胃癌は世界で癌死亡の3位であり、東アジアに多く、年間約70万人が亡くなっている。特に切除不能進行胃癌の予後は不良であり、有効な化学療法の開発が望まれている。しかしながら、胃癌は組織型や病因がヘテロであるため、胃癌の分子生物学的特徴に合わせたオーダーメイド治療は確立されていない。

Epstein-Barr virus (EBV) は、ほとんどの成人が保有しているヘルペスウイルス科の二本鎖DNAウイルスである。1960年代の発見当初より、EBVはBurkittリンパ腫や咽頭頸癌に強い関連を有するヒト腫瘍ウイルスと考えられてきた。1990年に初めて胃癌にEBVが発見され、世界各地から約10%の胃癌にEBVが陽性であることが報告されてきた。また、EBV陽性胃癌細胞において、全ての癌細胞にEBVが感染しており、検出されるEBVは全て単クローン性であることから、癌の初期段階でEBVが関与することが示唆されている。EBVの潜伏感染様式は、潜伏感染遺伝子の発現から2つのタイプに分けられる。EBV関連胃癌はBurkittリンパ腫と同じLatency Iに属し、EBNA1, EBERs, BARFO, LMP2Aなどの遺伝子群を包括した潜伏感染遺伝子と発現している。このウイルス遺伝子の発現調節にはウイルスDNAのメチル化が関与しており、Latency Iでは全てのEBNAを発現するが、Cp/Cpプロモーターがメチル化され不活化されている。近年、DNAのメチル化異常が胃癌の発生に関与していることが報告されているが、特にEBV関連胃癌においてはDNAメチル化の頻度が高頻度であることが報告されてきている。DNAメチル化の観点からEBV関連胃癌における分子生物学的特徴を解析することで、胃癌のオーダーメイド治療への扉が開かれるのではないかと考えられる。

今回我々は、EBV陽性胃癌細胞株およびEBV関連胃癌の臨床検体を用いてEBV関連胃癌に特異的なDNAメチル化遺伝子について検討した。

EBV陽性胃癌細胞株SU719を用いたMIRA-chip assayにてメチル化を認めた遺伝子のうち、腫瘍に関連する遺伝子は69遺伝子であった。そのうち、メチル化が特異的PCR（Methylation specific PCR: MSP）のプライマーが使用可能な遺伝子を対象とし、SU719のMSPを施行したところメチル化が確認されたのは22遺伝子であった。今回我々は、その22遺伝子のうちで臨床検体でのMSPが可能であったTP73, BLU, FSD1, BCL7A, MARK1, SCRN1, NKX3.1の7遺伝子を対象とした。我々はBisulfite処理を行ったSU719のDNAを用いたMSPの結果から、これらの遺伝子のプロモーター領域がメチル化していることを確認した。また、Bisulfite sequenceの結果から、これらの遺伝子のプロモーター領域の5-Aza-2'-deoxycytidine (DAC) とヒストン脱アセチル化酵素阻害剤（Trichostatin A: TSA）の2剤で処理すると、BCL7Aを除く6遺伝子はその発現が上昇することがQuantitative Real-time RT-PCRの結果から認められた。DACとTSAでSU719を処理することによって7遺伝子のプロモーター領域が脱メチル化することがMSPの結果から確認された。我々はEBV関連胃癌がDNAのメチル化を検討した。対象とした7遺伝子の臨床検体でEBV関連胃癌症例は早期癌から進行癌までの症例を含み、EBV関連胃癌1症例につき、年齢、性格、病理、微細度、組織型を厳密に一致させた2症例ずつのEBV陽性胃癌症例をControlとして設定した。EBVの有無はEBER1に対するin situ hybridization法を用いて同定した。その結果EBV関連胃癌においてはEBV陽性胃癌と比較して7遺伝子においてメチル化の頻度が有意に高かった。

