DNA hypermethylation of the ZNF132 gene participates in the clinicopathological aggressiveness of “pan-negative”-type lung adenocarcinomas

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Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; COPD, chronic obstructive pulmonary disease; FDR, false discovery rate; GO, gene ontology; N, adjacent normal lung tissue; PCA, principal component analysis; ROC, receiver operating characteristic; RT, reverse transcription; siRNA, small interfering RNA; T, tumorous tissue; TCGA, The Cancer Genome Atlas; TSS, transcription start site; 5'UTR, 5’ untranslated region.
Abstract

Although some previous studies have examined epigenomic alterations in lung adenocarcinomas, correlations between epigenomic events and genomic driver mutations have not been fully elucidated. Single-CpG resolution genome-wide DNA methylation analysis with the Infinium HumanMethylation27 BeadChip was performed using 162 paired samples of adjacent normal lung tissue (N) and the corresponding tumorous tissue (T) from patients with lung adenocarcinomas. Correlations between DNA methylation data on the one hand and clinicopathological parameters and genomic driver mutations, i.e. mutations of EGFR, KRAS, BRAF, and HER2 and fusions involving ALK, RET, and ROS1, were examined. DNA methylation levels in 12,629 probes from N samples were significantly correlated with recurrence-free survival. Principal component analysis revealed that distinct DNA methylation profiles at the precancerous N stage tended not to induce specific genomic driver aberrations. Most of the genes showing significant DNA methylation alterations during transition from N to T were shared by two or more driver aberration groups. After siRNA knockdown of ZNF132, which showed DNA hypermethylation only in the pan-negative group and was correlated with vascular invasion, the proliferation, apoptosis and migration of cancer cell lines were examined. ZNF132 knockdown led to increased cell migration ability, rather than increased cell growth or reduced apoptosis. We concluded that DNA hypermethylation of the ZNF132 gene participates in the clinicopathological aggressiveness of “pan-negative” lung adenocarcinomas. In addition, DNA methylation alterations at the precancerous stage may determine tumor aggressiveness, and such alterations that accumulate after driver mutation may additionally modify clinicopathological features through alterations of gene expression.

Keywords: DNA methylation, precancerous condition, driver mutation, lung adenocarcinoma, ZNF132.
Summary

Methylome analysis has revealed that DNA methylation alterations at the precancerous stage may determine the aggressiveness of lung adenocarcinomas and that such alterations accumulating after driver mutation, e.g. epigenetic silencing of ZNF132, additionally modify the clinicopathological features.
Introduction

It is well known that epigenomic alterations play a key role in carcinogenesis in various human organs (1-3). Aberrant DNA methylation is one of the most important epigenomic alterations resulting in chromosomal instability and altered expression of tumor-related genes (4). In the context of lung adenocarcinoma, various groups including The Cancer Genome Atlas (TCGA) (5) have reported the results of methylome analysis using appropriate screening tools, such as the Infinium assay (6), for large numbers of human tissue samples (7-9). For example, we have shown that epigenomic clustering of lung adenocarcinomas based on their genome-wide DNA methylation profiles is significantly correlated with carcinogenetic background factors (10) such as cigarette smoking and chronic obstructive pulmonary disease (COPD) (11). In addition, as in cancers of other organs (12-14), it has been revealed that the DNA methylation profiles of lung adenocarcinomas are significantly correlated with clinicopathological aggressiveness and poorer prognosis (15,16).

On the other hand, various genomic alterations, such as mutations of the EGFR, KRAS, BRAF, and HER2 genes and fusions involving the ALK, RET, and ROS1 genes are known to be mutually exclusive driver aberrations that are amenable to molecular targeted therapies (5,17). Genomic driver aberrations are known to be essential for growth, but alone they are insufficient for malignant progression of cancers through metastasis, for which molecular alterations other than driver aberrations, such as epigenomic lesions, are important (18). Therefore, in individual patients, epigenomic analyses and driver mutation screening are necessary to explain the clinicopathological diversity of lung adenocarcinomas.

As DNA methylation alterations are observed even in non-cancerous lung tissue of patients with lung adenocarcinomas, which have already been exposed to carcinogenetic factors such as cigarette smoking and COPD (11,19), we have focused on the correlations between DNA methylation profiles at the precancerous stage and subsequent driver mutations. In addition, during multistage carcinogenesis, epigenomic aberrations may further accumulate in the established cancers even after driver mutations have occurred. Since genomic polymorphism is known to affect DNA methylation profiles (20), the relationship between the driver mutation profile and the DNA methylation profile that accumulates in the later stage of carcinogenesis has also been a focus of interest. In the present study, based on the results of the Infinium assay using 364 lung tissue samples from patients who had
undergone screening of genomic driver aberrations, we examined the significance of DNA methylation profiles at the early and later stages of multistage lung adenocarcinogenesis.

