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Epigenetic regulation of human cancer/testis antigen gene, *HAGE*, in chronic myeloid leukemia

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ABSTRACT

Background and Objectives

Cancer testis antigens (CTA) provide attractive targets for cancer-specific immunotherapy. Although CTA genes are expressed in some normal tissues, such as the testis, this immunologically protected site lacks MHC I expression and as such, does not present *self* antigens to T cells. To date, CTA genes have been shown to be expressed in a range of solid tumors via demethylation of their promoter CpG islands, but rarely in chronic myeloid leukemia (CML) or other hematologic malignancies.

Design and Methods

In this study, the methylation status of the *HAGE* CTA gene promoter was analyzed by quantitative methylation-specific polymerase chain reaction (MSP) and sequencing in four Philadelphia-positive cell lines (TCC-S, K562, KU812 and KYO-1) and in CML samples taken from patients in chronic phase (CP n=215) or blast crisis (BC n=47). *HAGE* expression was assessed by quantitative reverse transcriptase-polymerase chain reaction.

Results

The TCC-S cell line showed demethylation of *HAGE* that was associated with overexpression of this gene. *HAGE* hypomethylation was significantly more frequent in BC (46%) than in CP (22%) (p=0.01) and was correlated with high expression levels of HAGE transcripts (p<0.0001). Of note, in CP-CML, extensive *HAGE* hypomethylation was associated with poorer prognosis in terms of cytogenetic response to interferon (p=0.01) or imatinib (p=0.01), molecular response to imatinib (p=0.003) and progression-free survival (p=0.05).

Interpretations and Conclusion

The methylation status of the *HAGE* promoter directly correlates with its expression in both CML cell lines and patients and is associated with advanced disease and poor outcome.

Key words: cancer testis antigens, *HAGE*, hypomethylation, CML.

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Chronic myeloid leukemia (CML) is a clonal disease of the hematopoietic stem cell, in which a reciprocal translocation, t(9;22)(q34;q11), forms the Philadelphia chromosome (Ph') and creates a new fusion gene, *BCR-ABL*. This chimeric gene is translated into a 210 kDa protein (p210), which has abnormal tyrosine-kinase activity that is central to the pathogenesis of the disease.[1] Treatment of CML has been notably improved by imatinib mesylate, a potent tyrosine-kinase inhibitor that blocks the kinase activity of p210, thus inhibiting proliferation of Ph'-positive progenitors.[2] In the past 5 years, the results of imatinib treatment of CML have established this drug as the first-line therapy for CML patients.[3] However, both the persistence of molecular disease in most imatinib-treated patients, as well as the observation that discontinuation of the drug usually results in a rapid loss of the response, indicate that it is unlikely that imatinib alone can cure CML.[2,3]

An alternative attempt to target CML cells is an active, specific immunotherapy (e.g. a vaccine). In fact, because of the unique amino acid sequence of p210 at the fusion point, the protein is a tumor-specific antigen to which an immune response can be induced. In some studies it has been shown that peptides derived from the p210 fusion point (b3a2) can bind to several HLA class I and class II molecules and thus generate peptide-specific dendritic cells (DC) and cytotoxic T lymphocyte (CTL) responses *in vitro*.[4,5] Preliminary clinical data suggest that the addition of a b3a2-specific vaccine to b3a2-CML patients treated with conventional treatment might favor a further reduction of residual disease, therefore increasing the number of patients who reach a molecular response.[6] In disagreement with these findings, it has been recently found that the CTL induced by DC transfected with RNA extracted from *BCR-ABL*-positive K-562 cells or CML blasts do not recognize epitopes derived from the chimeric BCR-ABL fusion protein. In contrast, they were able to lyse autologous DC electroporated with RNA from patients with acute myeloid leukemia (AML), indicating that some antigens shared by these malignant cells are involved and recognized by these CTL.[7] Therefore, BCR-ABL is processed and presented by Ph'-positive cells but is not the immunodominant antigen that induces $CD8^+$ T lymphocytes in competition with other tumor-associated antigens. However, the nature of these possible antigens is unknown.

Among the tumor antigens that are presented to human CTL by HLA class I molecules, several constitute safe targets for immunotherapy because they are absent in normal tissues. An important category of such tumor-specific antigens are those encoded by the *cancer testis antigen* (CTA) genes, such as the *MAGE, BAGE, CAGE, GAGE, HAGE, LAGE, PAGE, NY-ESO-1, SSX*, and *SCP* gene families.[8] These genes are expressed in several types of tumors but not in adult tissues with the exception of spermatogonia, which do not carry HLA molecules and therefore cannot present antigens to the T cells. Despite the fact that these antigens are expressed in a broad range of human tumors, few CTA genes are expressed in hematologic malignancies, although *SCP1* and, specially, *HAGE* have been found in a proportion of samples from both AML and CML patients.[9,10] Epigenetic events appear to represent the unique mechanism regulating the expression of CTA in cancer cells, and DNA methylation seems to play the major role; in fact, a correlation between hypomethylated CpG dinucleotides in CTA promoters and their expression has been found in neoplastic cell lines and tissues.[11-14] Moreover, unmethylated *MAGE* promoters drive the transcription of reporter genes in CTA-negative neoplastic cells, suggesting that availability of transcriptional factors is not involved in the heterogeneity of CTA expression and that DNA methylation is the main restricting factor for CTA expression.[15] Consistently, *in vitro* methylation of these reporter constructs is sufficient to block their transcriptional activity in *MAGE*-positive cells.[16] In addition, all CTA genes that are expressed in tumors or testis can be induced *in vitro* by DNA demethylation or by inhibitors of histone deacetylation.[17]

We have recently shown that sense and antisense transcription of the LINE1 retrotransposon (one of the most frequent repetitive sequences in the genome) is activated by promoter hypomethylation in CML and that this event is frequently associated with evolution of the disease to the advanced phase.[18] Since global hypomethylation is consistently associated with hypomethylation of the LINE1 elements,[19] it can be tentatively speculated that this genome-wide hypomethylation could lead to the reactivation of genes silenced by DNA methylation in CML patients, as for example, CTA genes. In the present study, we provide the first evidence showing the role of promoter methylation in the primary regulation of *HAGE* and its role in the progression and clinical behavior of CML.