以上の結果から、今回同定された7遺伝子はEBV関連胃癌において特異的にメチル化されていることが示された。今回対象とした7遺伝子の機能は全て明確になっているわけではないが、TP73とBLU, NKX3.1は癌抑制遺伝子としての機能が知られており、SCRN1は癌関連抗原としての機能が知られている。これらの遺伝子はメチル化による転写抑制によって機能が抑制されるため、EBV関連胃癌の発生や進展に関与しているかもしれない。また、今回の検討ではDACやTSAといったEpigenetic修飾薬剤によって対象遺伝子のプロモーター領域のメチル化が脱メチル化し、遺伝子の発現が回復した。このことは、EBV関連胃癌に対してEpigenetic修飾薬剤が抗腫瘍効果を有する根拠となりうると考えられる。
Identification of genes specifically methylated in Epstein–Barr virus-associated gastric carcinomas

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(Received January 14, 2013; Revised June 11, 2013; Accepted June 23, 2013; Accepted manuscript online July 6, 2013)

We studied the comprehensive DNA methylation status in the naturally derived gastric adenocarcinoma cell line SNU-719, which was infected with the Epstein–Barr virus (EBV) by methylated CpG island recovery on chip assay. To identify genes specifically methylated in EBV-associated gastric carcinomas (EBVaGC), we focused on seven genes, TP73, BLU, FSD1, BCL7A, MARK1, SCRN1 and NKKX3.1, based on the results of methylated CpG island recovery on chip assay. We confirmed DNA methylation of the genes by methylation-specific PCR and bisulfite sequencing in SNU-719. The expression of the genes, except for BCL7A, was upregulated by a combination of 5-Aza-2′-deoxycytidine and trichostatin A treatment in SNU-719. After the treatment, unmethylated DNA became detectable in all seven genes by methylation-specific PCR. We identified DNA methylation of the genes in 75 primary gastric cancer tissues from 25 patients with EBVaGC and 50 EBV-negative patients who were controls. The methylation frequencies of TP73, BLU, FSD1, BCL7A, MARK1, SCRN1, and NKKX3.1 were significantly higher in EBVaGC than in EBV-negative gastric carcinoma. We identified seven genes with pro-oncogenic function that were specifically methylated in EBVaGC. Inactivation of these genes may suppress their function as tumor suppressor genes or tumor-associated antigens and help to develop and maintain EBVaGC. (Cancer Sci, doi: 10.1111/cas.12228, 2013)

The Epstein–Barr virus is associated with a variety of tumors derived from B cells, such as Burkitt lymphoma,1 post-transplant lymphoproliferative disease,2 and Hodgkin’s disease3, T cells such as peripheral T-cell lymphomas;4 epithelial cells, including nasopharyngeal carcinoma and gastric carcinoma1,5; and natural killer cells, such as nasal natural killer/T-cell lymphomas.5 Burke et al. first identified EBV in gastric carcinomas by PCR in 1990,4 and since then, about 10% of gastric carcinomas have been identified as resulting from monoclonal proliferation of EBV-infected cells.5,6 In each EBV-positive case of gastric carcinoma, almost all carcinoma cells are infected with the virus,7–9 which suggests that EBV plays an important role in the development of gastric carcinoma.

In the EBV genome, DNA methylation has been studied intensively.10 The expression of EBV latent genes is regulated under strict epigenetic control through DNA methylation in host cells. It is well known that CpG/Wp EBNA promoters, which can transcribe all EBNA, are methylated in Burkitt lymphoma and nasopharyngeal carcinoma, whereas the Qp promoter, which induces only EBNA1 expression, is used in these tumors.11,12 Additionally, LMP1 expression is regulated by methylation in its promoter region in EBV-positive nasopharyngeal carcinoma.13 In EBV-positive gastric carcinomas, there are latency patterns similar to that of Burkitt lymphoma, in which only Qp is active.13 Thus, the latency type of EBV-positive malignancies is regulated by methylation status of the EBV genome.

The importance of the CpG Island methylator phenotype (CIMP), which is characterized by simultaneous methylation of CpG islands in multiple genes, has also been examined in gastrointestinal carcinogenesis.14 Kusano et al. showed a strong association between EBV-associated tumors and CIMP-High (CIMP-H), hypermethylation of tumor-related genes, and a lack of p53 or K-ras mutations.15 Chang et al. also revealed that EBVaGC showed global CpG island methylation and that they comprised a pathogenetically distinct subgroup of CIMP-H gastric carcinomas.16 Thus, hypermethylation of tumor-related genes might lead to the progression of EBVaGC, whereas methylation of viral DNA determines EBV latency type.