Materials and Methods

Patients and tissue samples

We employed 162 samples of adjacent normal lung tissue (N) and 162 samples of the corresponding tumorous tissue (T) obtained from patients with primary lung adenocarcinomas who underwent lung resection at the National Cancer Center Hospital, Japan. All of the patients had undergone complete resection and none had received any preoperative treatment or adjuvant therapy after surgery. The patients comprised 85 males and 77 females, and their median age was 60 years (range, 30-76 years). Histological diagnosis was based on the World Health Organization classification (21), and the follow-up period ranged from 65 to 4,904 days (mean, 1,653 days) after surgery. Recurrence was diagnosed by clinicians on the basis of physical examination and imaging modalities such as computed tomography, magnetic resonance imaging, and positron emission tomography, and was sometimes confirmed histopathologically by biopsy. The clinicopathological parameters of the 162 patients are summarized in Supplementary Table 1, available at Carcinogenesis Online. A proportion of this cohort had also been included in our previous studies focusing on the correlation with smoking history (11) and recurrence-related genes (16).

All of the 162 cases were screened for EGFR, KRAS, BRAF, and HER2 hot spot mutations by the high resolution melt method, and for EML4- and KIF5B-ALK, KIF5B- and CCDC6-RET, and CD74-, EZR- and SLC34A2-ROS1 fusions by reverse-transcription (RT)-PCR, as described previously (17).

Tissue samples were taken immediately after surgery, and then frozen and stored in liquid nitrogen at the National Cancer Center Biobank, Japan, until analysis in accordance with the “Japanese Society of Pathology Guidelines for the handling of pathological tissue samples for genomic research” (22). This study was approved by the Ethics Committees of Keio University School of Medicine and the National Cancer Center, Tokyo, Japan, and was performed in accordance with the Declaration of Helsinki. All patients included in this study provided written informed consent for use of their materials and data.
Infinium assay

Genomic DNA was extracted from all tissue samples and cell lines using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) or phenol-chloroform extraction followed by dialysis. Five-hundred-nanogram aliquots of DNA were subjected to bisulfite conversion using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). DNA methylation status at 27,578 CpG loci was examined at single-CpG resolution using the Infinium HumanMethylation27 Bead Array (Illumina, San Diego, CA). After hybridization, the specifically hybridized DNA was fluorescence-labeled by a single-base extension reaction and detected using a BeadScan reader (Illumina) in accordance with the manufacturer’s protocols. The data were then assembled using GenomeStudio methylation software (Illumina). At each CpG site, GenomeStudio provided the so-called β-value, which is the ratio of the fluorescence signal measured using a methylated probe relative to the sum of those measured using methylated and unmethylated probes. These data were deposited in the Integrative Disease Omics Database (iDOx DB, https://gemdbj.ncc.go.jp/omics/). In accordance with a previous study (23), the M-value was calculated by logit transformation of the β-value and used for further statistical analysis.

Gene ontology (GO) enrichment analysis

GO enrichment analysis was conducted using genes showing DNA methylation alterations during lung adenocarcinogenesis by the GeneGO MetaCore™ software package (version 6.7) (Thomson Reuters, NY), an analytical tool based on a proprietary manually curated database. Such genes were considered significantly enriched in protein classes for which the P value was less than 0.0001.

Quantitative analysis of DNA methylation using pyrosequencing

The PCR and sequencing primers for the promoter regions of the TRIM58 and ZNF132 genes were designed using PSQ Assay Design Software Version 1.0.6 (Biotage, Uppsala, Sweden). To overcome any PCR bias, we optimized the PCR conditions: 0%, 50% and 100% of the fully methylated control DNA (Epitect methylated human control DNA, QIAGEN) was used as a template, as described previously (24), and the linearity of the measured values and their consistency with the theoretical values were confirmed. As a result of this
optimization experiment, both PCR reactions were performed using HotStarTaq DNA polymerase (QIAGEN). The further optimized PCR conditions for each primer set are summarized in Supplementary Table 2, available at Carcinogenesis Online. The biotinylated PCR product was captured on streptavidin-coated beads, and quantitative sequencing was performed on a PyroMark Q24 (QIAGEN) using the Pyro Gold Reagents (QIAGEN). The experiment was conducted in triplicate and the mean DNA methylation levels for each set of three experiments were used as quantitative values.

In addition to the exact Infinium probe CpG sites, i.e. cg20855565 (position 1) for the TRIM58 gene and cg13877915 (position 1) for the ZNF132 gene, DNA methylation data for neighboring CpG sites (positions 2 and 3) within the promoter regions were obtained for both the TRIM58 and ZNF132 genes by pyrosequencing, as shown in Supplementary Table 2, available at Carcinogenesis Online. Then mean values for positions 1 to 3 were considered to be the DNA methylation levels (%) of the promoter region for each gene obtained by pyrosequencing.

**Cell lines**

The lung adenocarcinoma cell lines PC-9 (25), H1975 (26) and A549 (27) and a hepatocellular carcinoma cell line JHH-7 (28) were purchased from Immuno-Biological Laboratories (Fujioka, Japan), the American Type Culture Collection (Manassas, VA), and Japanese Collection of Research Bioresources (Osaka, Japan), and authenticated based on short tandem repeat analysis by Japanese Collection of Research Bioresources Cell Bank (certification number, KBN0734) in June 2020. PC-9, H1975 and A549 were maintained in RPMI 1640 medium (Gibco-Thermo Fisher Scientific, Waltham, MA) and JHH-7 was maintained in William's E medium (Gibco-Thermo Fisher Scientific), supplemented with 10% fetal bovine serum, under 95% air and 5% CO₂ at 37°C.

