Tissue-specific differentially methylated regions of the human VASA gene are potentially associated with maturation arrest phenotype in the testis

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Title

Tissue-specific differentially methylated regions (TDMRs) of the human *VASA* gene are potentially associated with maturation arrest phenotype in the testis

Short title: Hypermethylation of the human *VASA* gene

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Abstract

Numerous CpG islands containing tissue-specific differentially methylated regions (TDMRs) are potential methylation sites in normal cells and tissues. The *VASA* (also called *DDX4*) gene, is thought to be under the control of TDMRs. A total of 131 male patients with idiopathic azoospermia or severe oligospermia was evaluated histologically and screened the status of methylation of CpG islands in the *VASA* gene. Genome DNAs were obtained from testicular biopsy and modified with sodium bisulfite and were applied Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This system is capable to analyze the methylated and unmethylated CpG island in genome. The methylation analysis is performed by an epigram as graphic data. Upon histological assessment, seventeen of 131 patients revealed maturation arrest. Six of seventeen patients demonstrated particularly high *VASA* TDMR methylation rates, while the remaining eleven patients and control had low methylation rates. This study may imply *VASA* TDMR methylation is significantly higher among maturation arrest patients, in whom *VASA* gene expression was silenced. This finding represents an important contribution to the molecular basis of meiotic arrest as one possible cause of idiopathic infertility.

Key words

Spermatogenesis, CpG island,DDX4, male infertility, MALDI-TOF MS

Introduction

Spermatogenesis is a complex multi-step process, mediated by different molecules, that encompasses the entire sequence of meiotic events from spermatogonia to mature spermatozoa in males. There is increasing evidence for genetic and epigenetic regulation of meiosis in the spermatogenesis of many model organisms. However, few attempts have been made at clinical investigation of this mechanism in humans. In particular, epigenetic changes are thought to influence male fertility [1,2]. DNA methylation is an epigenetic modification that is related to changes in gene expression without accompanying changes in DNA sequence [3,4]. The DNA methylation state at CpG dinucleotides, also known as CpG islands, is occasionally found in the promoter regions of many genes. CpG islands are generally not methylated during normal cell development, with the exception of imprinted genes and tissue-specific differentially methylated regions (TDMRs). Although each cell of an organism contains basically the same genome, gene activation is cell-specific as well as temporally and positionally mediated. TDMRs are hypomethylated in specific tissues and methylated in all other tissues [5,6], in which DNA methylation is involved in gene silencing. As a result, genes with hypomethylated TDMRs are expressed in a tissue-specific manner. Several studies have revealed that there are TDMRs at upstream regulatory regions in the mouse [7,8]. In general, repeat sequences, promoters of tissue-specific genes, and gene-coding regions are hypermethylated. However, numerous CpG islands containing TDMRs are potential methylation sites in normal cells and tissues. Genome-wide epigenetic analysis recently revealed the evolutionary conservation of tissue-specific methylation in human brain cells, keratinocytes, and peripheral blood lymphocytes [9,10].

Furthermore, the *VASA* (also called *DDX4*) gene is thought to be under the control of TDMRs, Kitamura et al. found unmethylated as well as methylated CpG islands in human testis, since bisulphite sequencing results showed 50% of clones hypomethylated and 50% of clones hypermethylated[11]. *VASA* encodes a member of the DEAD (Asp-Glu-Ala-Asp) box family of ATP-dependent RNA helicases. This indicates that the methylation status of *VASA* promoter CpG islands with TDMs was associated with spermatogenesis and the regulation of *VASA* gene expression.

The *VASA* ortholog in *Drosophila*, *vasa,* is required for spermatogenesis and oogenesis by a mechanism that involves regulating the translation of mRNAs essential for differentiation [12]. The *Mouse Vasa Homolog* (*Mvh*) gene is expressed specifically in the developing germ cell lineage during embryogenesis [13]. It has been reported that male mice homozygous for a targeted mutation of *Mvh* exhibit a reproductive deficiency in which they produce no spermatozoa in the testes. Instead, premeiotic germ cells cease differentiation by the zygotene stage and undergo apoptotic death [14]. In humans, the gene is mapped to human chromosome 5q; its expression is restricted to the testes in males, and is undetectable in somatic tissues [15].

Moreover, *VASA* gene modulates RNA structure, which is a crucial step in many fundamental biological processes. A variety of RNA metabolic processes, including transcription, ribosomal biogenesis, RNA splicing, editing, and transport, and translational events, are also controlled by *VASA* [16-21]. Additionally, recent studies have revealed *VASA* gene involvement in RNA metabolism and micro RNA processing[22].

The aim of the present study was to test the possibility that DNA methylation states in *VASA* promoter CpG islands may contribute to some forms of human idiopathic male infertility. In the present study, we also applied Matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis to identify DNA methylation states using testicular genome DNA.

Materials and Methods

Patients

We recruited 131 patients with azoospermia or severe oligozoospermia (sperm concentration<1×10^6/mL), excluding abnormal karyotypes and Y chromosome microdeletion [23]. All 131 patients underwent either open testicular biopsy or retrieval of sperm from testicular tissues in order to evaluate histology while attempting to extract testicular sperm (TESE). The Ethics Committee of Kanazawa University Hospital approved the study, and informed consent was obtained from all participants.