Design and Methods

Cell lines and samples

Four human Ph'-positive CML cell lines (K562, KU812, KYO-1 and TCC-S) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in appropriate medium until harvested for extraction of DNA and RNA. Heparinized bone marrow cells were collected from patients with chronic phase CML (CP-CML), and from healthy marrow donors. Immediately after harvest, total white blood cells were obtained by dextran sedimentation or by red cell lysis of centrifuged buffy coat preparations. Mononuclear cells were isolated from patients with CML in blast crisis (BC-CML) and donors by sedimentation on Ficoll-Hypaque gradients. More

than 90% of the mononuclear cell populations from patients with acute phase CML were leukemic blasts. We studied 215 patients with Ph-positive CP-CML, diagnosed between August 1982 and December 2005. The patients were unselected with regard to the type of front-line therapy (39 patients received oral chemotherapy alone, 76 interferon plus cytarabine and 100 imatinib). A diagnostic sample in CP was available for all patients. Paired samples, when both a CP diagnostic sample and a BC sample were available, were analyzed in 47 patients (34 in myeloid BC and 13 in lymphoid BC). BC was defined by the presence of at least 30% blasts in the blood or bone marrow or extramedullary involvement. Risk categories according to the Sokal and Hasford score systems were determined as previously described.[20,21] Hematologic, cytogenetic and molecular responses to interferon and imatinib were evaluated. A complete hematologic response (CHR) was defined by a white cell count of less than 101×10^9/L, with no immature cells and less than 5% basophils in the peripheral blood, a platelet count of less than 450×10^9/L, and the absence of palpable splenomegaly or extramedullary involvement. Cytogenetic response (CgR) was assessed by G-banding in at least 20 cells in metaphase per sample and was defined as *major* CgR, including complete responders (CCgR, 0% Ph'-positive metaphases) and partial responders (PCgR, 1%-34% Ph'-positive metaphases) or *poor* CgR, including minor (MinCgR, 35%-94% Ph'-positive metaphases) or no response (NCgR, 95%-100% Ph'-positive metaphases).

Disease progression was defined by any of the following events, whichever came first: (i) death from any disease-related cause during treatment, (ii) the development of accelerated-phase CML (defined by the presence of at least 15% blasts in the blood or bone marrow, at least 30% blasts plus promyelocytes in the blood or bone marrow, ≥20% peripheral basophils, or thrombocytopenia $<100\times10^9$/L unrelated to treatment), (iii) blast-phase CML (defined by the presence of at least 30% blasts in the blood or bone marrow or extramedullary blastic involvement), iv) loss of complete hematologic response (defined by the appearance of any of the following findings in two blood samples obtained at least 1 month apart: a white-cell count $>20\times10^9$/L, a platelet count $\geq600\times10^9$/L, the appearance of extramedullary disease, the appearance of at least 5% myelocytes and metamyelocytes in the peripheral blood, or the appearance of blasts or promyelocytes in the peripheral blood), v) loss of major cytogenetic response (defined as ≥30% increase in the Ph'-positive cells in two studies performed at least 1 month apart), or vi) an increasing white-cell count (defined as a doubling of the count to more than 2×10^9/L on two occasions at least 1 month apart in a patient who had never strictly had a complete hematologic response despite the maximum tolerated doses of therapy).

Minimal residual disease was determined during follow-up using a standardized real-time quantitative reverse transcriptase–polymerase chain reaction (PCR) analysis on peripheral blood and/or marrow aspirate, as established in the framework of the EU concerted action.[22] Complete molecular remission (CMR) was defined as negative quantitative PCR confirmed by nested PCR for *BCR-ABL*. Major molecular response (MMR) was defined as a reduction in the *BCR-ABL/ABL* ratio of at least three logs with regard to the diagnostic baseline value. Some of the patients in the present study (n=140) had been previously typed for hypomethylation of LINE1 retrotransposons.[18]

Semiquantitative real time methylation-specific PCR (qrt-MSP) of HAGE promoter and exon 1

Analysis of the *HAGE* promoter and exon 1 (GeneBank: NM_018665) has revealed that HAGE possesses a 383bp CpG island located between nt48 and nt430, showing >60% C+G content and an observed-over-expected CpG frequency of >0.6. Promoter methylation of the *HAGE* gene was determined by the MSP method, as previously reported.[23] Briefly, 1 μg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. Qrt-MSP was performed as previously reported by our group[18] in a rapid fluorescent thermal cycler with three-color fluorescence monitoring capability (LightCycler, Roche), using 1 μL of bisulfite-modified DNA in a 10 μL reaction volume with 0.4 μmol/L of each primer, and 1 μL of 1× LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The final Mg^{2+} concentration in the reaction mixture was adjusted to 3.5 mmol/L. Amplification of the *HAGE* methylated sequences was used as target sequence (forward, 5'-GGAGGAGTTTTTAAGGTTTTTACGT-3'; reverse, 5'-GACAATTCCTCGTAACCAACG-3'). The following program conditions were applied for running the qrt-MSP: denaturation program, one cycle at 95°C for 10 minutes; amplification program, 45 cycles at 95°C for 10 s, 65°C for 10 s and 72°C for 10 s; melting program, one cycle at 95°C for 0 s, 40°C for 60 s and 90°C for 0 s; and cooling program, one cycle at 40°C for 60 s. The temperature transition rate was 20°C/s, except in the melting program, in which it was 0.4°C/s between 40°C and 90°C. Amplification of the *HAGE* unmethylated sequences for each sample was performed as the reference sequence (forward, 5'-GGAGGAGTTTTTAAGGTTTTTATGT-3'; reverse, 5'-ACAACAATTCCTCATAACCAACAA-3'). It was amplified in the same run and following the same procedure described above for methylated sequences. A procedure based on the relative quantification of target sequence (methylated sequences) vs their controls/calibrators in relation to the reference sequence (unmethylated sequences) was used to assesses the degree of *HAGE* promoter methylation. Calculations were automatically performed by the LightCycler software (RealQuant, version 1.0, Roche). The normalized ratio was obtained from the following equation and

expressed as a percentage of the control/calibrator:

$$\text{Normalized ratio } (N_{\text{HAGE-METHYLATION}}) = (E_{\text{target}})^{\Delta Cp \text{ target (control– sample)}} \div (E_{\text{ref}})^{\Delta Cp \text{ ref (control–sample)}}$$