**5-aza-2'-deoxycytidine (5-aza-dC) treatment of cell lines**

A549 and JHH-7 cells were seeded at a density of 9x10⁵ cells per 15-cm dish on day 0 and then allowed to attach for 24 h. Then, 5-aza-dC (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 1 μM. Cells were passaged at a subculture ratio of 1:2 on day 3. At 24 h after replacing, 5-aza-dC was added again to the same final concentration. Genomic DNA and total RNA were extracted from both cell lines on day 3 or day 6.
Quantitative real-time RT-PCR analysis

Total RNA was extracted from the cell lines using TRIzol reagent (Life Technologies, Carlsbad, CA). cDNA was then synthesized from the total RNA with random primers using Super-Script IV Reverse Transcriptase (Thermo Fisher Scientific) and preamplified using TaqMan PreAmp Master Mix (Thermo Fisher Scientific). Levels of mRNA expression for ZNF132 were analyzed using a custom TaqMan expression Assay probe (Hs01036387_m1, Thermo Fisher Scientific) on the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) employing the relative standard curve method. Experiments were performed in triplicate, and the mean value was used as the CT value. The CT value was normalized to that of GAPDH (Hs02758991_g1, Thermo Fisher Scientific) in the same sample.

Transfection with small interfering RNA (siRNA)

H1975 and PC-9 cells were seeded in 96-well plates at a concentration of 1x10^4 cells/well. When the cells had reached about 60% confluence, the medium was replaced with Opti-MEM® I Reduced Serum Medium (Thermo Fisher Scientific). The cells were then transfected with either the negative control siRNA (siNC) or a ZNF132 specific siRNA (s15162 and s15163) (Thermo Fisher Scientific) using Lipofectamine™ RNAiMAX reagent (Thermo Fisher Scientific). At 48 h after transfection, the levels of expression of mRNAs for ZNF132 were determined by quantitative real-time RT-PCR analysis, using GAPDH as the reference gene. The transfected cells were then subjected to cell proliferation, apoptosis and cell migration assays.

Western blotting

Cultured cells were lysed in RIPA buffer containing proteinase inhibitors (sc-24948, Santa Cruz Biotechnology), and loaded on 12.5% polyacrylamide gel. Proteins were fractionated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-human ZNF132 rabbit polyclonal antibody (PA5-41096, 1:500 dilution, Invrtogen, Carlsbad, CA) or anti-human β-actin rabbit monoclonal antibody (13E5, 1:1000 dilution, Cell Signaling Technology, Danvers, MA) and reacted with horseradish peroxidase-conjugated goat antibody (anti-rabbit IgG, 7074, Cell Signaling Technology), followed by incubation with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).
**MTS cell viability assay**

The MTS cell viability assay was performed as described previously (29). Briefly, 48 h after transfection with negative control or ZNF132 specific siRNAs, cells were treated with CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI). After 1 h of treatment, the optical density was measured at 490 nm on a GloMax®-Multi+ Detection System Glomax (Promega). Results were presented as the mean ± standard deviation for 3 separate determinations.

**Apoptosis assay**

The apoptosis assay was performed as described previously (29). Briefly, 48 h after transfection with negative control or ZNF132 specific siRNAs, cells were treated with a Caspase-Glo 3/7 assay kit (Promega). After 1 h of incubation, the luminescent signal was measured on a GloMax-Multi+ Detection System Glomax (Promega). Results were presented as the mean ± standard deviation for 3 separate determinations.

**Cell migration assay**

Cell migration was determined using 24-well transwell chambers with an 8-μm pore polycarbonate filter (Corning Inc., Corning, NY). Forty-eight hours after transfection with the negative control or ZNF132 specific siRNAs, 5x10⁴ H1975 and PC-9 cells were seeded onto the upper-side transwells in 100 μl of serum-free medium, and 500 μl of the complete medium was added to the lower chamber. The cells were incubated to allow migration for 48 h at 37°C and 5% CO₂. At the end of the assay, the non-motile cells on the top surface of the inserts were removed with cotton swabs. Cells that had passed through the polycarbonate membrane were fixed with 10% formalin and stained with 0.5% crystal violet to visualize the attached cells. The crystal violet was eluted with 10% acetic acid and the optical density was measured at 600 nm on a GloMax Multi Detection System (Promega). Results were presented as the mean ± standard deviation for 3 separate determinations.
**Statistics**

In the Infinium assay, all CpG sites on chromosomes X and Y were excluded to avoid any gender-specific methylation bias. The call proportions ($P$-values for detection of signals above the background <0.01) for 42 probes in all of the tissue samples examined were less than 90%. As such a low proportion may have been attributable to polymorphism at the probe CpG sites, these 42 probes were excluded from the present assay as described previously (30,31), leaving a final total of 26,444 autosomal CpG sites.

