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ID4 methylation predicts high risk of leukemic transformation in patients with myelodysplastic syndrome

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ABSTRACT

Epigenetic gene silencing due to promoter methylation is observed in human cancers like acute myeloid leukemia (AML). Little is known about aberrant methylation in myelodysplastic syndrome (MDS), a heterogeneous clonal stem cell disorder with a ~30% risk of transformation into secondary AML. Recent evidence demonstrated that ID4, a negative regulator of transcription, may act as a tumor-suppressor gene. To clarify the role of ID4 in MDS, we employed methylation-specific PCR (MSP) to examine the methylation status of ID4 in 144 adult de novo MDS patients. We found that ID4 methylation was present in 35.4% ($n = 51$) of these MDS patients and methylaiton was correlated significantly with World Health Organization (WHO) subtypes and International Prognostic Scoring System (IPSS) risk groups. Patients with advanced stages of WHO subtypes (45.8% vs. 21.3%, $P = 0.002$) and higher risk IPSS subgroups (45.7% vs. 26.0%, $P = 0.014$) exhibited a significantly higher frequency of ID4 methylation. The median survival of patients with ID4 methylation was shorter than patients without ID4 methylation (12.2 months vs. 26.9 months, $P = 0.005$). Multivariate analysis indicated that ID4 methylation status was the independent factor that impacted leukemia-free survival (LFS). Disease in patients with ID4 methylation progressed more rapidly than those without ID4 methylation ($P = 0.047$, HR = 2.11). Our results suggest that ID4 may be a therapeutic target in MDS.

1. Introduction

Myelodysplastic syndrome (MDS) is a clonal disorder characterized by dyshematopoiesis and high susceptibility to acute myeloid leukemia (AML) [1,2]. Patients with MDS have widely variable prognoses and the pathology of this disease is not well understood. The leukemic transformation from normal stem cells is believed to be a multistep process requiring the accumulation of both genetic and epigenetic alterations. Aberration of epigenetic regulation is regarded as a common event in human neoplasia and constitutes a functionally equivalent mechanism to classical genetic alterations, such as mutations, deletions and allelic losses. Alterations in methylation patterns of several genes have been observed in almost all cancer types, including solid tumors and hematological malignancies [3,4]. A number of studies indicate that hypermethylation of normally unmethylated CpG islands of tumor-suppressor genes is associated with transcriptional silencing and thus is assumed to play an important role in cancer development and progression [5,6]. In MDS, critical genes, including $p15^{INK4b}$, estrogen receptor (ER), death-associated protein kinase (DAPK), E-cadherin (CDH1), hypermethylated in cancer 1 (HIC1), SOCS1, and FHIT, are inactivated by promoter methylation and the status of methylation is associated with the patient prognosis [7–11]. A better understanding of methylation events in MDS will allow earlier detection, better assessment of prognosis and better prediction of therapy response [12,13].

One important target for methylation analysis in MDS is inhibitor of DNA binding/inhibitor of differentiation (ID). The ID proteins form hetero-dimers with transcription factors and act as dominant negative inhibitors of gene transcription [14]; ID proteins play the important role in early embryonic development [15–17] and are also involved in angiogenesis, lymphocyte development, cell cycle control, and cellular senescence as recently reviewed [14,18,19]. Four human ID proteins have been identified, ID1, ID2, ID3, and ID4. Expression of ID1 and ID2 is increased in several human tumor types, including colorectal adenocarcinoma, pancreatic cancer, and gastric adenocarcinoma [20–22]. Expression of ID3 has been shown to be both up-regulated [20,23] and down-regulated [24,25] in different tumor types. ID4 is the most recently discovered member of ID family. ID4 expression is decreased in a variety of human cancers [26]. The methylation of ID4 has been reported in gastric adenocarcinoma [22], lymphoma [27], breast carcinoma [28,29], Barrett's esophagus and esophageal adenocarcinomas [30]. Aberrant ID4 methylation is a marker of breast cancer recurrence and increased risk of lymph node metastasis in T1 stage breast cancer [29,31] and is associated with histopathological

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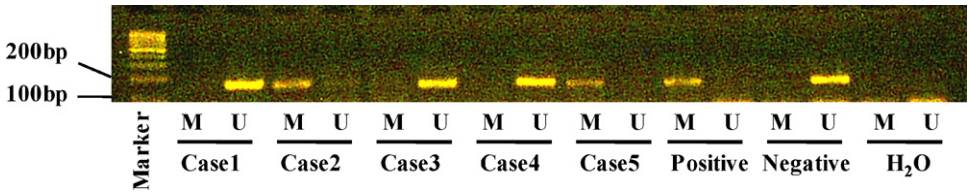

Fig. 1. The methylation-specific PCR of samples from MDS patients. Lanes of M and U represent PCR results by using primers for methylated and unmethylated ID4 gene, respectively. Cases 1–5 are results from five MDS patients. DNA from peripheral blood of healthy donors which was methylated by SssI CpG methylase enzyme was used as the positive control. DNA derived from the human breast cancer cell line MDA-MB231 and distilled water was used as a negative control.

tumor grade in colorectal carcinoma [32] and with tumor progression in prostate cancer [33]. A recent study utilizing a mouse model of acute lymphoblastic leukemia (ALL) of the T/natural killer cell lineage showed that ID4 gene expression was down-regulated by promoter methylation and identified ID4 as a putative tumor-suppressor gene [34]. Zhao et al. reported that the frequency of ID4 methylation in acute leukemia at diagnosis was 84.6%. The status of ID4 methylation in patients with complete remission (CR) correlated with the disease relapse [35].