We studied the comprehensive DNA methylation status in the naturally derived gastric adenocarcinoma cell line SNU-719, which is infected with EBV,17 by MIRA-chip assay. We succeeded in identifying several genes whose expression was regulated by DNA methylation in EBVaGC.

Materials and Methods

Cell cultures and drug treatment. The human gastric cancer cell lines SNU-719 (EBV-positive gastric cancer cell line), which was obtained from the Korean Cell Line Bank (Seoul, South Korea), and KATO-III (EBV-negative gastric cancer cell line) were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified 5% CO2 incubator.17

The SNU-719 cells were split 24 h before treatment and were then given one of the following treatments: (i) DAC (5 mM) (Sigma-Aldrich) or PBS for 72 h, with the medium changed every 24 h; (ii) TPA (300 nM) or ethanol (300 nM) for 24 h; or (iii) DAC (5 mM) for 72 h, with TPA (300 nM) for the last 24 h. The medium containing DAC was changed every 24 h. The dose of DAC (5 mM) was chosen based on preliminary studies showing optimal reactivation of gene expression. The timing and sequencing of DAC and/or TPA was based on similar preliminary studies as well as previously published studies.18

DNA and RNA extractions. Genomic DNA and total RNA from SNU-719 was isolated with an All Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. As described previously,19 DNA from paraffin-embedded samples was prepared. Briefly, we prepared 5-μm thick tissue sections from archival, formalin-fixed, paraffin-embedded tissue blocks. After the tissue sections were stained with H&E, the adenocarcinoma regions were microdissected

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with a 27-gauge needle. Tumor DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer’s protocol.

**MIRA-chip assay of SNU-719.** The methylated DNA was enriched with a MethylCollector Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s protocol. Fragments of CpG-methylated DNA prepared by MspI digestion specifically bind a His-tagged recombinant methyl-CpG-binding domain protein-2b. These protein–DNA complexes are captured with nickel-coated magnetic beads, and subsequent wash steps are performed with a stringent high-salt buffer to remove fragments with little or no methylation. The methylated DNA is then eluted from the beads in the presence of Proteinase K. Comparative genomic hybridization array slides (MacArray Karyo 4000; Macrogen Inc., Seoul, South Korea) were used in this study. The array was spotted with 4030 human bacterial artificial chromosome clones that covered the whole human genome at an average interval of 0.83 Mb. The experiments were performed according to the manufacturer’s protocol. Briefly, arrays were prehybridized with salmon sperm DNA to block repetitive sequences in the bacterial artificial chromosomes. Then, 500-ng input DNA (reference DNA) and cancer cell line immunoprecipitated DNA (test DNA) were labeled with Cy5-dCTP and Cy3-dCTP, respectively, by randomly primed labeling. The labeled probe and human Cot-I DNA were mixed and dissolved in hybridization solution. The probe mixture was denatured, cooled, and mounted on the array. Hybridizations were performed in a sealed chamber for 72 h at 37°C. After hybridization, array slides were washed and dried. Scanning was carried out using a GenePix 4000A two-color fluorescent scanner (Axon Instruments, Union City, CA, USA), and quantification was performed using MAC viewer software (Macrogen Inc.).

**Sodium bisulfite modification of DNA.** We performed bisulfite treatment as reported previously. In 50-μL water, 2-μg genomic DNA was denatured with 5.5-μL 2 M NaOH at 37°C for 10 min, which was followed by incubation with 30-μL 10 mM hydroquinone and 520-μL 3 M sodium bisulfite (pH 5.0) at 50°C for 16 h in darkness. Then, DNA was purified with 50-μL water and a DNA Cleanup Kit (Promega, Madison, WI, USA), which was used as recommended by the manufacturer. The DNA was incubated with 5.5-μL 3 M NaOH at room temperature for 5 min; precipitated with 1-μL 20 mg/mL glycogen, 33-μL 10 M ammonium acetate, and 260-μL 100% ethanol; washed with 70% ethanol; and finally resuspended in distilled water.