When the false discovery rate (FDR) of q was less than 0.01, differences in the $M$ values of Infinium probes between sample groups were considered significant. The receiver operating characteristic (ROC) curve was generated and the Youden index (32) was calculated in order to discriminate recurrence-positive patients from negative patients. Recurrence-free survival curves were generated by the Kaplan-Meier method, and differences were compared by the log-rank test. The DNA methylation profiles were analyzed using principal component analysis (PCA). Correlations between DNA methylation levels and clinicopathological parameters were examined using variance between groups (ANOVA) and Welch’s t-test at a significance level of FDR<0.5. Subsequently, multivariate analyses were performed using logistic regression. The mRNA expression levels, optical density assessed by MTS and cell migration assays, and luminescence for the apoptosis assay were examined by Welch’s t-test at a significance level of $P <0.05$. All statistical analyses were performed using programming language R.

**Results**

**Prognostic impacts of DNA methylation profiles**

Differences in the $M$-value for each of the 26,444 autosomal probe CpG sites in 162 N and 162 T samples were examined between recurrence-negative cases (n=39) and recurrence-positive cases (n=123) (FDR <0.01). In N samples, 9,048 probes, designed for 7,132 genes, showed significant differences between patients who were positive and negative for recurrence (Supplementary Table 3A, available at Carcinogenesis Online), indicating that a distinct DNA methylation profile with a prognostic impact had already been established at the precancerous N stage. As shown in Figure 1A, 1,041 probes (red dots) and 8,007 probes (blue dots) showed DNA hypermethylation and DNA hypomethylation in recurrence-positive patients relative to recurrence-negative patients, respectively.
In T samples, 5,344 probes, designed for 4,477 genes, showed significant differences between recurrence-positive and recurrence-negative patients (Supplementary Table 3B, available at Carcinogenesis Online). As shown in Figure 1B, 173 probes (red dots) and 5,171 probes (blue dots) showed DNA hypermethylation and DNA hypomethylation in recurrence-positive patients relative to recurrence-negative patients, respectively. Among the 5,344 probes showing a significant correlation with recurrence in T samples, 4,513 also revealed such a correlation even in N samples.

A Venn diagram summarizing the number of signatures from Figures 1A and 1B is shown as Figure 1C. This indicates that most of the DNA methylation alterations in T samples were shared with Ns and that the number of probes showing DNA methylation alterations unique to Ts was limited, suggesting that DNA methylation profiles at the precancerous N stages were inherited by the tumors themselves.

In addition, we compared the M-values of the present 162 N and 162 T samples with those of 32 samples of normal control lung tissue (C) obtained from patients without lung cancers, who may not have been exposed to lung carcinogenetic factors (the C samples had been analyzed and published in our previous paper [ref. 11]). Representative probes showing ordered differences in M-values from C to N, and then to T samples (P<0.05 by Jonckheere-Terpstra trend test), are shown in Supplementary Figure 1, available at Carcinogenesis Online. Supplementary Figure 1 again indicates that DNA methylation alterations in N samples relative to control C samples were inherited by or strengthened in T samples.

The prognostic impact of DNA methylation alterations at the precancerous N stage was further examined. ROC curves were generated for all 26,444 probe CpG sites using $M_N$ values. Youden indices were calculated to discriminate recurrence-positive from recurrence-negative patients. Recurrence-free survival curves for patients belonging to groups with higher ($M_N \geq$ Youden index) and lower ($M_N <$ Youden index) M-values were generated by the Kaplan-Meier method. For 12,629 probes, designed for 8,984 genes, the log-rank test showed that recurrence-free survival differed significantly between patients showing higher and lower DNA methylation levels in N samples (FDR <0.01) (Supplementary Table 4A, available at Carcinogenesis Online): the Kaplan-Meier curves for nine representative probes are shown in Figure 2A.

Next, to examine the significance of DNA methylation alterations during the N to T transition stage, ROC curves were generated for all 26,444 probe CpG sites using the $\Delta M_{T-N}$ values. Recurrence-free survival curves for patients belonging to groups showing larger (|$\Delta M_{T-N}$| $\geq$ |Youden index|) and smaller (|$\Delta M_{T-N}$| < | Youden index|) differences were
generated by the Kaplan-Meier method. For only 130 probes, designed for 124 genes, the log-rank test revealed that recurrence-free survival differed significantly between patients showing larger and smaller differences during the N to T transition (FDR <0.01) (Supplementary Table 4B, available at Carcinogenesis Online): the Kaplan-Meier curves for three representative probes are shown in Figure 2B.

**Correlations between DNA methylation profiles and genomic driver aberrations**

M-values for all 26,444 probes in all 162 N samples were analyzed using PCA. The patients showing each genomic driver aberration, i.e. mutations of the EGFR, KRAS, BRAF and HER2 genes and fusions including the ALK, ROS1 and RET genes, were scattered on the plot (Figure 3), indicating that the distinct DNA methylation profiles at the precancerous N stage did not appear to induce any specific genomic driver aberrations.

