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Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer

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Abstract

Hypomethylation of DNA repetitive elements is a common finding in cancer, but very little is known about the DNA methylation changes of different types of DNA repetitive elements, such as interspersed repeats (*LINE1* and *Alu Yb8*) and tandem repeats (*Sat-α*, *NBL-2* and *D4Z4*). We used bisulfite-PCR Pyrosequencing to quantitatively measure the DNA methylation of 5 different DNA repetitive elements in normal tissue and cancer. In all we studied 10 different tissues from 4 individuals undergoing autopsy, 34 paired normal and tumor tissues from patients with bladder cancer, 58 patients with chronic myelogenous leukemia and 23 patients with acute promyelocytic leukemia. We found that the DNA methylation of interspersed repeats (*LINE1* and *Alu Yb8*) was very consistent from person to person and tissue to tissue while tandem DNA repeats appeared more variable in normal tissues. In bladder cancer we found clear hypomethylation of *LINE1*, *Alu Yb8*, *Sat-α*, and *NBL-2*. Conversely, we found an increase in the DNA methylation levels of *D4Z4* from normal to cancer. In contrast leukemia showed no significant changes in the DNA methylation of *LINE1* and *Alu Yb8*, but DNA methylation increases in *NBL-2* and *D4Z4* tandem repeats. Our findings show that the changes in DNA methylation levels of individual DNA repetitive elements are unique for each repetitive element, which may reflect distinct epigenetic factors and may have important implications in the use of DNA methylation of repetitive elements as global DNA methylation biomarkers. Keywords: DNA methylation, DNA repetitive elements, bladder cancer, leukemia

Keywords

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Aberrant promoter hypermethylation of CpG islands is a common finding in cancer and is associated with gene silencing in human cancer¹. In contrast to CpG islands, DNA repetitive elements are normally heavily methylated in somatic tissues and account for more than one-third of the total DNA methylation in the human genome². However, during tumorigenesis and aging global DNA hypomethylation has been described³⁻⁵, and is attributable a decrease of DNA methylation in DNA repetitive elements, as there is an association of DNA repetitive element methylation and total genomic 5-methylcytosine⁶. The etiologies of this global decrease in DNA methylation is not known but may be induced by environmental factors and precede cancer. Hypomethylation of *LINE1* and Alu repetitive elements have been associated with exposure to the carcinogen benzene⁷, suggesting a link between environmental exposure and epigenetic consequence⁸.

The significance of this global hypomethylation of DNA repetitive elements in cancer is unclear, but it may play multiple roles in the pathogenesis of cancer. DNA hypomethylation of *LINE1* promoters may lead to an increase of its transcription *in vivo*^{9, 10}. This increase of *LINE1* transcription might result in increased transposition of *LINE1* elements into new genomic loci causing mutational events in cancer. *De novo* insertion of *LINE1* into the *c-Myc* gene and the adenomatous polyposis coli (APC) gene leads to their inactivation in somatic cells^{11, 12}. In addition, hypomethylation of the *LINE1* antisense promoter has been shown to increase transcription of *c-MET*, a proto-oncogene, which leads to the generation of a truncated form of *c-MET* in leukemia¹³. The link between repetitive element hypomethylation and cancer has also been modeled in mice carrying a hypomorphic allele and a null allele for DNA methyltransferase 1 (*Dnmt1*^{chip/-}). This mouse model shows hypomethylation-induced tumors that involve chromosome instability and retrotransposition of a intracisternal A particle into the *notch1* genomic locus¹⁴. Thus, the hypomethylation of DNA repetitive elements may promote tumorigenesis through chromosomal instability or overexpression of oncogenes¹⁴⁻¹⁶.