The efficiency (E) of each gene was calculated from the slope of the crossover points (Cp) versus DNA concentration plot, according to the formula $E=10^{(-1/\text{slope})}$. ΔCp corresponded to the difference between control/calibrator Cp and sample Cp, for either the target or the reference sequences. The selected control/calibrator was the bone marrow specimen from a healthy donor, which was considered as 100% (this is not an absolute value indicating a fully methylated patient but a relative value used as a measure for the relative level of *HAGE* methylation in the particular sample). Water blanks were included with each assay. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples. Occasionally, equal amounts of PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Expression of HAGE transcripts

Qrt-PCR for *HAGE* expression was performed with the LightCycler technology, using 1 μL of cDNA in a 20 μL reaction volume with 0.4 μmol/L of each primer (forward: 5'-CCTTTCAATGTTATCCTGAG-3'; reverse: 5'- TATTCTTCAGATTGACGAAG-3'), and 2 μL of 10× LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The Abelson gene (*ABL1*) was employed as the reference gene, and it was amplified in the same run and following the same procedure described above (forward: 5'-CCCAACCTTTTC-GTTGCACTGT-3'; reverse: 5'-CGGCTCTCGGAG-GAGACGTAGA-3'). In order to reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes versus their controls/calibrators in relation to the reference gene was used. Calculations were automatically performed by LightCycler software (RealQuant, version 1.0, Roche). The normalized ratio ($N_{\text{HAGE-EXPRESSION}}$), expressed as a percentage of the control/calibrator, was obtained by RealQuant software as described above for qrt-MSP of the *HAGE* promoter. The selected controls/calibrators were bone marrow specimens from healthy donors, which were considered as having 100% expression.

5-aza-2'-deoxycytidine treatment

CML-derived K562, KU812, KYO-1 and TCC-S cell lines were grown at a density of 750,000 cells/mL in 25 cm^2 flasks with 8 mL of RPMI 1640 medium supplemented with 20% fetal bovine serum and maintained at 37°C in a humid atmosphere containing 5% CO_2. Each of the cell lines was treated with 2 and 4 μM of 5-aza-2'-deoxycytidine (Sigma-Aldrich, Steinheim, Germany) for 4 days.

HAGE promoter and exon 1 analysis by sequencing after sodium bisulfite modification

The methylation status of *HAGE* promoter and exon 1 was analyzed by bisulfite genomic sequencing of its CpG island in TCC-S and KU812 CML derived cell lines. One microgram of genomic DNA was treated and modified using the CpGenomic™ DNA Modification Kit (Intergen Company, Purchase, NY, USA). Bone marrow DNA from healthy donors was used as a methylated or positive control for sequencing analysis. After bisulfite modification, the *HAGE* promoter was amplified by seminested-PCR. The first PCR was performed using 5 μL of modified DNA and HAGE1-SB1 (5´-TTTTTTTTGGAATAAT-GTTTTATTA-3´) and HAGE1-SB2 (5´-TAACCCCACC-TATCCTACCCTAC-3´) primers under the following conditions: 94°C for 10 min, 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, and a final elongation cycle at 72°C for 10 min. The second PCR was performed with 5 μL of the first PCR product using HAGE1-SB3 (5´-GGAGGAGTTTTTAAGGTTTTTA-3´) and HAGE1-SB2 primers under the following conditions: 94°C for 10 min, 20 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, and a final elongation cycle at 72°C for 10 min. Two PCR reactions were carried out in a total volume of 25 μL, with 1 U high fidelity Platinum Taq DNA polymerase (Invitrogen Life Technologies, Paisley, UK), 1.5 mM $MgCl_2$, 0.2 mM dNTP and 50 pmol of each primer. The second 280 bp PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Amplification products obtained in the second PCR reaction were subcloned into pCR® 4-TOPO® plasmid using TOPO TA Cloning® Kit for sequencing (Invitrogen Life Technologies, Paisley, UK) and transformed into *Escherichia coli* according to the manufacturer's recommendations. Colonies with recombinant plasmids containing the described PCR products were screened by digestion with *Eco*^{R} I (Amersham Biosciences, Buckinghamshire, UK). Candidate plasmid clones were sequenced with ABI-PRISM™ d-Rhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using T7 and T3 universal forward and reverse primers.

Statistical analysis

All calculations were performed with the SPSS statistical package (SPSS, Chicago, IL, USA). The medians, standard deviations (SD) and interquartile ranges for age and the most relevant clinical and laboratory findings at diagnosis were calculated for patients with and without *HAGE* hypomethylation and tested for any significant differences with the Mann-Whitney *U* test (for continuous variables) or χ^2 analysis and Fisher's exact test (for categorical variables). Overall survival (OS) was calculated from time of diagnosis to death from any cause and was censored only for patients known to be alive at last contact. Progression-free survival (PFS) was measured from

the diagnosis of CML to the appearance of BC or death without disease progression, and was censored only for those patients alive and without evidence of progression at last follow-up. For calculations of both OS and PFS, bone marrow transplant recipients were censored at the time of transplantation. Distributions of OS and PFS curves were estimated by the method of Kaplan and Meier, with the 95% confidence intervals being calculated using Greenwood's formula. Comparisons of OS and PFS between groups were based on the log-rank test. Comparisons adjusted for significant prognostic factors were based on Cox regression models and hazard regression models. All progression and survival data were updated as of March 31, 2006, and all follow-up data were censored at that date.