Next, the correlation between the DNA methylation profile and genomic driver aberrations was examined during the N to T transition. In patients with EGFR mutation (n=68), ALK fusion (n=7), KRAS mutation (n=12), ROS1 fusion (n=9) and HER2 mutation (n=4) and pan-negative patients (n=58), 9,583, 599, 792, 470, 102 and 8,907 probes showed significant differences in DNA methylation levels between N and T samples, respectively (FDR <0.01) (Supplementary Table 5, available at Carcinogenesis Online). Since the number of patients was so small, patients with BRAF mutation (n=2) and RET fusion (n=2) were excluded from further statistical analysis. Among these aberrantly methylated probes, DNA methylation alterations on 7,648 probes were shared by two or more driver aberration groups (Supplementary Table 6, available at Carcinogenesis Online). The numbers of probes shared by 2 to 6 driver aberration groups are summarized in Supplementary Table 7, available at Carcinogenesis Online. On the other hand, only 1,948, 18, 4, 4, 3 and 1,297 probes showed significant differences between N and T samples that were specific to the EGFR mutation-positive, ALK fusion-positive, KRAS mutation-positive, ROS1 fusion-positive, HER2 mutation-positive and pan-negative groups, respectively (Supplementary Table 7, available at Carcinogenesis Online).
DNA methylation alterations shared by multiple genomic driver aberration groups

Among the 7,648 probes aberrantly methylated during the N to T transition that were shared by two or more driver aberration groups, 1,404 probes were located within the CpG islands around the transcription start site (TSS), i.e. the region from the TSS to 1500 bp upstream of it, the 5’ untranslated region (5’UTR), and the first exon or the first intron of the 1,116 genes based on the UCSC Genome Browser (http://genome.ucsc.edu/). Among 1,116 genes, the levels of mRNA expression were inversely correlated with the levels of DNA methylation for 197 genes (259 probes) in lung cancers deposited in the TCGA database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) using the MethHC pipeline (http://MethHC.mbc.nctu.edu.tw) (Pearson correlation coefficient [r] <-0.2 and \( P <0.05 \)) (Supplementary Table 8A, available at Carcinogenesis Online).

These 197 genes were evaluated for protein function by enrichment analysis using the MetaCore software, and compared with the protein function distribution of genes within the GeneGo database. Genes showing DNA methylation alterations shared by multiple driver aberration groups were clearly overrepresented by “transcriptional factors” (\( P=3.734\times10^{-13} \)) and “enzyme” (\( P=2.016\times10^{-6} \)) (Table 1). In addition, known tumor-related genes, e.g. CDH1, CDH2 and CLDN4, which participate in cell adhesion and epithelial-mesenchymal transition, and FZD2, SOX2 and PAX7, which participate in Wnt/β-catenin signaling, have been included in Supplementary Table 8A.

Genomic driver aberration-specific occurrence of DNA methylation alterations

Among the aforementioned 1,948, 18, 4, 4, 3 and 1,297 probes showing EGFR mutation-specific, ALK fusion-specific, KRAS mutation-specific, ROS1 fusion-specific, HER2 mutation-specific and pan-negativity-specific DNA methylation alterations, respectively, levels of mRNA expression were inversely correlated with DNA methylation levels for only 41 EGFR-specific genes (43 probes) and 26 pan-negative-specific genes (29 probes) (67 genes in total), based on the TCGA database (\( r <-0.2 \) and \( P <0.05 \)) (Supplementary Table 8B, available at Carcinogenesis Online).

Among the 67 genes, the DNA methylation alterations of 66 genes occurring during the N to T transition (\( \Delta M_{T-N} \)) were significantly correlated with clinicopathological parameters reflecting tumor aggressiveness, i.e. a larger tumor size, pleural invasion, lymphatic invasion, vascular invasion, intrapulmonary metastasis and recurrence (FDR<0.5) (Supplementary Table 9, available at Carcinogenesis Online).
Since gender, age and smoking status are known to be associated with DNA methylation (33), multivariate analysis of these parameters in addition to the DNA methylation status ($\Delta M_{T-N}$) of the genes included in Supplementary Table 9, available at Carcinogenesis Online, were performed. The DNA methylation status of multiple genes was significantly correlated with a larger tumor size, pleural invasion, lymphatic invasion and intrapulmonary metastasis, independently from gender, age, and smoking status (Supplementary Table 10, available at Carcinogenesis Online).

Technical verification of DNA methylation levels in the promoter region of the TRIM58 and ZNF132 genes

Using pyrosequencing, technical verification was performed for representative genes, i.e. the TRIM58 gene, for which our Infinium assay had demonstrated DNA hypermethylation shared by multiple genomic driver aberration groups and an inverse correlation with its mRNA expression had been evident in the TCGA database (Supplementary Table 8A, available at Carcinogenesis Online), and the ZNF132 gene, for which DNA hypermethylation based on our Infinium assay had been observed only in the “pan-negative” group and was significantly correlated with vascular invasion (Supplementary Table 9, available at Carcinogenesis Online). Since tissue samples from the present 162 patients were exhausted after the Infinium assay, 26 paired N and their corresponding T samples in another cohort (validation cohort \([n=52]\)) of lung adenocarcinomas were subjected to pyrosequencing. The $\beta$-values of cg20855565 and cg13877915 for the TRIM58 and ZNF132 genes based on the Infinium assay and DNA methylation levels in the promoter regions of the TRIM58 and ZNF132 genes based on pyrosequencing were significantly correlated with each other in the validation cohort (Pearson’s correlation coefficient $r=-0.922$ and $0.900$, and $P=2.234 \times 10^{-11}$ and $3.939 \times 10^{-10}$, respectively) (Figure 4), indicating that the Infinium data had been successfully verified using pyrosequencing.