There are many different types of DNA repetitive elements, such as interspersed repeats and tandem repeats; combined these elements comprise at least half of the human genome¹⁷. The interspersed repeats are mostly composed of retrotransposable elements such as Long Interspersed Nuclear Elements (LINEs), Short Interspersed Nuclear Elements (SINEs), Long Terminal Repeats (LTRs), or DNA transposons¹⁷. The ability of these elements to “jump” has led to their abundance and their distribution throughout the genome. In contrast, tandem repeats are present as long and uninterrupted simple or complex clustered sequences, and duplicate as inter and intra chromosomal segments. Centromeric, pericentromeric, and subtelomeric regions of the human genome contain tandem repeats and reside mostly in heterochromatic regions¹⁷. Despite the plethora of DNA repetitive elements and the large contribution of these elements to the genome, little is known about the DNA methylation of different types of DNA repetitive elements in cancer. This study systematically and comprehensively examines the changes in DNA methylation patterns of multiple DNA repetitive elements in normal tissue and cancer.

Materials and Methods

Tissue samples

Ten non-cancerous somatic tissues (bladder, brain, colon, esophagus, heart, kidney, liver, lung, spleen, stomach) were collected from four individuals under going post-mortem examinations (Case 1: 52 year-old male; cirrohsis, Case 2: 35 year-old male; cirrohsis, Case 3: 58 year-old female; breast cancer, Case 4: 60 year-old female; diabetic complication). 34 bladder tumor samples along with adjacent normal bladder urothelium were obtained from 25 male and 9 female patients with bladder cancer undergoing cystectomy. 58 Chronic myelogenous leukemia (CML) and 23 acute promyelocytic leukemia (APL) samples were obtained from peripheral blood (CML) and bone marrow (APL) of patients at the time of diagnosis. See supplemental table 1 for the details about autopsy samples, bladder tumor and leukemia samples. All human tissue was collected through protocols approved by the Institutional Review Board of the University of Southern California.

DNA extraction and Sodium Bisulfite modification

DNA was extracted using standard Phenol/Chloroform methods. Bisulfite modification of genomic DNA was performed using the EZ-96 DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA, USA), according to the manufacturer's recommended protocol.

Quantification of DNA methylation by Pyrosequencing

The level of DNA methylation of *LINE1*, *Alu Yb8* subfamily, *Sat-a*, *D4Z4* and *NBL-2* was assessed using a previously developed bisulfite-DNA Polymerase Chain Reactions (PCR) Pyrosequencing assay (See supplemental table 2 for details)^{2, 18}. Primers, designed to a consensus sequence for each repetitive element, amplified a global pool of repetitive elements rather than a single element or genomic locus. In brief, bisulfite treatment of DNA leads to the formation of single nucleotide polymorphism, either a C, indicating methylated cytosine, or a T, indicating unmethylated cytosine, which can be quantitated by Pyrosequencing. Biotin labeled PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden). The Sepharose beads containing the immobilized PCR products were purified, washed, denatured using a 0.2 M NaOH solution, and washed again. Pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System (Biotage, AB, Uppsala, Sweden) per the manufacturer's protocol.

Statistical analysis

All values are reported as mean \pm SD. Data was analyzed using the paired or unpaired Student's *t* test using StatView software (Abacus, Berkeley, CA, USA). The significance level was set at $P < 0.05$.

Inter- and intra variability of DNA methylation of DNA repetitive elements in normal tissues

To understand the methylation of DNA repetitive elements in normal tissue, we first examined 40 unique tissues from 4 individuals without cancer undergoing autopsy (Fig. 1 and supplemental table 3). This allowed examination of the variability of DNA methylation