Results

HAGE is hypermethylated in normal bone marrow cells

We analyzed *HAGE* methylation in 30 normal bone marrow samples. As expected, *HAGE* promoter DNA sequences were strongly methylated in normal bone marrow cells. This is illustrated by the amplification of the methylated sequences with the complete lack of non-methylated sequence amplification in half of these samples (Figure 1A). However, 50% of the non-neoplastic marrow specimens displayed a slight amount of *HAGE* hypomethylation, as defined by the presence of specific products in the melting curves from both methylated (melting temperature 76.1°C) and unmethylated sequences (melting temperature 74.6°C, Figure 1B and 1C). Based on the background fluorescence intensity, a cut-off level was determined for specific fluorescence for both unmethylated and methylated sequences in normal individuals. This threshold was used to calculate the cycle threshold or crossing point (C_P) of each sample. The C_P value was directly proportional to the amount of target sequence present in the sample. The normal sample which showed the lowest difference in C_P between the target (methylated sequences) and the reference (unmethylated sequence) sequences was used as the control/calibrator sample for quantification of *HAGE* promoter methylation in both healthy individuals and CML samples. It was considered as 100%.

Based on these prerequisites and to determine the cut-off value for altered *HAGE* promoter methylation in CML samples, the methylation status of the *HAGE* promoter was quantified by means of qrt-MSP in the

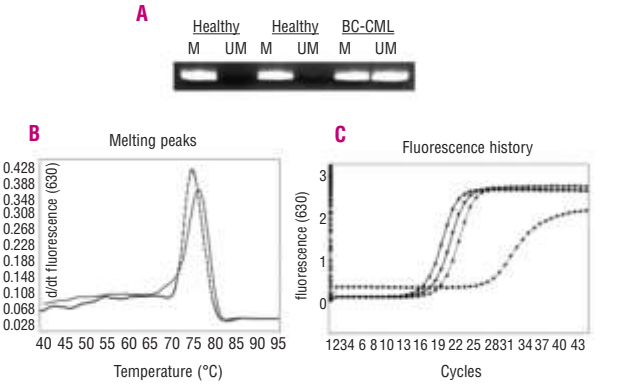

Figure 1. Methylation status of the *HAGE* promoter in healthy individuals and in a patient with CML. (panel A) MSP analysis of the CpG island within the *HAGE* promoter in healthy individuals and CML patient. BC indicates blast crisis CML; UM, unmethylated sequences; M, methylated sequences. (panel B) qrt-MSP melting curve analysis of a sample from a CML patient showing the presence of two specific PCR products: unmethylated (dotted line, melting temperature 74.6°C) and methylated *HAGE* sequences (solid line, melting temperature 76.1°C) (panel C) *HAGE* promoter methylation level of a representative CML case (lines with triangles) and a control/healthy individual (lines with circles) as measured by qrt-MSP. The difference between the crossing points of the methylated (bold lines) and unmethylated (fine lines) *HAGE* sequences was smaller for the CML patient than for healthy individual, indicating a higher level of hypomethylation in the CML patient.

healthy donors showing some degree of unmethylated sequence amplification. $N_{HAGE\text{-}METHYLATION}$ ratios fell between 100% and 217% (160± 40%). A $N_{HAGE\text{-}METHYLATION}$ ratio equal or below 80% (determined as the mean minus 2 SD) was chosen to define hypomethylation of *HAGE* promoter in CML DNA samples.

HAGE is hypomethylated in CML samples

By qrt-MSP, the CpG island of the *HAGE* promoter was revealed to be highly hypomethylated in the TCC-S Ph-positive CML cell line ($N_{HAGE\text{-}METHYLATION}$ ratio: 10%) whereas K562, KYO-1 and KU812 cell lines showed normal levels of HAGE methylation (median $N_{HAGE\text{-}METHYLATION}$ ratio: 98%, range: 100-97%, Figure 2 and Table 1). In order to confirm the results of the qrt-MSP, we screened bone marrow DNA from healthy donors and two CML-derived cell lines (TCC-S and KU812) using bisulfite genomic sequencing. As described in the *Design and Methods* section, the 280 bp amplification products of the *HAGE* promoter obtained in a second PCR, which are composed of 20 CpG dinucleotides, were subcloned and sequenced. Genomic sequencing after bisulfite modification revealed that bone marrow DNA

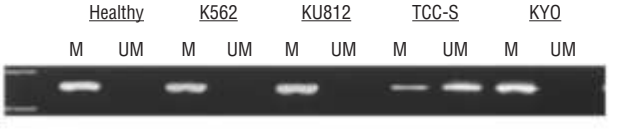

Figure 2. Methylation status of the *HAGE* promoter in Ph'-positive CML cell lines. MSP analysis of the CpG island within the *HAGE* promoter in four Ph'-positive CML cell lines. UM, unmethylated sequences; M, methylated sequences. Only the TCC-S cell line showed *HAGE* hypomethylation.

Table 1. HAGE methylation status and mRNA expression in Ph'-positive CML cell lines before and after treatment with 5-aza-2'-deoxycytidine.

Cell line	$N_{HAGE\text{-}METHYLATION}$	HAGE hypomethylation	$N_{HAGE\text{-}EXPRESSION}$	$N_{HAGE\text{-}EXPRESSION}$ + AZA	p
Tcc-s	10%	yes	1045±72%	1129±63%	0.8
K562	98%	no	10±1%	178±12%	0.01
Kyo-1	97%	no	20±2 %	160±14%	0.02
Ku812	100%	no	0%	142±11%	0.003

$N_{HAGE\text{-}EXPRESSION}$ represents the mean expression ± SD in cell lines from three different experiments in comparison with expression in healthy bone marrow cells (normalized ratio = 100%).

from healthy donors and DNA from the KU812 CML-derived cell line were almost completely methylated, showing methylation of the 20 analyzed CpG dinucleotides in all analyzed clones. In contrast, the TCC-S cell line showed a complete lack of methylation of *HAGE* promoter in all analyzed CpG dinucleotides and clones (Figure 3).