Silencing of ZNF132 due to DNA hypermethylation

We further focused on the ZNF132 gene, for which DNA hypermethylation during N to T transition ($\Delta M_{T-N}$) was significantly correlated with vascular invasion (Supplementary Table 9, available at Carcinogenesis Online). The levels of DNA methylation and mRNA expression of ZNF132 in PC-9, H1975, A549 and JHH-7 cells are shown in Figure 5A. In the top two cell lines showing the highest DNA methylation levels, A549 and JHH-7, the level of ZNF132 mRNA expression was low. These two cell lines were then subjected to 5-aza-dC treatment. This led to a marked reduction of DNA methylation and restored the expression level of
ZNF132 mRNA (Figure 5B), indicating that ZNF132 had been silenced due to DNA hypermethylation in lung adenocarcinomas.

Significance of ZNF132 in proliferation, apoptosis and migration of lung adenocarcinoma cells

Knockdown of ZNF132 using siRNA transfection was performed in the top two cell lines showing the highest mRNA expression levels, H1975 and PC-9. After transfection, marked reduction of ZNF132 expression was confirmed by quantitative real-time RT-PCR and Western blotting (Figure 5C and Supplementary Figure 2, available at Carcinogenesis Online). Although an increase of cell growth and a decrease of caspase-3/7 activities were observed only in s15162-treated H1975 cells (Figures 5D and 5E, respectively), cell migration ability was significantly enhanced by knockdown of ZNF132 using both s15162 and s15163 in both H1975 and PC-9 cells (Figure 5F and Supplementary Figure 3, available at Carcinogenesis Online).

Discussion

Here we have reported the results of the Infinium assay for 324 samples of lung tissue (162 N and 162 T samples) along with the genomic driver aberration data for T samples. According to the field cancerization concept (34), N samples obtained from patients with lung adenocarcinomas, which can be exposed to carcinogenic factors such as cigarette smoking and chronic persistent inflammation during COPD (10), are considered to be at precancerous stages. Therefore, we first focused on the DNA methylation profiles of N samples.

More probes for N samples than for T samples showed significant correlation with recurrence after surgery, reflecting the poorer prognosis associated with the former (Figure 1 and Supplementary Table 3, available at Carcinogenesis Online). In fact, a significant association between the level of DNA methylation at each of the CpG sites in N samples with recurrence-free survival rate had been confirmed by long-term follow-up (Figure 2A). Moreover, in T samples, most of the probes showing significant correlation with recurrence also showed such a correlation even from the N stages (Figure 1C and Supplementary Table 3, available at Carcinogenesis Online), indicating that DNA methylation profiles at precancerous N stages may already determine the aggressiveness of established cancers developing in the same individuals. However, this conclusion has a limitation in that DNA
methylation profiles of N samples may be affected by some paracrine factors derived from adjacent established tumors.

On the other hand, the number of probes showing a correlation between DNA methylation alterations during the N to T transition (ΔMT-N) and recurrence-free survival was very limited (Supplementary Table 4B, available at Carcinogenesis Online), suggesting that many passenger DNA methylation alterations may occur, at least partly due to genomic alterations such as copy number alterations, during progression from the precancerous N stage to established Ts. The finding that the number of probes showing DNA methylation alterations significantly associated with recurrence in T samples was smaller than that in N samples (Figure 1) may be attributable to the fact that passenger DNA methylation alterations can mask any clinicopathologically significant DNA methylation profiles in T samples.

Next, we focused on the interaction between epigenomic and genomic alterations during multi-stage lung adenocarcinogenesis. Our PCA analysis clearly revealed that patients with each type of genomic driver aberration were individually scattered on the scattergram (Figure 3), indicating that, in general, preceding distinct DNA methylation profiles at the precancerous N stage may not induce specific genomic driver mutations, although this conclusion has some limitation due to the very limited sample size. Since, in general, DNA methylation alterations are more dependent on environmental factors than genomic aberrations (35), it is feasible that DNA methylation profiles are generally independent of mutation profiles.

During multi-stage carcinogenesis, DNA methylation alterations are further accumulated in established cancers even after a driver mutation has arisen. The majority of DNA methylation alterations (on the 7,648 probes) occurring during transition from N to T, potentially after driver mutation, were shared in two or more genomic driver aberration groups (Supplementary Table 7, available at Carcinogenesis Online), indicating that, in general, each genomic driver mutation does not induce a specific DNA methylation profile. DNA methylation alterations again occur independently, regardless of the driver aberration profile. This conclusion again has some limitation as it was based on results from a very limited sample size.