**Methylation-specific PCR.** Primer sequences for amplification of the TP73, BLU, FSDI, BC17A, MARK1, SCHR1, NXX3.1 genes are listed in Table S1. The methylation status of these seven genes was determined by bisulfite treatment of DNA followed by MSP. In brief, 2-μl bisulfite-treated DNA in 10-μL PCR solution was used as the template for PCR reactions with primers specific for methylated and unmethylated alleles. The DNA used as positive controls for methylated and unmethylated alleles were SsIl methyltransferase-treated plasmid DNA (New England Biolabs, Beverly, MA, USA) and lymphocyte DNA, respectively. Next, PCR products from methylated and unmethylated reactions were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining. Each MSP was repeated at least twice. The prospectively established criterion for presence of hypermethylation was detection of a methylated band in all independent assays.

**Bisulfite sequencing.** We performed bisulfite sequencing. The primers are listed in Table S2. The PCR amplification was performed for a total of 40 cycles. The annealing temperature of each gene is shown in Table S2. The PCR products were gel purified from agarose gel using Wizard SV Gel (Promega) and PCR Clean-Up Kit (Promega), and cloned into the TA cloning vector pGEM-T Easy (Promega). At least six subclones were isolated and identified by direct sequencing.

**Quantitative real-time PCR.** The extracted total RNA was reverse-transcribed into single-stranded cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed using the cDNA with Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences of TP73, BLU, FSDI, BC17A, MARK1, SCHR1, NXX3.1, and β-actin are listed in Table S3. Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantitative PCR parameters for cycling were as follows: 95°C for 10 min followed by 40 cycles of PCR at 95°C for 15 s and 60°C for 1 min. All reactions were done in triplicate in a 20-μL reaction volume. The mRNA expression level was determined using the 2^{-ΔΔCt} method.

**Clinical materials.** We evaluated 75 primary gastric cancer tissues from 25 patients with EBVaGC and 50 patients with EBV-negative gastric carcinoma who underwent surgical resection between 1995 and 2007 at Yamaguchi University Hospital (Ube, Japan). All patients provided informed consent, and the research project was approved by the university and institutional review boards. The samples were formalin-fixed, paraffin-embedded tissues of gastric cancer. The EBVaGC were positive for EBER1 in situ hybridization. Age-, sex-, histological type-, and stage-matched patients with EBV-negative gastric carcinoma were selected. The clinicopathological characteristics of the patients are summarized in Table 1.

**In situ hybridization of EBER1.** With a digoxigenin-labeled 30-base oligomer using previously described procedures, EBER1 was detected. Paraffin-embedded sections of 5-μm thickness were deparaffinized, rehydrated, predigested with pronase, prehybridized, and then hybridized overnight at 37°C. After the sections were washed with 0.5 × SSC, hybridization was detected using an anti-digoxigenin antibody alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

**Statistical analysis.** The Mann–Whitney U-test was used to compare variables. Differences in methylation frequencies

| Table 1. Clinicopathological characteristics of EBVaGC and controls |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | EBVaGC (n = 50) | Controls (n = 50) |
| Sex              |                 |                 |
| Men              | 22              | 44              |
| Women            | 3               | 6               |
| Age              | 64.6 ± 12.1     | 67.7 ± 11.1     |
| Histological type|                 |                 |
| Differentiated   | 10              | 20              |
| Undifferentiated | 15              | 30              |
| Lymph node metastasis |         |                 |
| n0               | 15              | 29              |
| n1               | 6               | 13              |
| n2               | 4               | 7               |
| n3               | 0               | 1               |
| Macroscopic type |                 |                 |
| Elevated         | 19              | 43              |
| Depressed        | 6               | 7               |
| Stage            |                 |                 |
| I                | 14              | 28              |
| II               | 5               | 10              |
| III              | 4               | 8               |
| IV               | 2               | 4               |

EBVaGC, Epstein–Barr virus-associated gastric cancer.

doi: 10.1111/cac.12228
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were evaluated with the Fisher's exact test. A *P*-value of <0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and Instat3 statistical software (GraphPad Software).