Among CML patients, hypomethylation of *HAGE* promoter was more frequently observed in BC (22/47, 46%) than during C_P disease (47/215, 22%), with this difference being statistically significant (p=0.01). Furthermore, a more profound level of hypomethylation was observed in BC than in C_P samples (mean $N_{HAGE\text{-}METHYLATED}$ ratios: 16%±5% vs 32%±8%, p=0.06). There was a significant concordance between *HAGE* hypomethylation and presence of LINE1 hypomethylation; *HAGE* hypomethylation was present in 23 of 93 (25%) LINE1 methylated tumors versus 24 of 47 (52%) LINE1 hypomethylated tumors (p=0.01).

HAGE hypomethylation in CML is associated with over-expression of HAGE transcripts

Quantitative expression of *HAGE* transcripts was assessed by means of qrt-PCR using cDNA from a healthy donor as the control (considered to have a $N_{HAGE\text{-}EXPRESSION}$ ratio of 100%). Normalized ratios for *HAGE* expression were determined in bone marrow specimens from 30 healthy individuals. $N_{HAGE\text{-}EXPRESSION}$ ratios fell between 20% and 100% (mean $N_{HAGE\text{-}EXPRESSION}$: 35±25%). Among CML patients, those with methylated *HAGE* promoter showed a mean $N_{HAGE\text{-}EXPRESSION}$ of 34%±30%, similar to that found in healthy individuals; however, the mean $N_{HAGE\text{-}EXPRESSION}$ was significantly higher in those CML patients who showed *HAGE* promoter hypomethylation (319%±118%, p<0.0001). An $N_{HAGE\text{-}EXPRESSION}$ value equal or above 160% (determined as the mean $N_{HAGE\text{-}EXPRESSION}$ from normal individuals plus 5 SD) was chosen to define over-expression of *HAGE* in CML RNA samples. Using this cut-off value, over-expression of *HAGE* was found in 100% of CML patients with *HAGE* hypomethylation and in none of the CML patients with methylated *HAGE* promoter (p<0.0001).

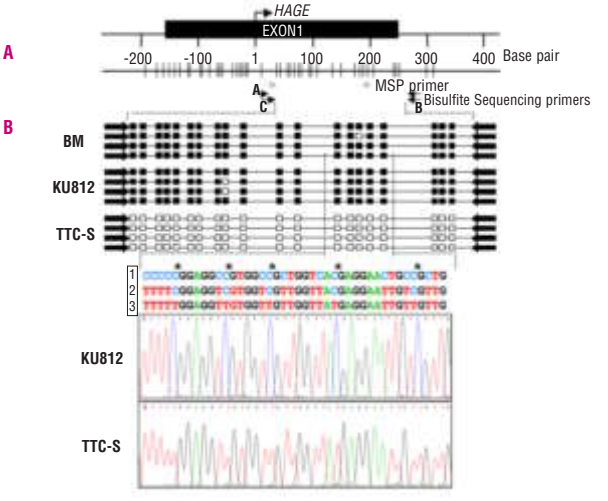

Figure 3. Analysis of *HAGE1* CpG island methylation status by bisulfite sequencing in bone marrow of healthy donors, TCC-S and KU812 CML derived cell lines. (Panel A) Schematic description of the *HAGE* CpG island. Long black arrow indicates the *HAGE* translation start site and each vertical bar represents a CpG dinucleotide. The gray arrows show the location of the MSP primers and the black arrows the location of bisulfite sequencing primers (A: HAGE1-SB1 primer; B: HAGE1-SB2 primer; C: HAGE1-SB3 primer). (Panel B) Bisulfite sequencing of the *HAGE* CpG island. Each box indicates a CpG dinucleotide (white box: unmethylated, black box: methylated) and each line represents the analysis of 20 CpG dinucleotides of a single clone of an *HAGE* analyzed region. 1: non-modified sequence of *HAGE1* CpG island; 2: methylated sequence of *HAGE* CpG island after bisulfite modification; 3: non-methylated sequence of *HAGE* CpG island after bisulfite modification. **CpG dinucleotide position.*

High levels of *HAGE* expression were observed in the *HAGE* hypomethylated TCC-S cell line ($N_{HAGE\text{-}EXPRESSION}$ ratio: 1045%) whereas *HAGE* hypermethylated Ph-positive CML (K562, KU812 and KYO-1) cell lines showed low levels of *HAGE* expression (mean $N_{HAGE\text{-}EXPRESSION}$ ratio: 10%, range: 0-20%, Table 1).

Exposure of CML cell lines to the demethylating agent 5-aza-2'-deoxycytidine restored expression of *HAGE* mRNA in cell lines that show hypermethylation of the *HAGE* promoter, such as K562, KU812 and KYO-1 (mean $N_{HAGE\text{-}EXPRESSION}$ ratio: 160%, range: 142-178%) but had no effect on *HAGE* mRNA expression in the hypomethylated CML-derived TCC-S cell line (Table 1). These results indicate that hypermethylation is a major mechanism through which *HAGE* expression is silenced in CML cells.

HAGE hypomethylation, response to treatment and clinical outcome

The clinical and laboratory characteristics at diagnosis of CP-CML patients with hypomethylated and normal *HAGE* at diagnosis are shown in Table 2. Individual factors such as sex, age, spleen size, percentage of blast cells in the peripheral blood, platelet count, hemoglobin level and white cell count were not significantly associated with the *HAGE* methylation status. When *HAGE* methylation status was correlated with pretreatment

Table 2. Clinical characteristics at diagnosis and outcome of 215 chronic myeloid leukemia patients divided according to *HAGE* methylation status.