Histological evaluation of testicular biopsies

Testicular specimens were fixed in Bouin's solution, and paraffin slides were prepared and stained by hematoxylin and eosin staining for histological evaluation. To diagnose the spermatogenic pattern, samples were obtained from at least three different (lower, middle, upper) areas in the testis. Diagnostic biopsies included evaluation of more than 100 sections per biopsy of seminiferous tubules per biopsy [24,25]. Testicular histology was classified into hypospermatogenesis (reduction in the degree of normal spermatogenic cells), maturation arrest (lack of late stage of spermatogenesis), Sertoli cell-only syndrome(lack of germ cells in seminiferous tubules), and tubular sclerosis (no germ cell or Sertoli cell present in the seminiferous tube) [26].

Isolation of total RNA and genomic DNA

All of testicular tissue specimens from biopsy were used for isolation of total RNA and testicular genomic DNA. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was available for extraction of both molecules from the same specimen. Total RNA was treated with the TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove any residual genomic DNA. Single-stranded cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).

RT-PCR

To define the testis-specific expression of the *VASA* gene, RT-PCR was performed on cDNA samples from normal human tissues, including testis, muscle, kidney, heart, brain, and colon tissues provided by Pro. Nagai, Nihon University. *Taq* DNA polymerase was used for PCR reactions (TaKaRa Bio, Shiga, Japan). PCR amplification for both *VASA* and *GAPDH* were performed with the following parameters: 95°C for 5 min; 29 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extention at 72°C for 30 sec; and a final incubation at 72°C for 10 min. The PCR products were resolved on 1.2% agarose gels. Specific primer pairs used for *VASA* (NM_024415) and *GAPDH* (NM_002046) amplification were 5'-CAAGAGAGGCGGCTATCGAGAT-3' and 5'-GTCAGAATTGCTGGTGGTGCATC-3' (483bp) for *VASA*, and 5'-GACCACAGTCCATGCCATCA-3' and 5'-TCCACCACCCTGTTGCTGTA-3' (453bp) for *GAPDH*.

Confirmation of TDMR

Kitamura et al. demonstrated that the presence of TDMRs upstream of the *VASA* gene is associated with testis-specific expression [11]. The region is -1127 to -760 (368bp amplicon) from the transcription start site (TSS), and contains a promoter CpG island with 30 CpG binucleotide, a G+C content of 63.5%, and an observed/expected CpG

ratio of 1.01 (http://genome.ucsc.edu/index.html?org=Human&db=hg18&hgsid=107086429). We analyzed this amplicon using patients' tissue samples.

Bisulfite treatment

Isolated genomic DNA (1µg) from testicular specimens was converted with sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions.

PCR and in vitro Transcription

The position of the *VASA* promoter CpG island and the amplicon analyzed in this study are also shown in Figure 1. Bisulfite treated DNA was subjected to PCR with the specific primer pair. A reverse primer of 5'-CAGTAATACGACTCACTATAGGGAGAAGGCTCATCCACACTTTAACCAAAAATC-3' was tagged with a T7 promoter sequence. The forward primer used was 5'-AGGAAGAGAGGTTTTGTATTTATAGGTTTAATAGGTTATT-3'. Amplification of 1µl bisulfite-treated DNA (~20 ng/µl) was performed with HotStar *Taq* Polymerase (Qiagen, Valencia, CA, USA) in a 5µl reaction volume, using PCR primers at a final concentration of 200 µM. PCR amplification was performed with the following parameters: 94°C for 15 min hot start, followed by 45 cycles of denaturing at 94°C for 20 s, annealing at 58°C for 45 s, and extention at 72°C for 1 min, and a final incubation at 72°C for 3 min. After shrimp alkaline phosphatase treatment, 2µl of the PCR products were used as a template for *in vitro* transcription to obtain RNA transcripts. The reverse product was subjected to base-specific cleavage and the resulting samples were conditioned and spotted on a 384-pad SpectroCHIP® (Sequenom) using a MassARRAY nanodispenser® (Samsung, Irvine, CA, USA),

followed by spectral acquisition on a MassARRAY Analyzer® Compact MALDI-TOF MS (Sequenom).

MALDI-TOF MS analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was utilized for high-throughput quantitative DNA methylation assay [27]. This system analyzed base-specific cleaved amplification products. In this study, we used the MassARRAY® Compact system (Sequenom, San Diego, CA, USA). The resultant methylation calls were performed by using EpiTyper software v1.0 (Sequenom,) to generate quantitative results per cleavage fragment (called CpG unit) including each CpG site or aggregate of multiple CpG sites. The graphic data obtained from methylation analysis using MassARRAY® was expressed as an epigram.