We considered that such commonly shared DNA methylation alterations in established cancers with various driver mutations might shed light on the significance of epigenomic events in the later steps of multistage carcinogenesis. Commonly shared DNA methylation alterations which could potentially result in expression abnormalities were enriched among “transcriptional factors” (Table 1). It is feasible that such factors affect the
transcriptional regulation of various downstream genes including tumor-related genes. In addition, some known tumor-related genes themselves have shown commonly shared DNA methylation alterations (Supplementary Table 8A, available at Carcinogenesis Online). These findings suggest that DNA methylation alterations in the later stages may further participate in the progression of cancers.

On the other hand, the number of genes showing DNA methylation alterations, which are specific to some mutation groups and could potentially result in expression abnormalities, was very small, i.e. only 67. However, such alterations were frequently correlated with tumor aggressiveness (Supplementary Table 9, available at Carcinogenesis Online), indicating that DNA methylation alterations in the later steps of carcinogenesis may further modify the clinicopathological diversity of lung adenocarcinomas.

For example, DNA hypermethylation of ZNF132 during progression from the precancerous stage to established lung adenocarcinoma was significantly correlated with vascular invasion. Although epigenomic silencing of ZNF132 has been reported in prostatic adenocarcinoma (36) and esophageal squamous cell carcinoma (37), its participation in lung cancer has never been confirmed previously. In addition to the inverse correlation between DNA methylation and mRNA expression in the TCGA database, our 5-aza-dC treatment revealed that ZNF132 is silenced by DNA methylation. Our knockdown study indicated that silencing of ZNF132 may participate in the malignant progression of lung adenocarcinomas, especially that of pan-negative cancers that have not yet been well studied, through an increase of cell migration ability, which can result in invasiveness and potential for metastasis, rather than increased cell proliferation or reduced apoptosis.

In summary, our study has shown that DNA methylation alterations at the precancerous stage, which precedes genomic driver aberration, largely determine the clinicopathological aggressiveness and outcome of lung adenocarcinomas. In general, such distinct DNA methylation alterations at the precancerous stage do not induce specific genomic driver mutations, and each driver mutation tends not to induce specific DNA methylation profiles. Moreover, DNA methylation alterations occurring after driver mutation at the later stages of multistage carcinogenesis again contribute to the further progression and clinicopathological diversity of lung adenocarcinomas by altering the expression of the affected genes.
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Table 1. Gene ontology enrichment analysis of the 197 genes commonly showing significant differences in DNA methylation levels between non-cancerous and cancerous tissue specimens in two or more driver aberration groups, for which DNA methylation alterations would potentially result in mRNA expression alterations (listed in Supplementary Table 8A, available at Carcinogenesis Online), according to protein function using MetaCore software.

| Protein class   | r   | n   | R   | N   | Expected | Ratio    | P       |
|-----------------|-----|-----|-----|-----|----------|----------|---------|
| Transcription factors | 32  | 220 | 1254| 40677| 6.782    | 4.718    | 3.734E-13 |
| Enzymes         | 36  | 220 | 2869| 40677| 15.52    | 2.32     | 2.016E-06 |
| Receptors       | 19  | 220 | 1700| 40677| 9.194    | 2.066    | 2.367E-03 |
| Kinases         | 9   | 220 | 685 | 40677| 3.705    | 2.429    | 1.279E-02 |
| Ligands         | 5   | 220 | 562 | 40677| 3.04     | 1.645    | 1.898E-01 |
| Phosphatases    | 2   | 220 | 242 | 40677| 1.309    | 1.528    | 3.769E-01 |
| Proteases       | 4   | 220 | 623 | 40677| 3.369    | 1.187    | 4.358E-01 |
| Other           | 115 | 220 | 32813| 40677| 177.5    | 0.648    | 1.534E-21 |

r, number of objects from the present data set for a given protein class; n, total number of objects from the present data set; R, number of background objects from the database for a given class; N, total number of background objects from the database; Expected, mean value for hypergeometric distribution (n x R / 40677); Ratio, the ratio of actual / expected. If the ratio is more than 1, P-values less than 0.0001 mean significant enrichment, and these are underlined.
**Figure legends**

Figure 1. Correlations between DNA methylation profiles and recurrence in samples of adjacent normal lung tissue (N) (A) and the corresponding tumorous tissue (T) (B) from patients with lung adenocarcinomas. All 26,444 probes are shown along the chromosome. Red and blue indicate probes showing significantly higher and lower DNA methylation levels observed in recurrence-positive (rec+) patients when compared to those in recurrence-negative (rec-) patients, respectively (false discovery rate < 0.01). (C) A Venn diagram summarizing the number of probes showing significantly higher and lower DNA methylation levels observed in recurrence-positive (rec+) patients relative to those in recurrence-negative (rec-) patients in panels (A) and (B). $\Delta M_{\text{rec+}-\text{rec-}} \geq 0$ means DNA hypermethylation (shown in red) and $\Delta M_{\text{rec+}-\text{rec-}} < 0$ means DNA hypomethylation (shown in blue) in rec+ patients relative to rec- patients. Most of the DNA methylation alterations in T samples were shared with Ns and the number of probes showing DNA methylation alterations unique to Ts was limited.

