DNA methylation marker to estimate ovarian cancer cell fraction

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Abstract

Evaluation of cancer cell fraction is important for accurate molecular analysis, and pathological analysis is the gold standard for evaluation. Despite the potential convenience, no established molecular marker for evaluation is available. In this study, we aimed to identify ovarian cancer cell fraction markers using DNA methylation highly specific to ovarian cancer cells. Using genome-wide DNA methylation data, we screened candidate marker genes methylated in 30 ovarian cancer FFPE samples and 14 high-grade serous ovarian cancer cell lines and unmethylated in two female leucocytes and two normal fallopian epithelial cell samples. Methylation levels of three genes, *PALLD*, *SIM1*, and *ZNF154*, showed high correlations with pathological cancer cell fractions among the 30 ovarian cancer FFPE samples (R = 0.56 for *PALLD*, 0.61 for *SIM1*, 0.71 for *ZNF154*). For cost-effective analysis of FFPE samples, pyrosequencing primers were designed, and successfully established for *SIM1* and *ZNF154*. Correlation between pathological cancer cell fraction and methylation levels obtained by pyrosequencing was confirmed to be high (R = 0.52 for *SIM1*, 0.64 for *ZNF154*). Finally, an independent validation cohort of 20 ovarian cancer FFPE samples was analyzed. *ZNF154* methylation showed a high correlation with the pathological cancer cell fraction (R = 0.81, P < 0.0001), and *SIM1* methylation retained its correlation (R = 0.50, P = 0.03). Therefore, the *ZNF154* methylation level was considered to be useful for the estimation of ovarian cancer cell fraction, and is expected to help accurate molecular analysis. (237 words < 250 words)

Introduction

Evaluation of cancer cell fraction is important for accurate molecular analysis of various cancer samples [1]. When a sample has a low cancer cell fraction, it is not suitable for detection of cancer cell-specific mutations and expression changes. Additionally, cancer cell fraction helps to distinguish a germline mutation from a somatic mutation. Currently, the standard method to estimate cancer cell fraction is pathological evaluation. At the same time, mutant allele frequency of driver oncogenes in a next-generation sequencing analysis that has recently been used [2], especially in DNA samples without pathological cancer cell fraction data. Regarding ovarian cancer, in which distinction between a *BRCA1/2* germline mutation from a somatic mutation is important, few driver oncogene mutations are present [3], while mutations of tumor-suppressor genes, such as *TP53* and *BRCA1/2*, are frequent [3, 4]. Since tumor-suppressor gene mutations are frequently accompanied by loss of heterozygosity, and their mutant allele frequencies are not suitable for the estimation of cancer cell fraction.

Instead of mutant allele frequency of oncogenes, the DNA methylation level of a specific gene represents the fraction of cells with its methylation, and can also be used as a cancer cell fraction marker. Such markers have been isolated for esophageal, gastric, breast, and pancreatic cancers [5, 6, 2, 7]. DNA methylation patterns are specific to cell lineage [8], and can also be used to predict the tissue of origin of cancers with an unknown primary cause [9]. Therefore, also for ovarian cancers, we might be able to isolate genes specifically methylated in ovarian cancer cells and unmethylated in background cells, such as normal fallopian epithelial cells and leucocytes. If it were possible to isolate such a marker for ovarian

cancers, it would make it easier to analyze methylation levels by using FFPE samples and reduce the workload of pathologists.

In this study, we aimed to isolate DNA methylation markers of ovarian cancer cell fraction by performing comprehensive DNA methylation analysis, and to validate the markers in an independent cohort.

Materials And Methods

Clinical samples

High-grade serous ovarian cancer samples were obtained from the National Cancer Center Hospital and the Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital. This study was approved by the Institutional Review Board of the National Cancer Center (approval no. 2018-024), Juntendo University (approval no. 2020151), and the Komagome Hospital (approval no. 2547). Written informed consents were obtained from all the patients. A certificated and experienced pathologist (H.Y.) performed microscopic examination of the specimens to evaluate the cancer cell fractions. FFPE samples of surgical specimens after neoadjuvant chemotherapy were used. For macroscopic dissection, samples more than 5 mm on the major axis were used. DNA was extracted and bisulfite-treated from 5-10 slices of 10 μm sections after macroscopic dissection using Deparaffinization Solution (QUAGEN) and an Epitect Bisulfite Kit (QUAGEN). One peripheral leucocyte sample was obtained from a female healthy volunteer.