Real-time quantitative RT-PCR

For expression analysis of the human *VASA* gene, we utilized real-time quantitative RT-PCR. The generated sample cDNA was amplified on a LightCycler system (Roche Applied Science, Switzerland) using a LightCycler TaqMan Master (Roche Diagnostics, Mannheim, Germany). TaqMan probe was purchased from Roche Diagnostics. Universal probes no. 62 and no. 60 were used for *VASA* and *GAPDH*, respectively. Each universal probe was designed according to the Universal Probe Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). An additional primer pair used for *VASA* expression was 5'-GGAGTTGGAAAGAGTTTTGGAA-3' and 5'-CAGAAACCAGAGCTATCACCATC3-' (74bp). The primer pair used for *GAPDH* expression was 5'-AGCCACATCGCTCAGACA-3' and 5'-GCCCAATACGACCAAATCC-3' (66bp). To normalize the *VASA* mRNA

expression with respect to sample-to-sample differences in RNA input, RNA quality, and reverse transcriptase efficiency, we amplified the housekeeping gene *GAPDH*. We obtained the copy numbers of *VASA* and *GAPDH* according to the respective standard curves. The results were determined by the ratio of *VASA*/*GAPDH* in each cDNA sample. All PCR reactions were performed in a total volume of 20μL containing 4μL of 5x LightCycler TaqMan Master (Roche Diagnostics, final concentration: 1x), 0.3μL of 10μmol/L TaqMan probe (Roche Diagnostics, final concentration: 150 μmol/L), 1μL of 10μmol/L each primer (final concentration: 500 μmol/L), 2μl of sample cDNA, and 11.6μl of DEPC-treated water. Water was used in the negative controls instead of cDNA template. Amplifications of *VASA* and *GAPDH* were performed in triplicate for each sample. The thermal cycling conditions were 10 min at 95°C, followed by 50 cycles at 95°C for 10 s and 50 cycles at 60°C for 20 s for both *VASA* and *GAPDH*. The number of *VASA* and *GAPDH* transcripts in each sample was calculated with the LightCycler software, using these standard curves.

Sample collection

Several normal human tissue samples were obtained by organ donation from autopsy cases at the Pathology Division of the Nihon University School of Medicine in Tokyo, Japan. This study was approved by the Ethics Committees of both the Kanazawa University Graduate School of Medical Science, and the Nihon University School of Medicine. Informed consent was obtained from all study participants. In cases where tissues were received through organ donation, bereaved families or relatives provided informed, written consent.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences statistical software version 11.0 (SPSS, Chicago, IL, USA). Mann-Whitney's U test was used

to compare the average methylation rate between patients within the MA and control groups. Welch's t-test was used to compare *VASA* mRNA expression between two groups of maturation arrest. Studen's t-test was used to examine the correlation between levels of hormones. Differences were considered as statistically significant at $P < 0.05$.

Results

Patients' characterization by histological evaluation

Upon histological assessment, spermatogenesis was classified as normal in 39 patients. In the group with normal spermatogenesis, 26 patients had obstructive azoospermia, 6 patients had retrograde ejaculation, and 7 patients presented with unknown causes. We defined these patients as the control group. An additional 25 patients were diagnosed with hypospermatogenesis. These hypospermatogenetic patients were excluded from this study, because testicular biopsy revealed the presence of late spermatogenesis and a few spermatozoa.

Histology also revealed maturation arrest (MA) in testicular biopsy results from 17 patients, who were defined as the MA group in this study. Although all MA patients lacked late spermatogenesis when examined histologically, examination of ejaculate revealed that some had a few spermatozoa in the ejaculate (MA4, 8, 27), as shown in Tables 1 and 2. The mean ages of the control and MA groups (±SD), were 36.3±7.1 and 35.5±5.9 years, respectively. Additionally, the remaining 50 patients had either a Sertoli cell only phenotype or tubular sclerosis by histologic analysis and were also excluded in this study because of absence of germ cells.

VASA gene expression

VASA expression was detected *via* RT-PCR in human testicular tissues. No *VASA* expression was detected in the other five tissue types (Figure 2). Thus, the *VASA* gene was specifically expressed in testis tissue.

Confirmation of TDMR in normal tissues

After bisulphate treatment PCR, these amplicons were screened using MassARRAY® in order to evaluate the methylation status of *VASA* promoter CpG islands in different normal tissues.

We screened normal tissues, including testis; the graphic data illustrated the methylation status within each amplicon (Figure 3). *VASA* promoter CpG islands were specifically hypomethylated in the testis, whereas five other tissue types were highly methylated.

The evaluation of *VASA* TDMR methylation state in the testicular genome

We analyzed *VASA* gene TDMRs (that is, *VASA* promoter CpG islands) using MassARRAY®. All samples were evaluated using testicular genome DNA. All controls (n=39) are hypomethylated in the TDMR of the *VASA* gene; the average rate is 16.3 ±4.83% (Figure 4). In six patients from the MA group (n=17), the methylation rate of each CpG unit within an amplicon is especially high; the average rate is 77.4 ±3.07%. The methylation rate was low in the remaining eleven patients, with an average of 29.3 ±2.68% (Figure 5). The methylation rate of the former was significantly higher than that of the latter ($p<0.0001$). It has been suggested that some maturation arrest might account for the methylated TDMRs in this study.