from person to person and tissue to tissue for the first time. We employed bisulfite-PCR Pyrosequencing to quantitatively assess the level of DNA methylation of interspersed repeats (*LINE1* and *AluYb8*) and tandem repeats (*Sat-α*, *NBL-2* and *D4Z4*). We studied DNA methylation of *LINE1* since it is known to be hypomethylated in many types of cancer and is associated with global hypomethylation^{2, 6, 19}. We also have measured the DNA methylation of several *Alu* subfamilies including *AluYb8*, *Alu Sx*, and *Alu J* in normal tissues using specific polymorphisms unique to each *Alu* subfamily. We found the mean level of DNA methylation as measured by bisulfite PCR Pyrosequencing is 85.5%, 24% and 18.8% for *Alu Yb8*, *Alu Sx*, and *Alu J* respectively (data not shown). The higher DNA methylation levels of *Alu Yb8* reflect the younger evolutionary age of these elements and the lower degree of C to T transition mutations compared to the older *Alu Sx* and *Alu J* elements. As previous studies have shown hypomethylation of *Alu* elements in cancer we chose to study the *Alu Yb8* subfamily as the higher methylation levels would allow a technical advantage to study hypomethylation changes. We analyzed chromosome 1 satellite-α (*Sat-α*) repeats whose DNA methylation is known to be hypomethylated in cancer and associated with global DNA methylation^{6, 20, 21}. We also studied *NBL-2* and *D4Z4* known to be hypomethylated in ICF (*I*mmunodeficiency, *C*entromeric instability, and *F*acial abnormalities) syndrome patients²². Schematic structures and estimated copy numbers of the DNA repetitive studied are shown in supplement figure 1. The bisulfite-PCR Pyrosequencing assay for the repetitive elements proved very consistent with the mean of standard deviation (SD) for *LINE1*, *Alu Yb8*, *Sat-α*, *NBL-2* and *D4Z4* DNA repeats was 2.47%, 0.93 %, 2.3 %, 0.71 % and 2.2 %, respectively.

The relative standard deviation (RSD) of ten different tissues (*Tissue RSD*, *TRSD*) and RSD of four individuals (*Individual RSD*, *IRSD*) were calculated to assess inter and intra individual variability of each DNA repetitive element. The level of DNA methylation of interspersed repeats, *LINE1* (*TRSD*=2.8%, *IRSD*=2.5%) and *Alu Yb8* (*TRSD*= 1.2%, *IRSD*=1.1%) was more consistent among tissues and individuals than tandem repeats, *Sat-α* (*TRSD*=9.4%, *IRSD*=8.7%), *NBL-2* (*TRSD*=1.7%, *IRSD*=4.7%) and *D4Z4* (*TRSD*=9.6%, *IRSD*=13%), (Fig. 1F). This may be attributable to a sampling effect as the copy number of interspersed repeats is greater than tandem repeats, and interspersed repeats are spread more diffusely in the genome whereas tandem repeats tend to be localized to specific areas of chromosomes. DNA methylation of interspersed repeats, *LINE1* and *Alu Yb8*, were consistent from tissue to tissue and person to person. In contrast, the DNA methylation of one of the tandem repeats, *NBL-2* was more consistent from tissue to tissue within an individual ($p<0.0001$). It is possible that the level of DNA methylation might be determined before tissue differentiation by genetic factors such as copy number variation or an unknown genetic determinant. Thus a genetic factor or very early stem cell event would determine the level of *NBL-2* methylation in all tissues within an individual. These differences between methylation of DNA repetitive elements may have important implications if DNA repetitive elements are to be used as a biomarker in population based studies.

DNA methylation of DNA repetitive elements in bladder cancer

In order to determine the level of DNA methylation of DNA repetitive elements in two distinctive tumor types, we chose bladder tumor as an example of a solid tumor and

leukemia as an example of a liquid tumor, both of which undergo tumor progression changes. First, we study the changes in the DNA methylation of multiple repetitive elements in cancer by studying normal bladder urothelium from people without bladder cancer with paired bladder tumors and adjacent normal tissue. We compared the level of DNA methylation using adjacent normal and tumor samples from the same individual (Fig. 2). The methylation level of interspersed repeats *LINE1* and *Alu Yb8* significantly decreased in bladder cancer in comparison to adjacent normal bladder urothelium. Methylation of *LINE1* and *Alu Yb8* decreased from 69.8 ± 3.8 % to 57.3 ± 10.6 % ($p < 0.0001$) and from 85.9 ± 2.4 to 80.5 ± 6.8 ($p = 0.0006$), respectively, which is consistent with previous reports^{19, 23} (See supplemental table 4 for details).