Ovarian cancer cell lines and fallopian tube epithelial cells

Methylation data of 14 human high-grade serous ovarian cancer cell lines (CAOV3, CAOV4, FU.OV, HEY, JHOS2, JHOS4, OAW28, OV90, OVCAR3, OVCAR4, OVCAR5, OVCAR8, OVKATE, and OVMIU), one female leucocyte, and two human fallopian tube epithelium cells using HumanMethylation450 were obtained from the GEO database (GSE179759, GSE68379, GSE146552) [10,11].

Genome-wide DNA methylation analysis

Genome-wide DNA methylation analysis of 30 ovarian cancers and one peripheral leucocyte sample was conducted using an Infinium HumanMethylationEPIC BeadChip array (Illumina, San Diego, CA, USA), as previously reported [12]. The DNA methylation level of an individual probe was obtained as a β value that ranged from 0 (unmethylated) to 1 (fully methylated). In this study, we used 452,453 probes common between HumanMethylationEPIC BeadChip and HumanMethylation450 located on autosomes and X chromosomes.

Measurement of DNA methylation levels of specific genomic regions

Gene-specific DNA methylation levels were measured by bisulfite pyrosequencing. Specifically, bisulfite modification was conducted using 100-1,000 ng of genomic DNA, as previously reported [12]. Using one of 10-20 μl modified DNA, a target genomic region was amplified using biotinylated primers (Supplementary Table. S1). The PCR product labelled with biotin was annealed to a 0.2 μM pyrosequencing primer, and pyrosequencing was carried out using a PyroMark Q24 Advanced machine (QIAGEN, Valencia, CA, USA). Methylation levels were obtained using PSQ Assay Design software (QIAGEN). Partially methylated DNA was prepared by mixing methylated and unmethylated control DNA (QIAGEN).

Statistical analysis

The correlation between pathological cancer cell fractions and methylation levels was analyzed using Pearson's product-moment correlation coefficients. Statistical analysis was performed using JMP software version 11 (SAS Institute Inc, USA).

Results

Identification of genomic regions specifically methylated in ovarian cancers

To identify genomic regions specifically methylated in ovarian cancers, from the 408,263 probes evaluable with adequate signals, we first selected 125,630 unmethylated probes (β value < 0.2) in two normal fallopian tube epithelial samples and two female leukocyte samples (Fig. 1). Then, from these 125,630 probes, we selected 15 probes in eight genomic regions methylated in 11 or more of 14 high-grade serous ovarian cancer cell lines (β > 0.8) and 21 or more of 30 ovarian cancer clinical samples (β > 0.2). From the eight genomic regions, we selected five genomic regions from four genes that had multiple (≥ 2) flanking probes which met the above criteria.

In addition, we evaluated copy number alterations of the four genes in ovarian cancers from a previous report [3] because such alterations could affect methylation levels and estimation of cancer cell fractions. Copy number alterations of the four genes were confirmed to be infrequent (< 2%). As a result, the four genes, *PALLD*, *SIM1*, *OTX2*, and *ZNF154*, were considered to be good candidate markers for ovarian cancer cell fractions (Table 1). Target CpG sites were located at a CpG island (CGI) or an N shore of a CGI (Fig. 2).

Methylation levels of the candidate marker genes in clinical samples

We next analyzed methylation levels of the four candidate marker genes in 30 clinical samples with information on their pathological cancer cell fractions, to evaluate whether these genes could reflect ovarian cancer cell fractions (Supplementary Fig. S1). The methylation level of *OTX2* tended to be lower than those of the other three candidate marker genes, suggesting that *OTX2* was not methylated in all of the ovarian cancer cells, and we excluded this gene from further analysis. For the remaining three genes, a correlation between the pathological cancer cell fraction and methylation level of a candidate marker gene was analyzed (Fig. 3). Correlation coefficients of *PALLD*, *SIM1*, and *ZNF154* were 0.56, 0.61, and 0.71, respectively.

Implementation of pyrosequencing and confirmation of correlation

To estimate methylation levels more efficiently and cost-effectively, we tried to establish primers for pyrosequencing. However, we could not establish a primer set for *PALLD* because CpG sites in the region were too dense to establish primers with adequate specificity and sensitivity. For *SIM1* and *ZNF154*, we were able to establish primers that linearly amplified partially methylated DNA samples with high correlations ($R = 0.97$ and $P = 0.0004$ for *SIM1*, and $R = 0.98$ and $P = 0.0002$ for *ZNF154*) (Fig. 4).