In 25 patients from the hypospermatogenetic group, the variability of the methylation rate is great, the average rate is 45±21.4 %. All of the remaining 50 samples of *VASA* TDMRs are methylated; the average methylation rate of patients with Sertoli cell only and tubular sclerosis was 88.0 ±8.7%.

Expression study of methylated and unmethylated TDMRs in the MA group

To examine the expression of the *VASA* gene, *VASA* mRNA was measured by real-time quantitative RT-PCR. The average methylation rate and *VASA* gene expression level are depicted as a scatter plot in Figure 6a. The expression level of *VASA* in the methylated group (mean±SEM: 0.00132±0.00033) was significantly lower than in the remaining eleven patients (0.01391±0.00428, $P = 0.0150$), Figure 6b. These results demonstrate that highly methylated TDMRs result in low levels of gene expression. Thus, the alteration of methylation within the *VASA* promoter may greatly affect expression, resulting in meiotic failure.

Clinical characteristics of patients with methylated and unmethylated TDMRs

The general characteristics and clinical parameters of patients with the MA phenotype are summarized in Table 1. Methylation state is classified into two groups: high methylation (HM) and low methylation (LM) shown in Fig. 6a,b. In the HM group (n=6), all patients but one (MA4) had azoospermia in the ejaculate. In the LM group (n=11), all patients except two (MA8, MA15) had azoospermia in the ejaculate. Levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) in the HM group were 23.4±5.5, 7.1±2.8, and 4.6±0.7, respectively. In contrast, Levels of FSH, LH, and T in the LH group were 6.1±2.1, 3.3±0.9, and 5.2±0.4, respectively. The FSH level in the HM group was significantly higher compared to that of the LM group (p=0.037). There was no significant difference in levels of LH (p=0.25) or T (p=0.52) between the HM and LM groups. This finding leads to the suggestion that high methylation rates in the *VASA* TDMR may be more harmful to spermatogenesis than low methylation rates.

Discussion

In this study, we identified patients with MA had a relative high methylation status, a reduction of *VASA* expression due to its promoter CpG island methylation is potentially responsible for the maturation arrest phenotype.

It is well known that human *VASA* expression is germ cell- specific. To evaluate methylation status of patients' DNA, we should collect pure population of germ cell, however, this is extremely difficult because of heterogeneity appearance for clinical samples. Therefore, we applied MALDI-TOF MS system, which was evaluated the rate of methylation levels in total cell population mixture, not in individual cloned DNA methylation.

In this study, we confirmed moderate spermatocyte count in all 17 samples by histological diagnosis. To characterized the effect of methylation rate and expression on *VASA* in MA patiets, a total of 17 testicular biopsy samples were examined, six patients had a relative high and also indicaed a significantly lower VASA expression compared to the remaining eleven MA patient samples. Needless to say, the genomic DNA extracted from these testicular biopsy may not represent the methylation level in only germ cells. These testicular biopsy is contained many of somatic cells such as Leydig cells and Sertoli cells and so on. In extreme case of Sertoli only or tubular screlosis in the remaining 50 patients, *VASA* TDMR methylation rate indicated high as expected. These observations are consistent with the results obtained by Kitamura et al.[11]. Therefore, abrerrant methylation of *VASA* led to the molecular basis of meiotic arrest as one possible cause of idiopathic infertility.

In animal experiments, Tanaka et al. generated a mutation in the mouse *Vasa homolog* gene (*Mvh*) and demonstrated that male knockout mice for *Mvh* produced no spermatozoa in the testes. Histological findings revealed that spermatogenesis was arrested in *Mvh*-deficient mice early in meiosis [14]. Interestingly, these histological

findings were consistent with those observed in our six patients with histologically confirmed MA. Despite an apparently high level of *VASA* TDMR methylation, very few spermatozoa were present one patient in the ejacurate (MA4). Because *Mvh*-deficient mice are genetically homogenous, the histology of testis may have a homogenous appearance. However, histology in most of testicular biopsies clinically is heterogenous[28].

In general, maturation arrest is clinically characterized by an absence of late spermatogenesis, as detected upon testicular biopsy. Testicular biopsy appears not to reflect the function of whole testicular areas [29], because the production of spermatozoa occurs in a multifocal (or "patchy") distribution [30,31]. Patients with maturation arrest have no or very few spermatozoa in their ejaculate. This may also imply that heterogeneity in testicular histology can cause heterogeneity in DNA methylation status of the *VASA* gene.

In males, meiosis occurs by two successive divisions. In the first meiosis, primary spermatocytes develop into secondary spermatocytes and the second meiotic division yields round spermatids. Genetic regulation of meiosis during spermatogenesis remains unclear, although some meiotic failure is thought to result from the meiotic recombination frequencies [32] or the methylation state of genes' promoter regions [1,2]. On the one hand, ejaculate spermatozoa contain a complex of mRNAs and can now be assessed in a non-invasive examination of testis-specific infertility [31]. It has been reported that the expression of *VASA* mRNA is significantly decreased in the ejaculate sperm of oligozoospermic patients[33]. However, the cause of the absence of *VASA* mRNA expression was not investigated in these reports. The results from this study demonstrate that aberrant methylation of TDMRs may be the underlying cause of meiotic arrest, leading to infertility. This study also may imply heterogeneous

testicular expression of *VASA* gene. These data raise the possibility that TDMR methylation in human *VASA* may contribute to some forms of idiopathic male infertility.

