays an important role in the regulation of many cancer-related genes (1,2). The investigation of DNA methylation patterns contributed to the understanding of various biological mechanisms in human cancer. As compared with previous methods (3–6), the pyrosequencing technique represents a highly sensitive method for quantification of DNA methylation (7,8). All these approaches are based on sodium bisulfite conversion of genomic DNA (gDNA). However, analysis of DNA methylation in clinical specimens is restricted by limited availability of starting gDNA, bisulfite-dependent DNA degradation, and unstable cytosine-to-uracil transformation (9,10). Therefore, whole-genome amplification (WGA) might represent a suitable method to overcome these limitations. One WGA method is the non-PCR-based isothermal multiple displacement amplification (MDA). In our study, we tested one of the commercially available kits for MDA-based WGA. The starting material was obtained from hematopoietic cells of 100 subjects, comprising patients with various hematological diseases as well as healthy volunteers. In order to assess the applicability to sodium bisulfite-converted

DNA, the DNA methylation of three genes (*CSX*, *p15*, and *CALR*) was quantified at three different stages: after sodium bisulfite conversion (BC), after subsequent WGA (AMP), and after nucleic acid purification (SC) of the whole genome-amplified DNA. The evaluation comprised assessment of overall quality from pyrosequencing readings, comparison of the CpG methylation values before and after WGA, and data reproducibility (for details, see the Supplementary Material).

First, we observed a considerable reduction of the overall quality of the *CSX* methylation analysis results for the WGA-treated samples AMP and SC as compared with the initial BC samples (Figure 1A). The percentages of *passed* CpG sites matching the pyrosequencing quality parameters for reliable methylation results were as follows: 79% of the BC CpGs, 19% of the AMP CpGs, and 36% of the SC CpGs. Sixteen percent of the BC CpGs, 45% of the AMP CpGs, and 29% of the SC CpGs matched the quality criteria for *check* CpGs. Finally, 36% of the analyzed CpG sites after WGA and 34% after subsequent purification were detected as *failed*, while this failure occurred only in 6% of the CpGs in non-WGA-treated samples (for detailed

information on pyrosequencing quality criteria, see the Supplementary Material). Furthermore, electrophoretic separation of PCR products revealed a specific band in BC-amplified samples and additional unspecific PCR fragments in reactions carried out with AMP or SC DNA input (Figure 1B), resulting in a high increase of signal quality warnings and divergent peak patterns in the pyrograms of WGA samples (Figure 1C).

The evaluation of absolute methylation values showed substantially different results after WGA. The differences were random and did not follow a specific pattern. We detected average differences in single CpG methylation of $11\% \pm 11\%$ (mean $\pm$ SD) in AMP and $10\% \pm 11\%$ in SC samples for *CSX*, $6\% \pm 9\%$ in AMP and $6\% \pm 8\%$ in SC samples for *p15*, and $3\% \pm 0\%$ in AMP and $3\% \pm 5\%$ in SC samples for *CALR* compared with BC specimens. For *CSX*, 42% of the total CpGs after WGA and 45% after subsequent purification showed a methylation difference of $>10\%$ relative to the sodium bisulfite-converted samples (Figure 2A).

Reproducibility of the pyrosequencing results was determined using a set of 25 bisulfite-converted DNA samples and

Figure 1. WGA leads to decreased signal quality of the pyrosequencing reactions. (A) The results of the *CSX* methylation analysis were utilized. The distributions of the different quality assessments of BC, AMP, and SC DNA are given in colored graph bars. Blue: *passed* if there were no *failed* or *check* quality assessments. Yellow: *check* if there were no *failed*. The trouble report comprised uncertainty due to low signal-to-noise ratio (overall) and high CpG sum deviation, possible dispensation error at a specific dispensation position, and uncertain reference sequence pattern (overall) Red: *failed* due to low signal-to-noise ratio (overall), not analyzed due to lack of data (overall) or failed bisulfite conversion at specific dispensation position. (B) Representative PCR products (samples 1–3) obtained from BC, AMP, and SC DNA samples. (C) Representative pyrograms of BC, AMP, and SC DNA samples illustrating the divergent peak pattern of the pyrosequencing-based methylation analysis results.