Using the primers, we performed pyrosequencing of the screening cohort samples with the remaining samples. Correlations between the b values in DNA methylation microarray and methylation levels in pyrosequencing were moderate for *SIM1* ($R = 0.53$ and $P = 0.55$) and high for *ZNF154* ($R = 0.92$ and $P < 0.0001$) (Supplementary Fig. S2). Therefore, we considered that, for *ZNF154*, methylation levels of the CpG sites analyzed by the pyrosequencing had a high correlation with b values of the CpG site analyzed by a DNA methylation microarray. In contrast, for *SIM1*, methylation levels of the CpG sites analyzed by the pyrosequencing had only a moderate correlation with b values of the CpG site analyzed by DNA methylation microarray.

Validation of the cancer cell fraction maker in an independent sample set

To validate whether *ZNF154* methylation levels by pyrosequencing and pathological cancer cell fraction coincide, we performed pyrosequencing of 20 independent additional samples. Correlation between pathological cancer cell fraction that had already been determined by pathology of histological sections and the *ZNF154* methylation level was 0.81 ($P < 0.0001$) (Fig. 5). This successful validation in an

independent sample set showed that *ZNF154* methylation level is as a good marker of ovarian cancer cell fraction as that determined from histological sections.

Discussion

In this study, we screened and validated a DNA methylation marker gene, *ZNF154*, which could be used to estimate ovarian cancer cell fraction. Its use in pyrosequencing will enable the estimation of cancer cell fraction efficiently and cost-effectively. DNA methylation alterations are widely used as diagnostic and prognostic marker in various cancer types [13–15]. *ZNF154* has been reported to be methylated in a variety of epithelial human cancers, including ovarian cancer, as well the usefulness of its methylation in liquid biopsy as a diagnostic marker in various cancers [16–18]. Our data here also supported that *ZNF154* was methylated specifically in ovarian cancer cells, not in normal fallopian tube epithelial cells or leucocytes, and that it is methylated in almost all cancer cells, though not heterogeneously. Therefore, the methylation level of *ZNF154* is useful as a cancer cell fraction marker, and considered to be useful to detect ovarian cancers, as reported [16, 18].

DNA methylation as a cancer cell fraction marker has several advantages. First, DNA methylation analysis could be performed using FFPE samples, the easiest samples to obtain in clinical settings. Second, cancer cell fraction could be assessed even after macroscopic dissection. The quality of macroscopic dissection depends on an individual's technique and also on sample characteristics, such as to what degree background cells are contaminated within cancer mass area according to histology and coexisting inflammation and fibrosis. If the methylation level of a sample is much lower than its pathological cancer cell fraction, it might be better to exclude the sample. Third, compared with DNA mutation analysis using a next-generation sequencer, DNA methylation analysis is cost-effective and not affected by allelic status or copy number alteration, a common feature in ovarian cancer [3].

There are some limitations in our study. First, although this marker gene was validated in an independent cohort, the number of patients was relatively small. However, previous reports on the utility of *ZNF154* [16–18] alleviate this concern. Second, the utility of this methylation marker in samples with high cancer cell fractions has still not been established, because we used samples after neoadjuvant chemotherapy. Additionally, neoadjuvant chemotherapy induces changes of gene expression and intra-tumor heterogeneity [19, 20], which may affect *ZNF154* methylation level. Third, methylation levels of *ZNF154* tended to be higher than in pathological cancer cell fractions, suggesting underestimation of pathological cancer cell fraction in samples after neoadjuvant chemotherapy, or methylation in some background cells. Nevertheless, we should be careful about the risk of overestimation of ovarian cancer cell fraction by this marker gene.

In conclusion, we established a DNA methylation marker gene, *ZNF154*, to estimate ovarian cancer cell fraction using FFPE samples. This marker gene will be helpful for suitable molecular analysis in ovarian cancer

Declarations

Acknowledgements

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Conflicts of interest

The authors state no conflicts of interest regarding this work.

Ethical approval

This study protocol was approved by the institutional review board at each participating center. This study was performed in accordance with the ethical standards of 1964 Declaration of Helsinki and its later amendments.

Consent

Written informed consent was obtained from all participants.

Data availability

Not available.