In conclusion, we report the novel finding that *VASA* TDMR methylation is significantly higher among maturation arrest patients, in whom *VASA* gene expression was silenced. This finding represents an important contribution to the molecular basis of meiotic arrest as one possible cause of idiopathic infertility.

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Table 1　Clinical characteristics of MA patients with high methylation

	Age(yr)	Semen (Sperm counts)	FSH(mlU/mL)	LH(mlU/mL)	Testosterone(ng/mL)
MA1	31	0	13.8	1.9	3.6
MA2	26	0	36.4	9.1	7.1
MA3	44	0	25.7	7.3	2.9
MA4	33	a few*	8.5	1.6	5.8
MA5	34	0	12.4	2.8	5.8
MA6	35	0	43.33	19.83	2.78

MA; maturatin arrest *; a few spermatozoa in the ejaculate after centrifugati

Table 2　Clinical characteristics of MA patients with low methylation

	Age(yr)	Semen (Sperm counts)	FSH(mlU/mL)	LH(mlU/mL)	Testosterone(ng/mL)
MA7	35	0	3.63	1.06	4.99
MA8	39	a few*	6.17	4.4	4.02
MA9	37	0	2.99	2.78	5.77
MA10	32	0	5.69	4.22	6.51
MA11	32	0	2.14	1.15	4.09
MA12	27	0	23.7	11.0	4.3
MA13	42	0	7.66	2.06	4.6
MA14	48	0	3.34	1.85	5.93
MA15	27	a few*	N/A	N/A	N/A
MA16	31	0	2.8	1	4.11
MA17	41	0	3.4	3.29	8.15

*; a few spermatozoa in the ejaculate after centrifugati

MA; maturatin arrest N/A;not available

Figure Legends

Fig. 1 Chromosomal location of *VASA*

This map is based on the March 2006 human reference sequence (NCBI Build 36.1). *VASA* is shown as an arrow, with an arrowhead indicating the orientation of the gene. CpG islands are shown with the number of CpGs. The amplicon is designed from -1127 to -760 (368bp) from the transcription start site (TSS).

Figure 2. Tissue-specific expression of *VASA* in normal human tissues.

cDNAs were prepared from testis, kidney, muscle, heart, brain, and pancreas. *GAPDH* was used as a positive control.

Figure 3. Epigram of the *VASA* TDMR methylation state in normal human tissues. The upper ruler indicates the location of amplicon (368 bp), and the lower scale indicates the CpG site. Circles mark the positions of CpG sites, and dashed lines annotate CpG sites that could not be analyzed. The color scale in each top right corner indicates the methylation level. Red circles indicate that the TDMR is unmethylated. Gradation of circles from red to yellow indicates rates of methylation from 0% to 100%. The epigram is shown for six normal human tissues (testis, kidney, muscle, heart, brain, and colon). TDMRs, tissue-specific differentially methylated regions.

Figure 4. Epigram of the *VASA* TDMR methylation state in control patients (n=39). The upper ruler indicates the location of amplicon (368 bp) and the lower scale indicates the CpG site. Circles mark the positions of CpG sites, and dashed lines annotate CpG sites that could not be analyzed. The color scale in each top right corner

indicates the methylation level. Red circles indicate that the TDMR is unmethylated. Gradation of circles from red to yellow indicates rates of methylation from 0% to 100%.