Feature	*HAGE hypomethylated (n=47)*	*HAGE methylated (n=168)*	*p*
Sex (M/F), %	58/42	60/40	NS
Age, median (IQR)	48 (37-59)	49 (37-60)	NS
Palpable spleen, %	69	63	NS
Median hemoglobin (g/L) (IQR)	120 (96.7-129.1)	110 (83.1-119.6)	NS
WBCx10^9/L, median (IQR)	162 (60.4-326.1)	143 (52.1-226.5)	NS
Median platelet count 10^9/L (IQR)	372 (262-614)	400 (280-650)	NS
Median peripheral blood blast as % WBC (IQR)	1 (0-4.2)	1 (0-3.8)	NS
Sokal score, %			NS
High	35	27	
Low/Intermediate	65	73	
Hasford score, %			NS
High	19	26	
Low/Intermediate	81	74	
Treatment type, %			NS
Chemotherapy	22	17	
Interferon	36	35	
Imatinib	42	48	
Transplantation	20	17	
Response to treatment, %			
CHR	72	89	0.05
CCgR with IFN	0	28	0.01
CCgR at 6m with IM	23	66	0.01
CCgR at 12m with IM	55	90	0.02
MMR at 12m with IM	18	58	0.003
Disease progression, %	53	62	NS
Death, %	42	55	NS

IQR: interquartile range; CHR: complete hematologic response; CCgR: complete cytogenetic response; MMR: major molecular response; IFN: interferon; IM: imatinib mesylate.

risk groups, no significant association between *HAGE* hypomethylation and high-risk patients, as assessed by the Sokal and Hasford scoring systems, was found. In the present study, CML patients were treated with oral chemotherapy (mainly, hydroxyurea, n=39), interferon-based regimens (n=76) or imatinib (n=100). Thirty-seven patients received stem cell transplantation (5 autologous, 32 allogeneic). The treatment modality and number of patients who received transplantation were similarly distributed between the two *HAGE* methylation groups (Table 2). Moreover, mean time of interferon administration was similar in methylated (31 months) and hypomethylated (30.4 months) patients. Hypomethylation of the *HAGE* promoter was correlated with a poor response to treatment (Table 2). Thus, the CHR rate was significantly lower among hypomethylated patients than in patients with methylated *HAGE* promoter (72% versus 89%, p=0.05). Among 76 patients under interferon therapy, a CCgR was observed in 28% of those with methylated *HAGE* and in none of the hypomethylated patients (p=0.01). Among the 100 CP-CML patients receiving imatinib as front-line therapy, the rates of complete cytogenetic response at 6 and 12 months were 66% and 90% in the *HAGE* methylated group, as compared with 23% and 55% in the *HAGE* hypomethylated group (p=0.01 and p=0.02, respectively). In addition, the major molecular response rate at 12 months was better for patients with methylated *HAGE* (58%) than for *HAGE* hypomethylated patients (18%, p=0.003). Moreover, the time to achieve a CCgR was significantly longer for hypomethylated patients (mean: 11.1±7.4 months) than for methylated patients (mean: 7.3±3.3 months, p=0.01).

Survival data were available from all patients. Kaplan-Meier analysis revealed differences in the duration of the CP among patients under interferon therapy (Figure 4). The estimated median PFS time for hypomethylated patients was 72.2 months (95% CI [confidence interval], 42.6-101.5) compared to 112.6 months (95% CI, 87.8-137.4) for patients with methylated *HAGE* (p=0.05). Among patients receiving imatinib therapy, only two patients progressed, both of whom had hypomethylation of the *HAGE* gene.

Discussion

In this study, we provide the first evidence that promoter hypomethylation is the molecular mechanism directly responsible for the high expression levels of the *HAGE* gene in CML. We have demonstrated that *HAGE* mRNA is expressed in 22 and 46% of CML patients in chronic phase and blast crisis, respectively. *HAGE* mRNA was previously reported to be expressed in half of 43 CML patients at presentation.[10] However, this study did not verify the methylation status of the *HAGE* promoter CpG islands. The evidence of *HAGE* expression, induced by hypomethylation, was provided by the re-expression of *HAGE*, after treatment with 5-aza-2'-deoxycytidine, in CML cell lines not expressing HAGE. In addition, our results clearly indicate a significant correlation between the expression and hypomethylation of the CpG sites of *HAGE*. The methylation status analysis of the *HAGE* promoter in CML cell lines and patients showed a perfect correlation between the expression of *HAGE* and hypomethylation. Interestingly, the key finding of increased *HAGE* expression in BC-CML with hypomethylated *HAGE* promoter seems to be contradicted by the fact that three out of four CML cell lines had low or no detectable *HAGE*

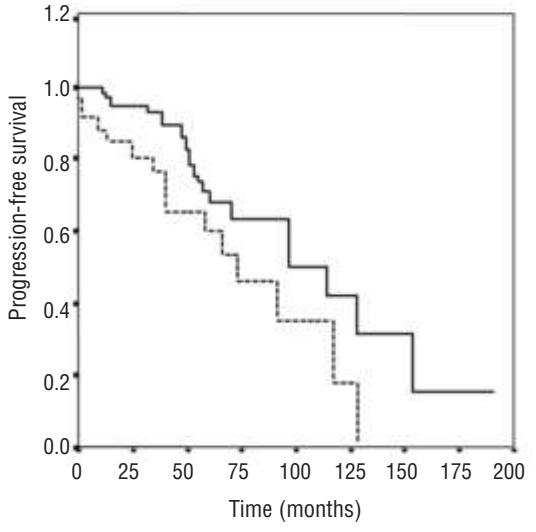

Figure 4. Kaplan-Meier progression-free survival curve for CML patients. PFS curve according to the *HAGE* methylation status for 76 CML patients treated with interferon. Solid line, patients with methylated *HAGE*; dashed line, patients with hypomethylated *HAGE*.

expression. However, we have observed[18] that cancer cell lines (including CML-derived cell lines) have an increased rate of hypermethylation of certain CpG islands and less global genomic hypomethylation. Fortunately, some of them (TCC-S) retain the specific epigenetic profile of each tumor cell type and are, therefore, very useful for *in vitro* experiments while not contradicting the results obtained in clinical samples.