Author contributions

All authors contributed to the study conception and design. Data collection and analysis was performed by TE, SY and HY. The first draft of the manuscript was written by TE. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, Table 1 is only available as a download in the Supplemental Files section.

Figures

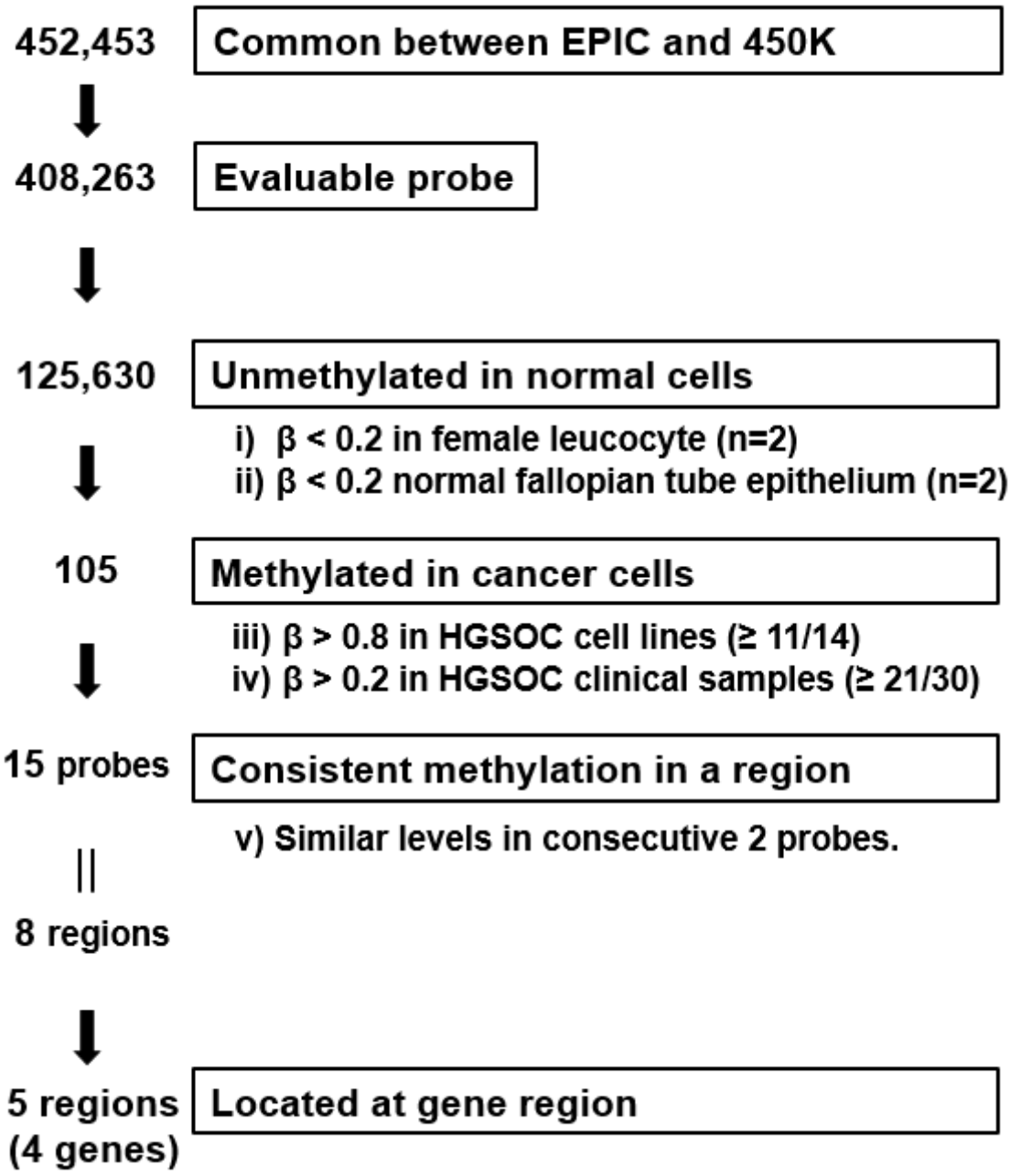

Figure 1

Screening flow of marker genes. Comprehensive DNA methylation analysis data of i) female leucocyte (n = 2), ii) normal fallopian epithelial cells (n = 2), □) ovarian cancer cell lines (n = 14), and □) ovarian cancer samples (n = 30) were used. Probes unmethylated in normal cells and methylated in cancer cells were first identified using the criteria in the figure. Then, gene regions with two consecutive probes with methylation were selected as candidate marker genes.