In tandem repeats, the level of DNA methylation of *Sat-a* and *NBL-2* also significantly decreased from 71.6 ± 3.9 % to 58.2 ± 17.8 % ($p = 0.0073$), and from 83.2 ± 3.9 % to 67.3 ± 19.7 % ($p = 0.0012$), respectively. In contrast we found that DNA methylation of the tandem repeat *D4Z4* increased in bladder cancer. The level of *D4Z4* methylation increased from 51.3 ± 9.3 % to 58.1 ± 12.4 % ($p = 0.016$) in bladder cancer, in comparison to adjacent normal tissue. Our results are consistent with a previous report that showed *D4Z4* elements are hypermethylated in some cancers²⁴. Interestingly, we found that in adjacent normal bladder tissue from patients with cancer, DNA methylation changes could be detected in tandem repeats when compared to normal bladder tissue in people without cancer. *Sat-a* showed a significant decrease of 5.7 % ($p = 0.0019$) while *D4Z4* showed a mean increase of 3.0 % ($p = 0.45$) in adjacent normal bladder from individuals with bladder cancer, compared to normal bladder. This presents the possibility that these repetitive elements can be used as an early biomarker to predict cancer. However, there is an increase in variability of DNA methylation levels of DNA repetitive elements in bladder cancer, and DNA methylation changes do not occur in every cancer, which may limit its predictive ability as early cancer marker for individual patients.

To understand the relationship among different repetitive elements within an individual, we examined the correlation between two different DNA repetitive elements in 34 pair of normal and bladder tumor tissues. Two interspersed DNA repetitive elements, *LINE1* and *Alu Yb8* methylation strongly correlated each other ($r = 0.630$, $p < 0.0001$) and also correlated with *Sat-a* methylation (*Sat-a* vs. *LINE1*; $r = 0.687$, $p < 0.0001$ and *Sat-a* vs. *Alu Yb8*; $r = 0.489$, $p = 0.002$), but not *D4Z4* and *NBL-2* (See supplemental table 5 for details). Although *NBL-2* showed hypomethylation in bladder cancer, *NBL-2* hypomethylation did not correlate with hypomethylation of *LINE1*, *Alu Yb8* and *Sat-a*. Thus the mechanism responsible for the hypomethylation of *NBL-2* is independent of hypomethylation of the other DNA repetitive elements. Again, these data support the finding that DNA methylation of tandem repeats differs from that of interspersed repeats and that different DNA repetitive elements have unique changes in methylation in bladder cancer

It is known that a germline mutation of DNMT3B causes ICF syndrome which is characterized by hypomethylation of *NBL-2* and *D4Z4*²², thus linking the DNA methylation of these two tandem repeats. However, the discordant DNA methylation changes of *NBL-2* and *D4Z4* in bladder cancer indicate that epigenetic machinery other than DNMT3B must be involved in bladder cancer. One may also consider that DNMT3B has different functions,

where it is involved in increasing methylation of *D4Z4* and decreasing DNA methylation of *NBL-2* in bladder cancer, respectively²⁵.

DNA methylation changes of repetitive elements in leukemia

We next studied the DNA methylation changes of repetitive elements in leukemia (Fig. 3). This allowed us to compare two different cancers of the myeloid hematopoietic system and to study the changes in methylation during the progression of CML, which shows a well-defined progression from chronic to accelerated to blast phase leukemia. It has been reported that the level of *LINE1* methylation decreases during CML progression^{13, 27}, or does not significantly change in CML patients²⁶. In our study, the level of DNA methylation in *LINE1* and *Alu Yb8* did not significantly change in CML and APL (See supplemental table 6 for details). Our finding is consistent with the previous report by Issa *et al.*²⁶, but is different from the hypomethylation of DNA repetitive elements found during CML progression^{13, 27}. This difference may be attributable to different techniques used to measure the level of DNA methylation: bisulfite-PCR Pyrosequencing versus methylation specific PCR (MSP).