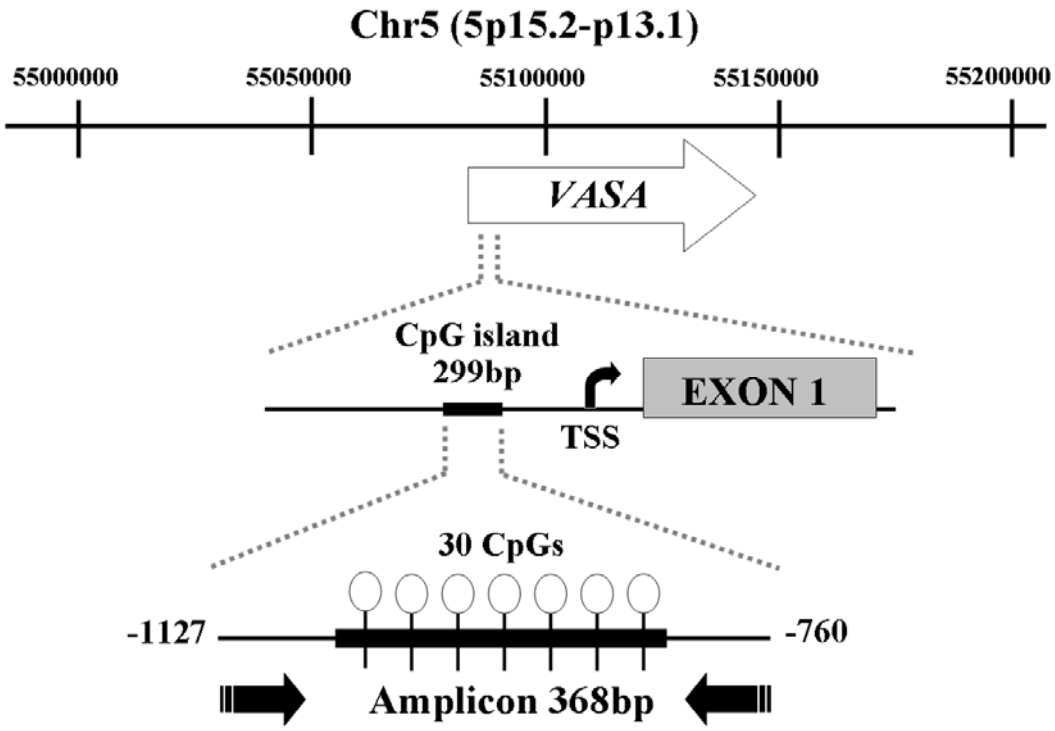
Figure 5. Epigram of methylation status in TDMR of *VASA* in 17 patients with maturation arrest.

The upper ruler indicates the location of amplicon (368 bp) and the lower scale indicates the CpG site. Circles mark the positions of CpG sites, and dashed lines annotate CpG sites that could not be analyzed. The color scale in each top right corner indicates the methylation level. Red circles indicate that the TDMR is unmethylated. Gradation of circles from red to yellow indicates rates of methylation from 0% to 100%.

Figure 6. Classfication between high and low methylated TDMRs group in patients with maturation arrest.

a. Scatter plot of the methylation rate and relative expression of *VASA* in patients with maturation arrest. closed circles; low methylation of TDMR (LM) group, closed triangles; high methylation of TDMR (HM) group b. The relative expression level of *VASA* between HM (n= 6) and LM group (n=11) in patients with maturation arrest.

Figure 1


Chr5 (5p15.2-p13.1)
55000000
55050000
55100000
55150000
55200000
VASA
CpG island
299bp
EXON 1
TSS
30 CpGs
-1127
-760
Amplicon 368bp

Figurure 2

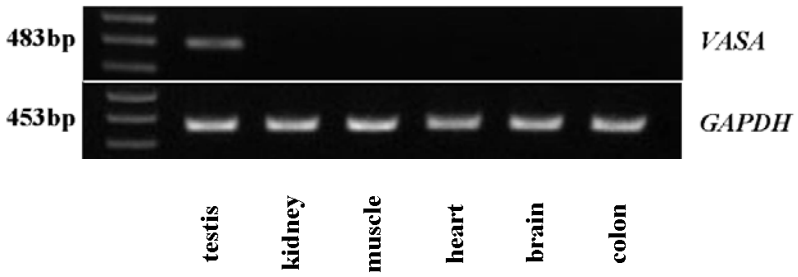

483bp
453bp
VASA
GAPDH
testis
kidney
muscle
heart
brain
colon

Figure 3

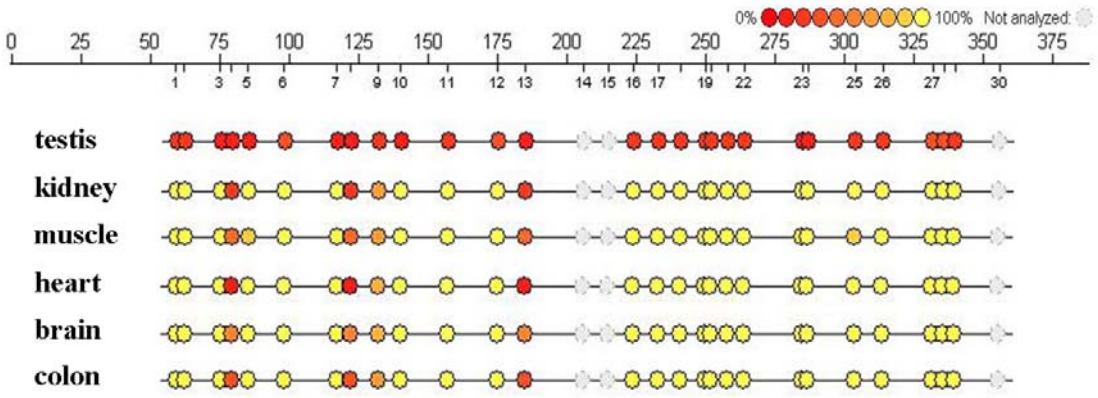

0%
100%
Not analyzed
0
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
1
3
5
6
7
9
10
11
12
13
14
15
16
17
19
22
23
25
26
27
30
testis
kidney
muscle
heart
brain
colon