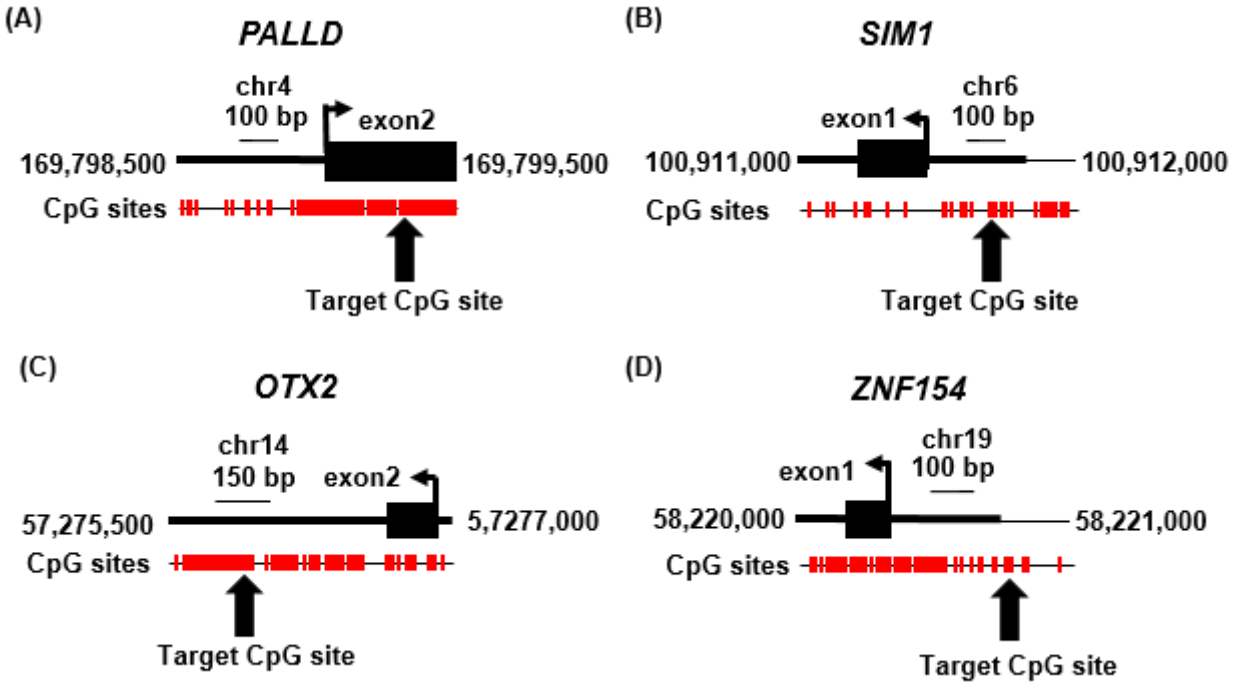

Figure 2

Gene structures of candidate marker genes and target CpG sites. Gene structure of the four candidate marker genes; (A) PALLD, (B) SIM1, (C) OTX2, and (D) ZNF154. An arrow shows the locations of a target CpG site identified by genome-wide DNA methylation analysis. For SIM1 and ZNF154, the locations of primers for pyrosequencing are also shown by arrow heads.