As shown in bladder cancer, the tandem repeats behaved differently than the interspersed repeats in leukemia. However, in leukemia both *NBL-2* and *D4Z4* methylation increased in APL and during the progression of CML; the increase was slightly greater in APL. Thus, the methylation of *NBL-2* decreases in bladder tumor progression, but increases during leukemogenesis. Our findings are consistent with the previous report, showing that *NBL-2* repeats can be either hypomethylated in neuroblastoma and hepatocellular carcinoma or hypermethylated in ovarian epithelial carcinomas²⁸. Nishiyama *et al.* also reported that hyper- and hypomethylation at different individual CpG sites within same *NBL-2* repeat in ovarian cancer²⁹. However, in our study we found the methylation at different individual CpG sites with *NBL-2* was concordant in both bladder cancer ($R^2= 0.81$) and in leukemia ($R^2= 0.50$). This indicates that the behavior of DNA methylation of tandem repeats may vary from cancer to cancer and deserves further investigation.

Here we demonstrated that changes in DNA methylation of tandem repeat elements behave differently than interspersed repeats, and that the methylation changes vary between bladder cancer and leukemia. Due to the variability of DNA methylation levels in different DNA repetitive elements, methylation assessment of DNA repetitive elements may possess limited ability as a biomarker for cancer stage or disease outcome in an individual patient. However, these DNA repetitive element assays have been used as a biomarker previously in population based studies. For example, *LINE1* has been used as a surrogate marker of demethylating agent treatments for MDS (myelodysplastic syndrome) and leukemia patients^{30–32}. *LINE1* and *Alu* methylation assays have also been used as a biomarker of environmental exposure⁷. Therefore we believe our results add to an understanding of how DNA methylation of repetitive elements change in different diseases, and can lead to a better understanding of their use as biomarkers for epigenetic drugs and population based epigenetic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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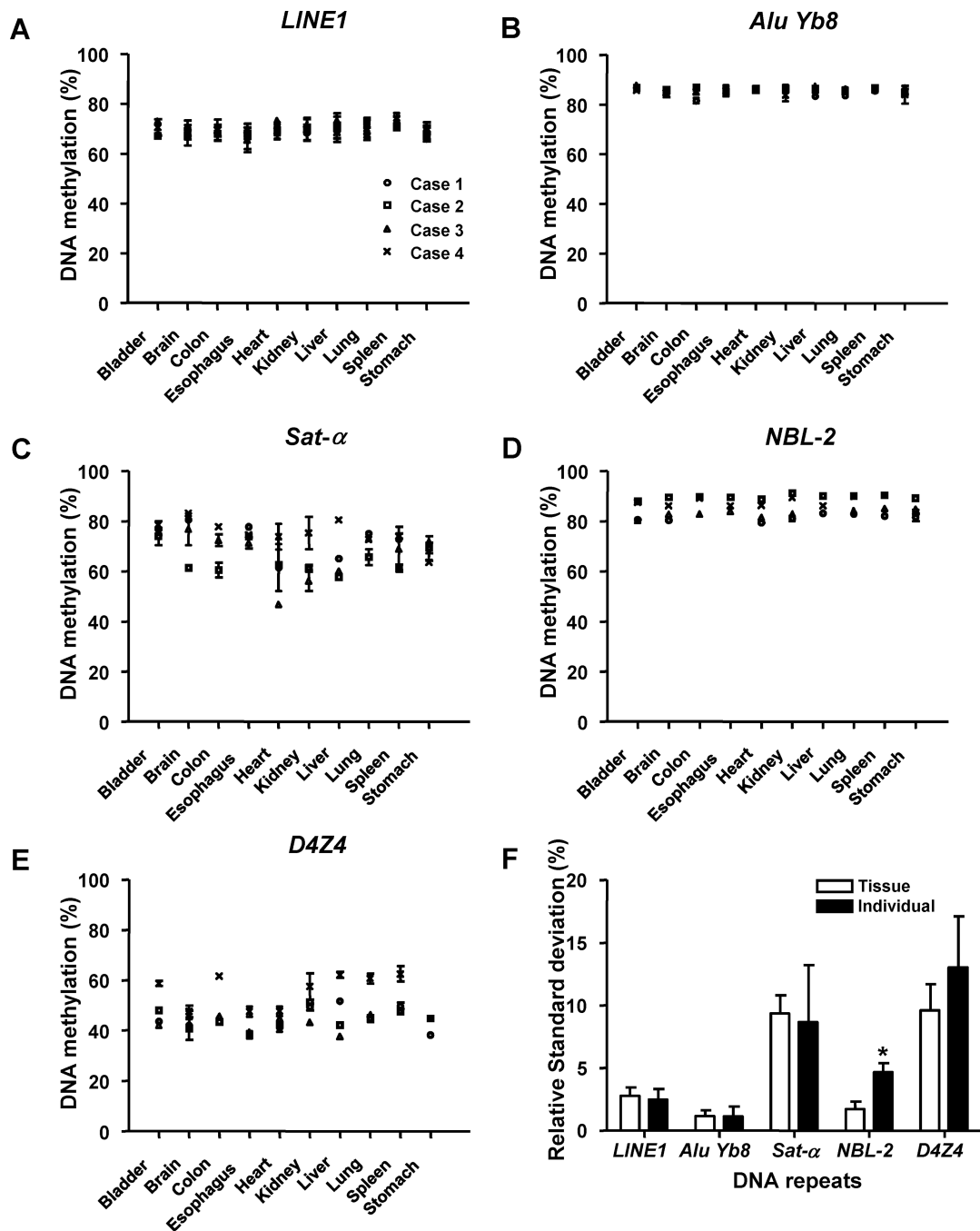