Figure 4

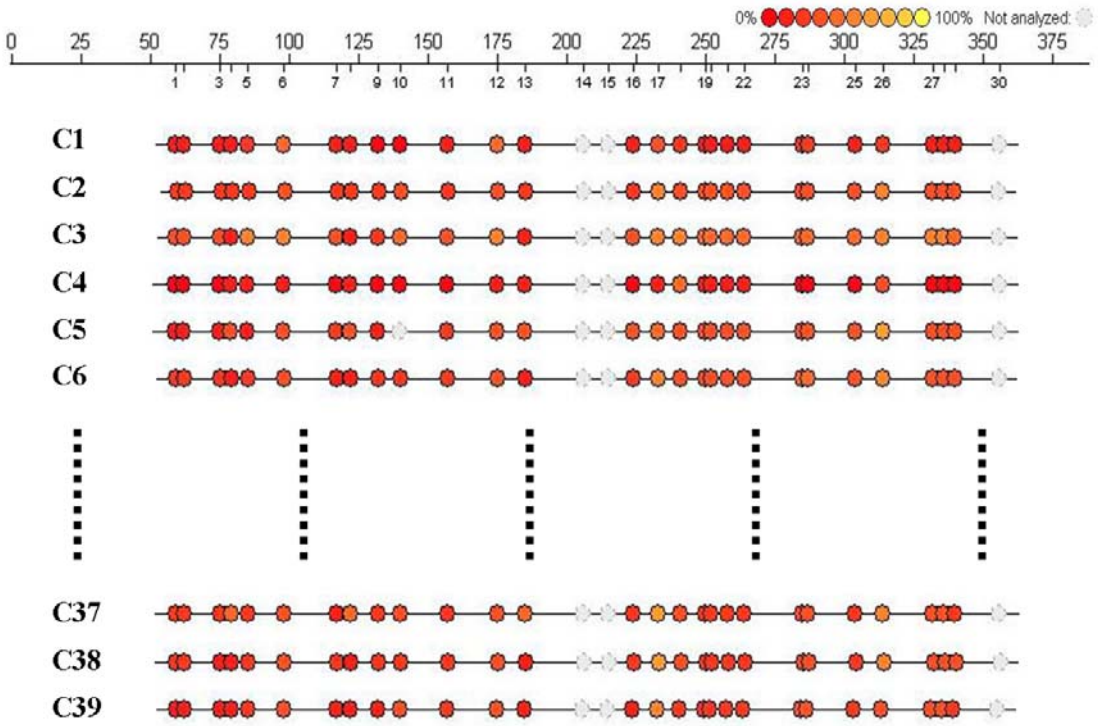

0%
100%
Not analyzed
0
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
1
3
5
6
7
9
10
11
12
13
14
15
16
17
19
22
23
25
26
27
30
C1
C2
C3
C4
C5
C6
C37
C38
C39