Recently, we showed that LINE1 sense and antisense transcription was activated by promoter hypomethylation in CML and that this event was frequently associated with the evolution of the disease to an advanced phase.[18] Because LINE1 hypomethylation is an indicator of global genomic hypomethylation and LINE1 undermethylation is closely correlated with that observed in the *HAGE* gene, we hypothesize that in CML, demethylation of the *HAGE* gene occurs during progressive stages of leukemogenesis in CML, probably after global DNA hypomethylation, as seen in many advanced cancers. Moreover, all these findings indicate that hypomethylation is an important feature in CML. Firstly, hypomethylation increased from non-neoplastic marrow cells toward CML cells; secondly, an increase in the hypomethylation status was observed in CML patients with advanced disease; and finally, high levels of hypomethylation would be a marker of poor prognosis in a subset of patients with CP-CML.

What could be the functional significance underlying the hypomethylation of *HAGE* in CML? The transition of CML from CP to BC is characterized by genomic instability leading to the accumulation of molecular and chromosomal abnormalities in addition to the Ph'-chromosome.[24] Several reports have shown that mammalian DNA methylation plays an important role in maintaining genomic stability unrelated to the effects of DNA methylation on gene expression and that hypomethylation of DNA in tumor cells is associated with genomic instability.[25] Moreover, genomic hypomethylation causes tumorigenesis in mice associated with the acquisition of additional genomic changes.[26] Our results suggest that hypomethylation affecting not only repetitive DNA sequences but also specific gene promoters (i.e, CTA genes) could be one of the mechanisms employed by CML cells to generate an unstable genome.

It is evident that *HAGE* hypomethylation occurs in many BC-CML patients but whether this epigenetic change is a cause or a consequence of the progression is unclear. Our data argue against the second alternative because *HAGE* hypomethylation was not only associated with BC but was also observed in a proportion of CML patients in CP. Interestingly, these patients responded slowly to treatment and progressed to BC much more rapidly than CP patients lacking hypomethylation, suggesting that hypomethylated patients *ab initio* may be more prone to genomic instability, similarly to patients with deletions of the derivative chromosome 9.[27] In support of the role of this epigenetic event in the progression of cancer, genome-wide hypomethylation has been shown to increase progressively in parallel to advanced grade in breast, ovarian, cervical, and neural cancers[28-30] and it has also been associated with the progression of lung cancer.[31] Therefore, it is not surprising that CTA gene expression has also been reported to be associated with less-differentiated, higher-grade tumors, later stages of cancer, and worse outcome.[32-34] In fact, *MAGEA3*, *MAGE-A10*, and *MAGE-A1* promoters were shown to undergo progressive demethylation in gastric cancer in parallel to disease progression.[35]

On the other hand, although *HAGE* expression could be a passive bystander in a more hypomethylated genome, the *HAGE* gene (located on 6q12-q13) encodes a protein that shows 55% similarity with the human p68 protein, a DEAD-box protein which has been demonstrated to have ATP-dependent RNA helicase activity.[36] The protein encoded by the *HAGE* gene seems to be a new member of the family of DEAD-box proteins,[37] which contain the highly conserved Asp-Glu-Ala-Asp (D-E-A-D) motif. These proteins are involved in many aspects of RNA metabolism, spermatogenesis, embryogenesis, and cell growth, functioning as important transcriptional regulators. The amino acids that are highly conserved in all of the DEAD-box proteins are also present in the HAGE protein, suggesting that HAGE may also be an ATP-dependent RNA helicase. DEAD-box proteins are up-regulated in cancer cells but not in the corresponding normal cells/tissues.[38] In addition, it has been recently reported that expression of an uncharacteristically large proportion of genes involved in pre-mRNA processing (including RNA helicases) is

increased in primary p210BCR/ABL-positive CD34+ cells, which correlates with altered splicing of gene products.[39] Taken together, these data suggest that *HAGE* is involved in both altered gene expression and RNA editing. One can, therefore, speculate that CML cells that overexpress *HAGE* may have a selective advantage over *HAGE*-negative cells, which would provide an explanation for why *HAGE* expression is positively selected during CML progression, even though its presence elicits a cytotoxic T-cell-mediated immune response. However, we have not performed functional analyses and therefore, the possible role of *HAGE* in the transformed status of CML cells should be clarified in further studies.

Besides the possible role of the *HAGE* gene in the pathogenesis and prognosis of CML, most CTA antigens are immunogenic, and their use as therapeutic cancer vaccines is currently being evaluated.[40,41] The DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine) has been used as part of chemotherapy protocols, primarily to reverse hypermethylation of tumor suppressor gene promoters, and has been shown to be beneficial in CML.[42] This drug, capable of inducing *HAGE* expression, could increase patient eligibility and treatment effectiveness in CTA-targeted immunotherapy. Our results suggest that the level of *HAGE* gene expression increases as CML progresses. This in turn suggests a potentially important therapeutic role for CTA-based cancer vaccines in the management of the late stages of CML that should be addressed in future studies.

In conclusion, the results of the present study show that the methylation status of the *HAGE* promoter directly correlates with its expression status in CML cell lines and patients and that in CML high levels of *HAGE* transcripts are associated with advanced disease and poor outcome.