Figure 1. DNA methylation of different DNA repetitive elements in normal tissue. DNA methylation of 5 different DNA repetitive elements was measured in ten non-cancerous somatic tissues from four individuals under going post-mortem examinations using bisulfite-PCR Pyrosequencing. Interspersed repeats: (A) *LINE1*, (B) *Alu Yb8*; Tandem repeats: (C) *Sat-α*, (D) *NBL-2*, (E) *D4Z4*, (F) inter and intra variation of DNA methylation of different DNA repetitive elements. * $p < 0.0001$. DNA methylation (%) represents the percentage

of $mCpG/mCpG + CpG + TpG$; mC (methylated cytosine), C (unmethylated cytosine) and T (thymine that are generated from CpG by spontaneous evolutionary deamination).

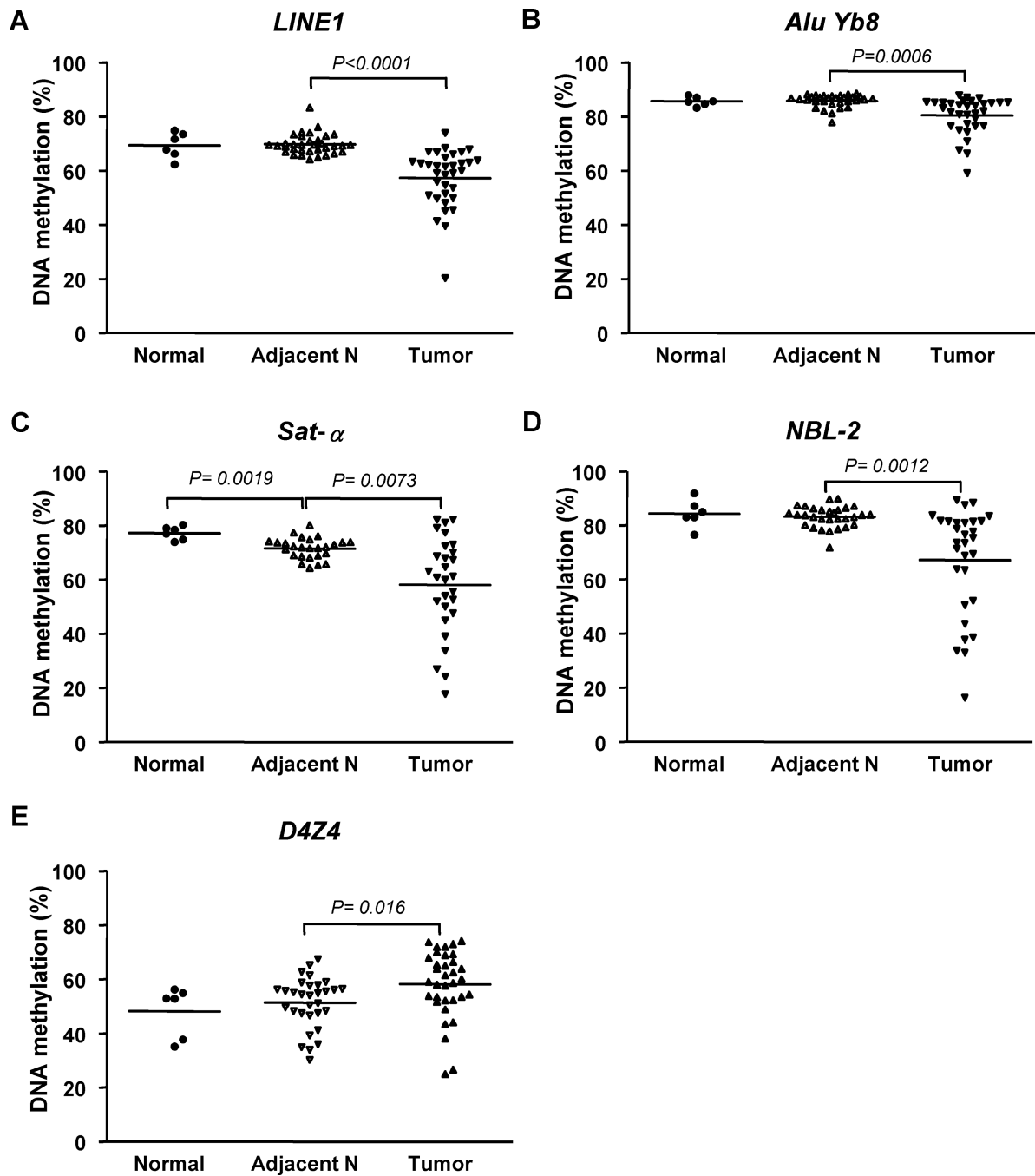


Figure 2.

DNA methylation changes of different DNA repetitive elements in bladder cancer. DNA methylation of 5 different repetitive elements was measured in normal bladder urothelium from people without cancer (Normal), and paired samples of normal bladder urothelium from patients with bladder cancer (Adjacent N) and bladder cancers (Tumor). Interspersed repeats: (A) *LINE1*, (B) *Alu Yb8*; Tandem repeats: (C) *Sat-α*, (D) *NBL-2*, (E) *D4Z4*.