Figure 2. Decreased concordance and reproducibility of CpG methylation analysis results after WGA. (A) The graph bars display the deviations of the CSX CpG methylation results generated by whole genome–amplified DNA (black bars) and purified DNA (gray bars) as compared with bisulfite-converted DNA without further modifications (BC versus AMP, BC versus SC). The deviations were graded into 5 ranks and were as follows: (0–10) 58% and 55%, (11–20) 19% and 21%, (21–30) 11% and 13%, (31–40) 5% and 6%, and (≥41) 7% and 5%. (B) The examination of the reproducibility of each CpG methylation data of the CSX pyrosequencing was generated by bisulfite-converted DNA (BC, black bars), bisulfite-converted, whole genome–amplified DNA (AMP, gray bars) and SureClean purified DNA after WGA (SC, white bars) in independent, duplicate pyrosequencing experiments. The proportions of differences of the duplicate measurements for the bisulfite-converted samples, the whole genome–amplified samples, as well as the amplified and purified samples were as follows (BC, AMP, SC): (0–5) 87%, 59%, and 55%, (6–15) 12%, 27%, and 24%, (16–25) 1%, 6%, and 11%, (26–35) 0%, 6%, and 2% as well as (≥36) 0%, 2%, and 8%. (C–F) The assessment of the CSX and *p15* methylation showed that the CpG methylation values of the AMP samples after WGA and after subsequent nucleic-acid purification (SC) strongly deviated from their corresponding BC samples depending on the mean sample methylation value of the BC samples without WGA (graded into four ranks). Error bars indicate the standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

their corresponding WGA-treated samples in duplicate CSX pyrosequencing experiments. Methylation deviation of 5% between single experiments was considered a threshold for reproducibility. While 87% of CpGs from bisulfite-converted samples were highly reproducible, the methylation values of 41% of the CpGs after WGA and 45% after subsequent purification deviated >5% (Figure 2B), indicating a considerable decrease of reproducibility after WGA.

Additionally, our study showed that WGA caused a strong deviation of the methylation results of DNA targets, with initial methylation levels of 20–80% as detected for *p15* and *CSX*, representing a broad methylation range. WGA treatment of these BC samples resulted in significantly increased CpG methylation differences of $16\% \pm 15\%$ in AMP and $16\% \pm 14\%$ in SC samples for *CSX*, as well as $17\% \pm 13\%$ in AMP and $16\% \pm 10\%$ in SC samples for *p15* (Figure 2, C–F). In contrast, WGA-treated samples of hypomethylated DNA (<20%) exhibited significantly fewer deviations as observed in all three analyzed genes,

specifically in the strongly hypomethylated *CALR* gene. A previous report compared DNA methylation profiles obtained from bisulfite-converted DNA treated with WGA to those without WGA treatment using the ABI Prism SNaPshot approach (10). The overall methylation difference was determined as $4\% \pm 5\%$. Of note, the vast majority of the analyzed samples in this study showed strong hypo- (<20%) or hypermethylation (>80%) in the non-WGA fraction. However, an exclusive analysis of the few samples harboring average methylation values of 20–80% revealed a mean CpG methylation difference of $13\% \pm 5\%$ between WGA and non-WGA specimens, indicating a trend toward higher methylation deviation in these samples. A further study reported the quantitative detection of DNA methylation by the qMAMBA technique after genome-wide amplification using a kit optimized for bisulfite-modified DNA (11). The authors observed a general agreement of methylation values in four genes before and after WGA. However, two of three genes exhibiting initial

average methylation of 40–60% demonstrated distinct variations with differences of ≤25%.

As noted above, additional unspecific PCR fragments in reactions carried out with AMP or SC DNA input might cause a high increase of signal quality warnings and divergent peak patterns in the corresponding pyrograms. Gel extraction and pyrosequencing of the specific PCR band resulted in improved methylation results of the WGA-treated samples (see Supplementary Figure S1 and Supplementary Table S1). Therefore, inaccuracy of methylation results after the WGA step may be caused by unspecific sequencing primer binding due to additional PCR products presenting off target binding sites. Therefore, an optimization of the PCR reaction or gel extraction might contribute to an improvement of this method and must be evaluated in detail before application of WGA for pyrosequencing-based DNA methylation quantification.

In summary, pyrosequencing of three independent gene loci demonstrated a

critical impact of WGA on quantitative DNA methylation when using a WGA kit not specifically optimized for bisulfite-converted DNA. This modification potentially leads to PCR interfering effects resulting in a considerable loss of pyrosequencing quality, impaired DNA methylation results and a decreased reproducibility. However, the suitability of this method depends on the experimental context. It still might be applicable for semiquantitative discrimination between very high or low DNA methylation, but not for exact quantification of small differences (which are frequently observed, for example, in clinical specimens to receive important diagnostic and prognostic informations). Therefore, we point out that the application of whole-genome amplification as a standard technique for subsequent quantitative DNA methylation analysis should be carefully evaluated with respect to the experimental studies planned.

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Competing interest statement

The authors declare no competing interests.

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