**Results**

**MIRA-chip analysis of SNU-719.** Analysis of SNU-719 by MIRA-chip showed that 1071 spots were determined to be hypermethylated, with a log ratio of >0.25. Of genes on the spots, 69 genes were known to be methylated in cancer, and 29 genes were chosen for further examination. The methylation status of the 29 genes was confirmed by MSP in SNU-719. Similar results were observed by MIRA-chip analysis and MSP in 22 of 29 genes (78.3%) (Fig. 1a). To identify genes specifically methylated in EBVaGC, we focused on seven genes, TP73, BLU, FSD1, BCL7A, MARK1, SSCR1, and NKKX3.1, because their MSP primers were available in primary gastric cancer tissues. Methylation DNA was not detectable in BCL7A, NKKX3.1, and BLU in the KATO-III EBV-negative gastric cancer cell line (data not shown). However, BCL7A and FSD1 were hypermethylated in SNU-719 by bisulfite sequencing (Fig. 1b), and the results of MSP were validated by bisulfite sequencing. The methylation status of TP73, BLU, FSD1, BCL7A, MARK1, and NKKX3.1 in SNU-719 according to bisulfite sequencing is shown in Fig. S1.

**mRNA expression of SNU-719 by treatment with DAC and/or TSA.** SNU-719 was treated with DAC and/or TSA, and change of mRNA expression of the seven genes was evaluated by quantitative RT-PCR (Fig. 2). Treatment with DAC restored TP73 expression, and the expression of other genes, except for BCL7A, was upregulated by a combination of DAC and TSA treatment. The expression levels of BCL7A in mock sample were higher than those in DAC and/or TSA-treated samples.

**MSP analysis of SNU-719 treated with DAC and/or TSA.** Analysis was performed by MSP on SNU-719 cells treated with DAC and/or TSA. After treatment with the combination of DAC and TSA, unmethylated DNA became detectable in all seven genes. The DAC was adequate to demethylate and express the TP73 gene. In other genes except for BCL7A, treatment with a combination of DAC and TSA had the greatest effect on demethylating the promoter regions (Fig. 3).

**Histopathology and MSP in clinical samples.** A representative EBVaGC lesion is shown in Figure 4. The ulcerated carcinoma was located on the posterior wall of the upper stomach (Fig. 4a). The tumor histological type was that of a moderately differentiated adenocarcinoma, and EBER1 signals were detected in almost all cancer cells by *in situ* hybridization, suggesting EBVaGC (Fig. 4b,c).

Methylation frequencies of TP73, BLU, FSD1, BCL7A, MARK1, SSCR1, and NKKX3.1 were significantly higher in EBVaGC than in EBV-negative gastric carcinoma (Table 2). A representative MSP image is shown in Figure 5.

**Discussion**

It has been reported that hypermethylation of tumor-related genes may be involved in the development of EBVaGC. We found that six genes, including TP73, BLU, FSD1,
Fig. 2. Expression analysis of the EBV-associated cancer-specific hypermethylated genes found by the MIRA-chip approach. The effects of treatment with the DNA methyltransferase inhibitor DAC and/or the histone deacetylase inhibitor TSA on TP73, BLU, FSD1, BCL7A, MARK1, SCR1, and NKK3.1 expression as well as reactivation evaluated by real-time PCR are shown. The expression level of DAC-treated sample was standardized to 1 in each targeted gene. Error bars represent standard deviation. DAC, 5-Aza-2'-deoxycytidine; EBV, Epstein-Barr virus; MIRA-chip, methylated CpG island recovery on chip; Mock, treatment with PBS; TSA, trichostatin A.

Fig. 3. Effects of treatment with the DNA methyltransferase inhibitor DAC and/or the histone deacetylase inhibitor TSA on TP73, BLU, FSD1, BCL7A, MARK1, SCR1, and NKK3.1. Methylation evaluated by methylation-specific PCR. The presence of a PCR band indicates methylated (lane M) or unmethylated (lane U) genes. DNA from normal lymphocytes (NL) and in vitro methylated DNA (IVD) were used as negative and positive controls, respectively, for methylated DNA. DAC, 5-Aza-2'-deoxycytidine; TSA, trichostatin A.