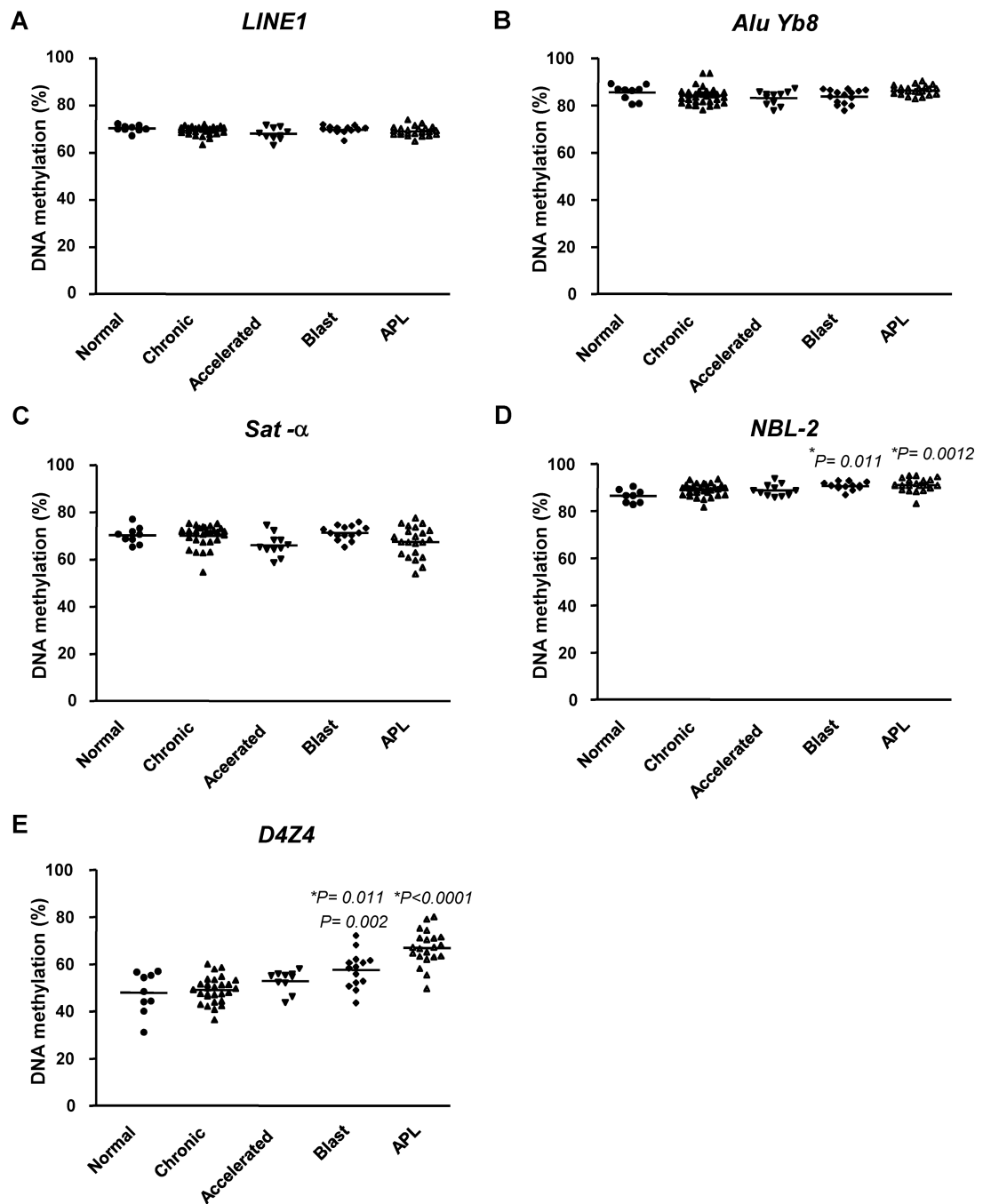


Figure 3.

DNA methylation changes of different repetitive elements in leukemia. DNA methylation of 5 different DNA repetitive elements was measured in Normal peripheral blood (Normal), Chronic phase (Chronic) CML, Accelerated phase (Accelerated) CML, Blast crisis (Blast) CML, and APL. Interspersed repeats: (A) *LINE1*, (B) *Alu Yb8*; Tandem repeats: (C) *Sat-α*, (D) *NBL-2*, (E) *D4Z4*. $*p$ vs. normal blood, $\dagger p$ vs. chronic phase.