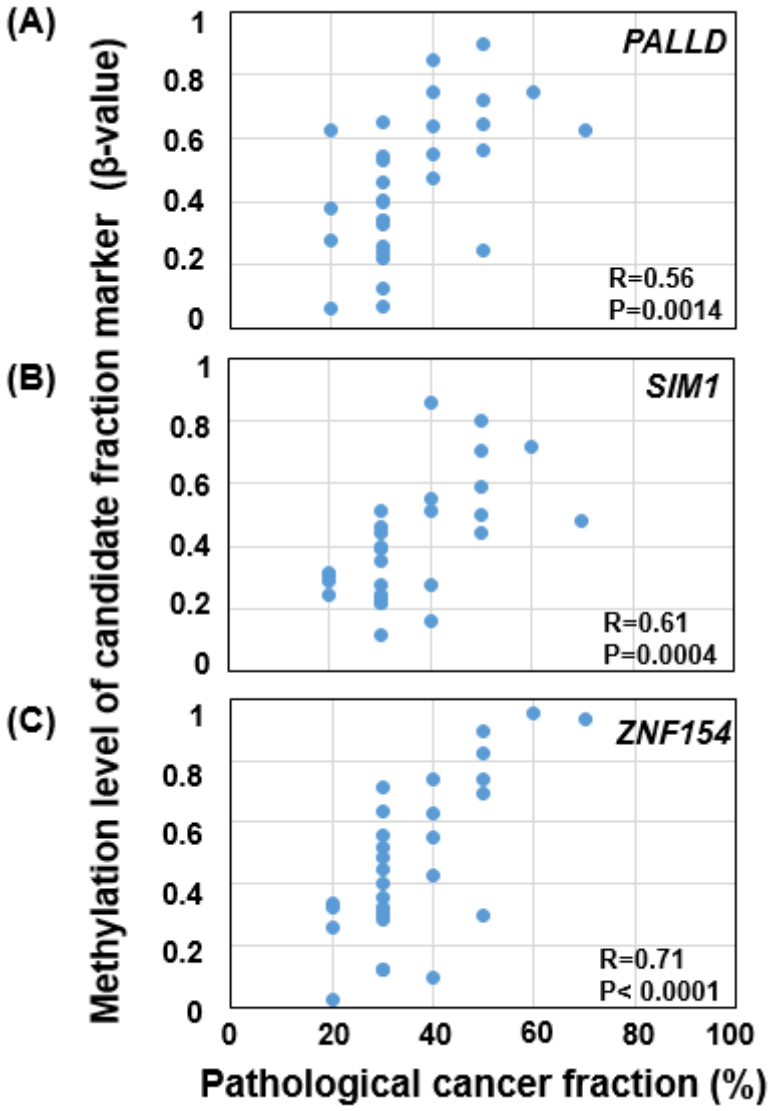

Figure 3

Correlation between the pathological cancer cell fraction and methylation level of candidate marker genes in screening cohort. Cancer cell fraction of 30 samples analyzed by a pathologist is plotted on the x-axis, and that assessed by a DNA methylation marker is on the y-axis. There was a significant correlation between pathological cancer cell fraction and methylation level for all three candidate markers.