Figure 5

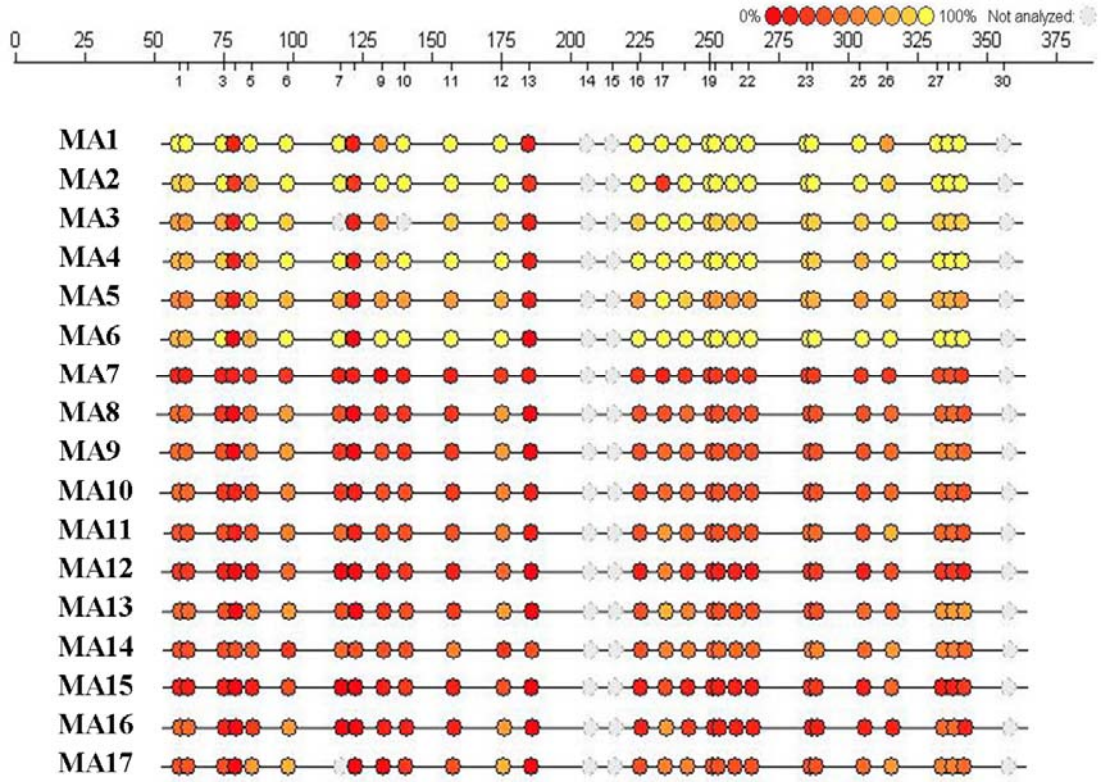
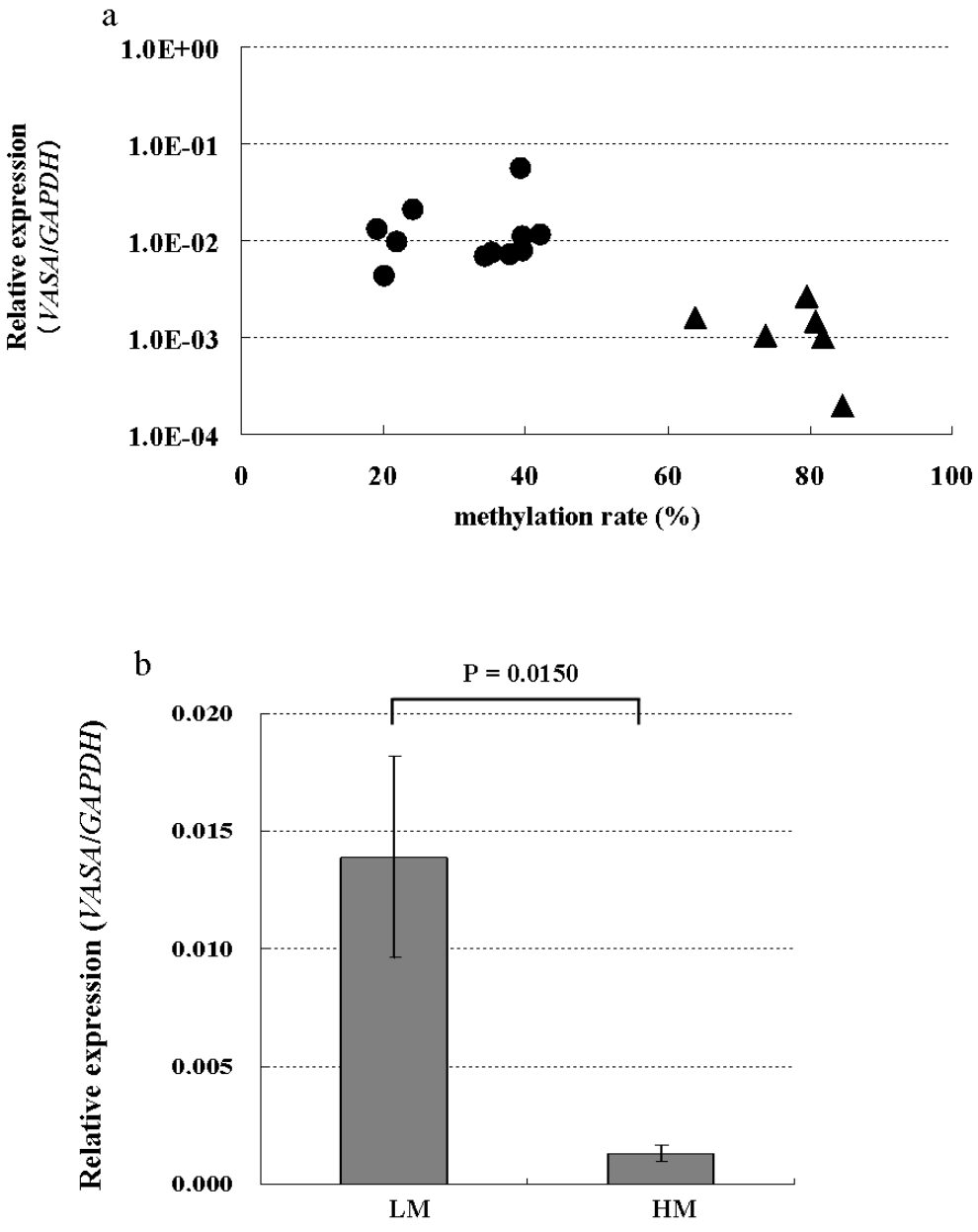

0%
100%
Not analyzed
0 25 50 75 100 125 150 175 200 225 250 275 300 325 350 375
1 3 5 6 7 9 10 11 12 13 14 15 16 17 19 22 23 25 26 27 30
MA1
MA2
MA3
MA4
MA5
MA6
MA7
MA8
MA9
MA10
MA11
MA12
MA13
MA14
MA15
MA16
MA17

Figure 6


a
1.0E+00
1.0E-01
1.0E-02
1.0E-03
1.0E-04
Relative expression
(VASA/GAPDH)
0
20
40
60
80
100
methylation rate (%)
b
P = 0.0150
0.020
0.015
0.010
0.005
0.000
Relative expression (VASA/GAPDH)
LM
HM