Authors' Contributions

JR-G, AJ-V and XA contributed to the conception of this study. JAC, GN, FC, FP, AH and AT were responsible for the patients studied and clinical data. JR-G, AJ-V and XA performed and processed most experiments, assisted by ESJ-E, LG and LC. JR-G, AJ-V and XA contributed to study design, data analysis and the final version of the manuscript, which was drafted by JR-G and XA, and approved by all authors. All the figures and tables of the paper have been created by JR-G.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer 2005;5:172-83.
2. Deininger M, Buchdunger E, Druker BJ.The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood 2005;105: 2640-53.
3. O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 2003;348:994-1004.
4. Bocchia M, Korontsvit T, Xu Q, Mackinnon S, Yang SY, Sette A, et al. Specific human cellular immunity to BCR-ABL oncogene-derived peptides. Blood 1996;87:3587-92.
5. Yasukawa M, Ohminami H, Kojima K, Hato T, Hasegawa A, Takahashi T, et al. HLA class II-restricted antigen presentation of endogenous bcr-abl fusion protein by chronic myelogenous leukemia-derived dendritic cells to CD4+ T lymphocytes. Blood 2001;98:1498-505.
6. Bocchia M, Gentili S, Abruzzese E, Fanelli A, Iuliano F, Tabilio A, et al. Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. Lancet 2005;365:657-62.
7. Grünebach F, Mirakaj V, Müller MR, Brümmendorf T, Brossart P. BCR-ABL is not an immunodominant antigen in chronic myelogenous leukemia. Cancer Res 2006;66:5892-900.
8. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 2005;5:615-25.
9. Lim SH, Austin S, Owen-Jones E, Robinson L. Expression of testicular genes in haematological malignancies. Br J Cancer 1999;81:1162-4.
10. Adams SP, Sahota SS, Mijovic A, Czepulkowski B, Padua RA, Mufti GJ, et al. Frequent expression of HAGE in presentation chronic myeloid leukaemias. Leukemia 2002; 16:2238-42.
11. Cho B, Lee H, Jeong S, Lee HJ, Hwang KS, Kim HY, et al. Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma. Biochem Biophys Res Commun 2003;18;307: 52-63.
12. Grunau C, Sanchez C, Ehrlich M, van der Bruggen P, Hindermann W, Rodriguez C, et al. Frequent DNA hypomethylation of human juxtacentromeric BAGE loci in cancer. Genes Chromosomes Cancer 2005; 43:11-24.
13. Lim JH, Kim SP, Gabrielson E, Park YB, Park JW, Kwon TK. Activation of human cancer/testis antigen gene, XAGE-1, in tumor cells is correlated with CpG island hypomethylation. Int J Cancer 2005;116:200-6.
14. Wang Z, Zhang J, Zhang Y, Lim SH. SPAN-Xb expression in myeloma cells is dependent on promoter hypomethylation and can be upregulated pharmacologically. Int J Cancer 2006;118:1436-44.
15. De Smet C, Courtois SJ, Faraoni I. Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 1995;42:282-90.
16. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, et al. Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother 2002; 25:16-26.
17. Sigalotti L, Fratta E, Coral S, Tanzarella S, Danielli R, Colizzi F, et al. Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-aza-2'-deoxycytidine. Cancer Res 2004;64:9167-71.
18. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L, et al. Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. Oncogene 2005; 24:7213-23.
19. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, et al. Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. Cancer Sci 2004;95:58-64.
20. Sokal JE, Cox EB, Baccarani M, Tura S, Gomez GA, Robertson JE, et al. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. Blood 1984;63:789-99.

21. Hasford J, Pfirrmann M, Hehlmann R, Allan NC, Baccarani M, Kluin-Nelemans JC, et al. A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon α. J Natl Cancer Inst 1998;90:850-8.
22. Gabert J, Van Der Velden VH, Bi W, Grimwade D, Pallisgaard N, Barbany G, et al. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for residual disease detection in leukemia: a Europe Against Cancer Program. Leukemia 2003;17:2318-57.
23. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93: 9821-6.
24. Calabretta B, Perrotti D. The biology of CML blast crisis. Blood 2004; 103:4010-22.
25. Rizwana R, Hahn PJ. CpG methylation reduces genomic instability. J Cell Sci 1999;112:4513-9.
26. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, et al. Induction of tumors in mice by genomic hypomethylation. Science 2003;300:489-92.
27. Huntly BJ, Bench A, Green AR. Double jeopardy from a single translocation: deletions of the derivative chromosome 9 in chronic myeloid leukemia. Blood 2003; 102: 1160-8.
28. Kim YI, Giuliano A, Hatch KD, Schneider A, Nour MA, Dallal GE, et al. Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. Cancer 1994;74:893-9.
29. Qu G, Dubeau L, Narayan A, Yu MC, Ehrlich M. Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. Mutat Res 1999;423:91-101.
30. Soares J, Pinto AE, Cunha CV, Andre S, Barao I, Sousa JM, et al. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer 1999;85:112-8.
31. Piyathilake CJ, Frost AR, Bell WC, Oelschlager D, Weiss H, Johanning GL, et al. Altered global methylation of DNA: an epigenetic difference in susceptibility for lung cancer is associated with its progression. Hum Pathol 2001;32:856-62.
32. KurashigeT, NoguchiY, SaikaT, Ono T, Nagata Y, Jungbluth A, et al. NY-ESO-1 expression and immunogenicity associated with transitional cell carcinoma: correlation with tumor grade. Cancer Res 2001;61: 4671-4.
33. Yakirevich E, Sabo E, Lavie O, Mazareb S, Spagnoli GC, Resnick MB. Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens in serous ovarian neoplasms. Clin Cancer Res 2003;9:6453-60.
34. Otte M, Zafrakas M, Riethdorf L, Pichlmeier U, Loning T, Janicke F, et al. MAGE-A gene expression pattern in primary breast cancer. Cancer Res 2001;61:6682-7.
35. HondaT, Tamura G, Waki T, Kawata S, Terashima M, Nishizuka S, et al. Demethylation of MAGE promoters during gastric cancer progression. Br J Cancer 2004;90:838-43.
36. Iggo R, Picksley S, Southgate J, McPheat J, Lane DP. Identification of a putative RNA helicase in E.coli. Nucleic Acids Res 1990;18:5413-7.
37. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T. Identification on a human sarcoma of two new genes with tumor-specific expression. Cancer Res 2000;60:3848-55.
38. Yang L, Lin C, Liu ZR. Phosphorylations of DEAD box p68 RNA helicase are associated with cancer development and cell proliferation. Mol Cancer Res 2005;3: 355-63.
39. Salesse S, Dylla SJ, Verfaillie CM. p210BCR/ABL-induced alteration of pre-mRNA splicing in primary human CD34+ hematopoietic progenitor cells. Leukemia 2004;18: 727-33.
40. Chen Q, Jackson H, Parente P, Luke T, Rizkalla M, Tai TY, et al. Immunodominant CD4+ responses identified in a patient vaccinated with full-length NY-ESO-1 formulated with ISCOMATRIX adjuvant. Proc Natl Acad Sci USA 2004;101: 9363-8.
41. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, et al. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 2005;201:249-57.
42. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, et al. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 2004; 103:1635-40.