Figure 2. Prognostic impact of DNA methylation profiles at the precancerous stage (A) and DNA methylation alterations during progression from the precancerous stage to established cancers (B). (A) Representative Kaplan-Meier survival curves of patients showing higher ($M_N \geq \text{Youden index}$) and lower ($M_N < \text{Youden index}$) DNA methylation levels in adjacent normal lung tissue (N) samples. (B) Representative Kaplan-Meier survival curves of patients showing larger ($|\Delta M_{T-N}| \geq |\text{Youden index}|$) and smaller ($|\Delta M_{T-N}| < |\text{Youden index}|$) differences in DNA methylation levels between N and the corresponding tumorous tissue (T) samples. FDR, false discovery rate.

Figure 3. Principal component analysis using DNA methylation levels of all 26,444 probes in adjacent normal lung tissue samples from patients with lung adenocarcinomas harboring **EGFR** mutation (red) (n=68), **ALK** fusion (blue) (n=7), **KRAS** mutation (brown) (n=12), **ROS1** fusion (pink) (n=9), **HER2** mutation (orange) (n=4), **BRAF** mutation (light blue) (n=2), **RET** fusion (green) (n=2) and none (pan-negative cases, black) (n=58). The horizontal and vertical axes show the first (PC1) and second (PC2) principal components, respectively.
Figure 4. Pyrosequencing verification of the β-values of the *TRIM58* (A) and *ZN132* (B) genes based on the Infinium assay. Since tissue samples from the present 162 patients were exhausted after the Infinium assay, pyrosequencing and the Infinium assay were performed using 26 paired samples of adjacent normal lung tissue and their corresponding cancerous tissues in another cohort (validation cohort [n=52]) of lung adenocarcinomas. The β-values of cg20855565 and cg13877915 for the *TRIM58* and *ZN132* genes and the DNA methylation levels in the promoter regions based on pyrosequencing were significantly correlated with each other in the validation cohort (Pearson’s correlation coefficient $r=-0.922$ and 0.900, and $P=2.234\times10^{-11}$ and $3.939\times10^{-10}$, respectively), indicating that the Infinium data had been successfully verified using pyrosequencing.

Figure 5. Treatment with 5-aza-2'-deoxycytidine (5-aza-dC) and *ZN132* gene knockdown experiments in lung adenocarcinoma cell lines. (A) DNA methylation levels of the promoter region based on pyrosequencing and mRNA expression levels based on reverse-transcription-PCR analysis of *ZN132* in PC-9, H1975, A549 and JHH-7 cells. A549 and JHH-7 cells showing higher DNA methylation levels were subjected to 5-aza-dC treatment and H1975 and PC-9 showing higher mRNA expression levels were subjected to knockdown experiments. (B) DNA methylation and mRNA expression levels of *ZN132* on day 3 or 6 (AZA3 or AZA6) of 5-aza-dC treatment and untreated control cells (AZA0). After 5-aza-dC treatment, reduction of DNA methylation levels and restoration of the mRNA expression levels (9.1-fold and 15.8-fold increase) were observed in A549 and JHH-7 cells, respectively. (C) Knockdown of *ZN132* by transfection with a *ZN132*-specific small interfering RNA (dashed bar) and a negative control siRNA (siNC) (clear bar) in H1975 and PC-9 cells. mRNA and protein expression levels were reduced in both cell lines. (D) Effects of *ZN132* knockdown on cell proliferation. Forty-eight hours after transfection of H1975 and PC-9 cells with siNC and siZN132, the MTS assay was performed. (E) Effects of *ZN132* knockdown on apoptosis. Twelve, 24 and 48 h after transfection with siNC (solid line) and siZN132, s15162 (broken line) and s15163 (dotted line), caspase -3/7 activity was evaluated in H1975 and PC-9 cells. (F) Effects of *ZN132* knockdown on cell migration ability. Forty-eight hours after transfection of H1975 and PC-9 cells with siNC and siZN132, transwell assay was performed. Although cell growth and apoptosis were altered only in H1975 using s15162 (panels D and E), cell migration ability was significantly enhanced by reduction of *ZN132* in both H1975 and PC-9 using both s15162 and s15163 (panel F).
Figure 1
Figure 2
Figure 3: Scatter plot of Principal Component Analysis (PCA) for various mutations.

- Red triangle: EGFR_Mutation
- Blue triangle: ALK_Fusion
- Brown triangle: KRAS_Mutation
- Green triangle: RET_Fusion
- Orange triangle: HER2_Mutation
- Light blue triangle: BRAF_Mutation
- Purple triangle: ROS1_Fusion
- Black triangle: Pan-negative
Figure 4

A

DNA methylation levels of the promoter region based on pyrosequencing (%) vs. β-value based on Infinium assay

$r = 0.922$

$P = 2.234\times10^{-11}$

B

DNA methylation levels of the promoter region based on pyrosequencing (%) vs. β-value based on Infinium assay

$r = 0.900$

$P = 3.939\times10^{-10}$
Figure 5