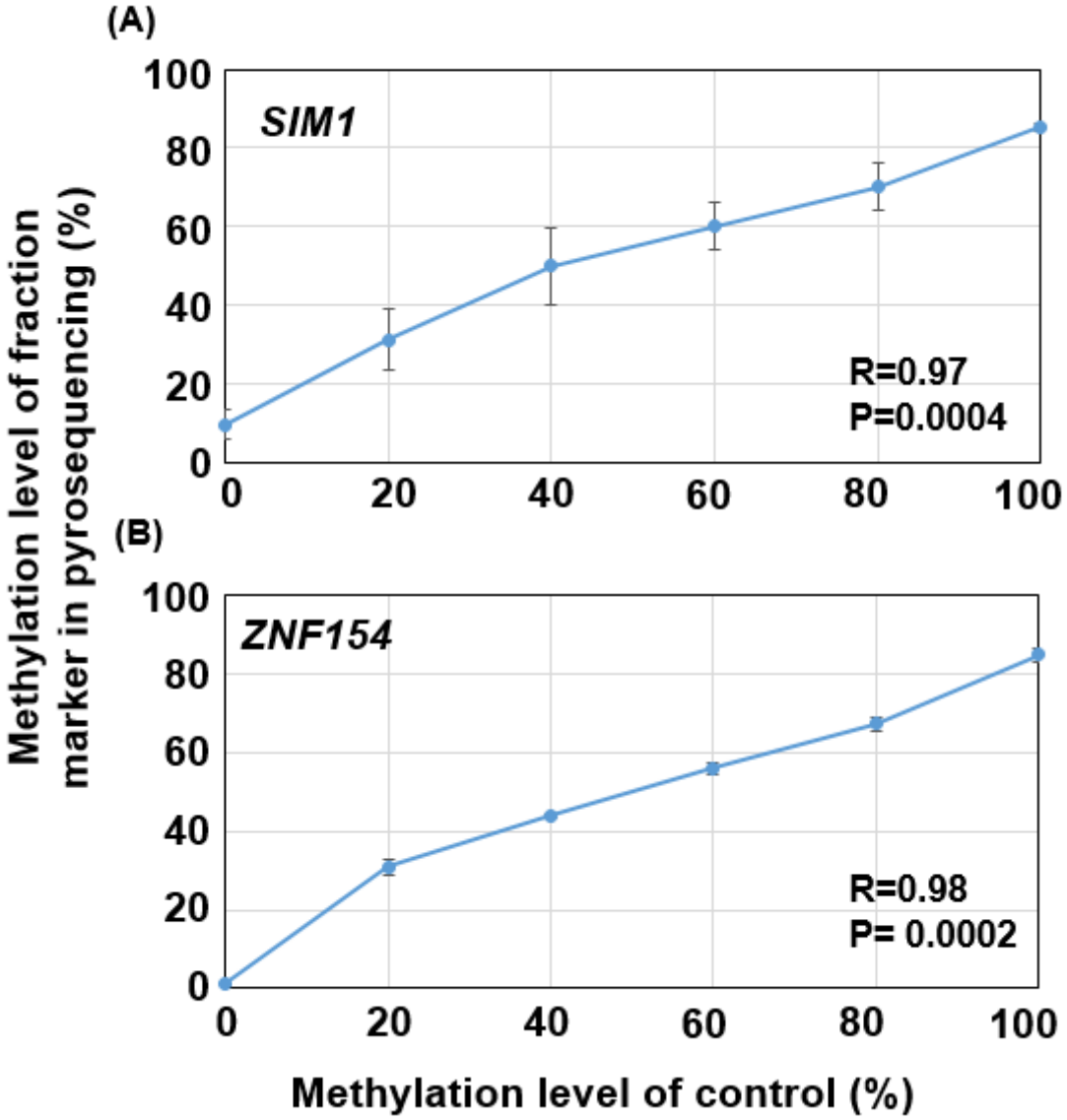

Figure 4

Technical confirmation of pyrosequencing. Methylation levels of artificially prepared partially methylated DNA were analyzed by pyrosequencing. Average value and SD of three independent results are shown. There were high correlations between the methylation levels of control DNA and methylation levels measured by pyrosequencing for SIM1 (R = 0.97, P = 0.0004) and ZNF154 (R = 0.98, P = 0.0002).

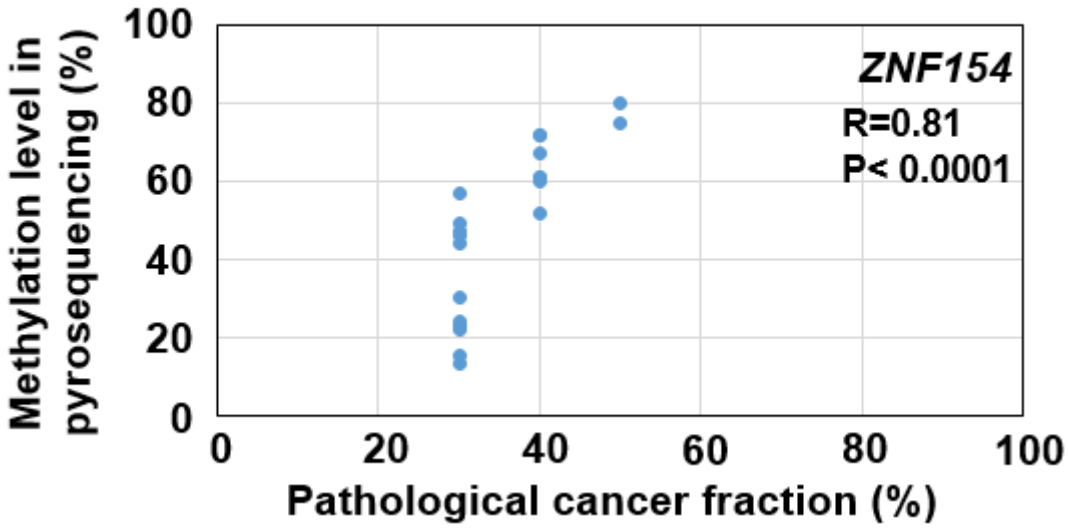

Figure 5

Correlation between the pathological cancer cell fraction and methylation level in a validation set. To validate the utility of the ZNF154 methylation marker, 20 additional samples were analyzed by a pathologist and by ZNF154 pyrosequencing. There was a significant correlation between pathological cancer cell fraction and ZNF154 methylation level (R = 0.81, P < 0.0001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.xlsx
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- Suppfig1.png
- Suppfig2.png