MARK1, SCR1, and NKK3.1 were hypermethylated more frequently in EBVaGC than in EBV-negative gastric carcinoma, and their expression was restored by DAC and TSA treatment in the SNU-719 EBVaGC cell line. Additionally, TP73 has been identified as a transcription factor with structural and functional homology to a tumor suppressor. Ushiku et al. found that loss of TP73 expression through aberrant methylation of the TP73 promoter occurred specifically in EBVaGC, together with the methylation of p14 and p16. Transcriptional inactivation of the TP73 gene by promoter methylation has been reported in other EBV-associated lymphoid malignancies such as natural killer cell lymphoma and Burkitt lymphoma. Therefore, TP73 methylation might be an EBV-specific mechanism that leads to the development of malignancies.

The DNA methylation of FSD1, MARK1, and SCR1 in gastric carcinoma cell lines and the upregulation of the genes by 5-Aza-2'-deoxycytidine treatment have been reported by Yamashita et al. Because the function of these genes in carcinogenesis is not fully understood, Suda et al. reported that SCR1 might be a novel immunotheraphy target. Thus, EBVaGC cells might evade immune reaction against SCR1 by downregulation of SCR1 expression by DNA methylation. In the present study, BLU and NKK3.1 were methylated specifically in EBVaGC as well. To date, DNA methylation of BLU and NKK3.1 has not been reported in gastric carcinomas, whereas BLU methylation in neuroblastoma and nasopharyngeal carcinoma and NKK3.1 methylation in prostatic cancer have been reported previously. Because the genes act as tumor suppressor genes in vitro, silencing of these genes by epigenetic mechanisms can play an important role in the development of EBVaGC.

We found that methylation of BCL7A was specific to EBVaGC and could be a useful marker to differentiate EBVaGC from EBV-negative gastric carcinoma. To our knowledge, this is the first report of this finding. Additionally, BCL7A promoter methylation has been reported in T-cell lymphoma. Because SNU-719 showed BCL7A expression

doi: 10.1111/j.12222
without DAC and/or TSA treatment in the current study. CpG methylation of BCL7A could be indifferent to transcriptional silencing in clinical samples as well.

The molecular mechanism underlying EBV-associated aberrant methylation has not been elucidated. One of the EBV latent genes, LMP2A, could be a candidate gene to induce aberrant DNA methylation. We previously showed that LMP2A mRNA was detected in both EBVaGC samples and EBV-infected gastric carcinoma cell lines that we generated with recombinant EBV.  Hino et al. reported that LMP2A activated DNA methyltransferase 1 through STAT3 phosphorylation and led to promoter hypermethylation of PTEN in gastric carcinoma.  This result suggests that LMP2A plays an important role in the epigenetic abnormalities in the development of EBVaGC. Another EBV-latent membrane protein, LMP1, was reported to activate DNA methyltransferase 1 via JNK/AP1 signaling in nasopharyngeal carcinoma, but LMP1-positive cells are rarely seen in EBVaGC and gastric epithelium by immunohistochemical analysis. We considered the influence of treatment with DAC and/or TSA for EBV gene expression, but LMP1 expression was not detected in SNU-719 by DAC treatment (data not shown).

In conclusion, we identified several genes with promoter regions that were specifically methylated in EBVaGC. Inactivation of these genes may suppress their function as tumor suppressor genes or tumor-associated antigens and help to develop and maintain EBVaGC.

Acknowledgment
This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 23591918 to J.N.).

Disclosure
The authors have no conflict of interest.

Abbreviations

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<th>Azacitidine</th>
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<td>CIMP</td>
<td>CpG island methylator phenotype</td>
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<td>DAC</td>
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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers for methylation-specific PCR.
Table S2. Primers for bisulfite sequence analysis.
Table S3. Primers for real-time quantitative PCR.