Nothing is currently known about the methylation of ID4 in MDS patients and the possible clinical significance remains unclear. The purpose of the present study was (i) to examine the status of ID4 methylation in 144 adult de novo MDS cases, (ii) to explore the possible correlation between the ID4 methylation status and clinicopathological variables, and (iii) to determine the prognostic value of ID4 methylation.

2. Materials and methods

2.1. Patients and samples

Bone marrow samples were obtained from 144 adult patients with primary MDS who were diagnosed at eight hospitals in Shanghai between June 2003 and April 2007. The diagnosis was made according to WHO criteria [36]. Patients included 84 males and 60 females with a median age of 64 years (range 18–90 years). According to WHO classifications, eight patients had refractory anemia (RA), four patients had refractory anemia with ring sideroblasts (RARS), 28 patients had refractory cytopenia with multilineage dysplasia (RCMD), 13 patients had RCMD with ring sideroblasts (RCMD-RS), 36 patients had refractory anemia with excess of blasts type 1 (RAEB-1), 47 patients had RAEB type 2 (RAEB-2), six patients had MDS unclassifiable (MDS-U) and two patients had 5q− syndrome. Cytogenetic examination was carried out on samples from 143 patients; the criteria defined by the International System for Human Cytogenetic Nomenclature were used for identification of abnormal clones [37]. The prognostic score for each patient was calculated using the International Prognostic Scoring System (IPSS) [38]. Bone marrow samples from all patients were harvested at the time of diagnosis and bone marrow mononuclear cells were isolated using Ficoll solution and washed twice in PBS. All samples were collected after informed consent had been obtained in accordance with the institutional guidelines of leukemia cooperative group. All patients were followed until death from any cause or until the last follow-up date (4/30/2008). The median follow-up time was 25.1 months (range 5.5–53.2 months). At the last follow-up date, 82 (56.9%) patients had died and 30 (20.8%) patients had progressed to AML.

2.2. DNA isolation and sodium bisulfite conversion

Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit following the manufacturer's instructions. Sodium bisulfite modification of the DNA was performed using the CpGenome™ DNA Modification Kit S7820 (Chemicon International). This kit converts unmethylated cytosine to uracil but does not alter methylated cytosine.

2.3. Methylation-specific PCR (MSP)

For MSP, sodium bisulfite-treated DNA was amplified using either a methylation-specific or a non-methylation-specific primer set. The sequences of the methylation-specific primers were 5′-TAG TCG GAT TTT TCG TTT TTT AGT ATC-3′ (forward) and 5′-CTA TAT TTA TAA AAC CGT ACG CCC CG-3′ (reverse). Sequences of the non-methylation-specific primers were 5′-GGT AGT TG GAT TTT TTGTTT TTT AGT ATT-3′ (forward) and 5′-AAC TAT ATT TAT AAA ACCATA CAC CCC A-3 (reverse) [31]. As a positive control, DNA extracted from peripheral blood of healthy donors was methylated at CpG sites using SssI CpG methylase enzyme (New England Biolabs) according to the manufacturer's recommendations. DNA derived from the human breast cancer cell line MDA-MB231 and distilled water was bisulfite-treated to serve as a negative control [31]. MSP was performed under the following cycling conditions: 95 °C for 5 min; 39 cycles of 95 °C for 1 min; specific annealing 59 °C for 1 min; and 72 °C for 1 min; and a final extension of 7 min at 72 °C. TaKaRa Taq™ Hot Start Version was used in the experiment. The PCR mixture contained 50 ng of bisulfite-treated DNA, 4 μL (2.5 mM) of deoxynucleoside triphosphate mixture, 0.5 μL (20 μM) of each primer, 10× PCR buffer, and 1.25 units of TaKaRa Taq HS in a final volume of 50 μL. PCR was performed in a PTC-200 cycler (Bio-Rad, USA). The amplification products were analyzed on 2.2% agarose gels and visualized under UV illumination.

2.4. Statistical analysis

The correlation between the frequency of ID4 promoter methylation and the clinical and hematologic parameters was analyzed using the x^2 test and Fisher's exact test. Overall survival (OS) was measured from the day of diagnosis until death from any cause or until the last follow-up date (4/30/2008). Leukemia-free survival (LFS) was calculated from diagnosis to progression to acute leukemia or end of follow-up. Patients who died as a result of any cause before leukemic evolution were considered as censored at the time of death. Distributions of OS and LFS curves were estimated according to the Kaplan–Meier method. Comparisons of OS or LFS between groups were evaluated by the log-rank test. The Cox regression model was used for multivariate survival analysis in order to identify the significant independent prognostic factors affecting OS or LFS. For all analyses, the *P*-values were two-tailed and a *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Distributions of ID4 methylation among WHO subtypes

Representative results of methylation-specific PCR are shown in Fig. 1. The sequencing of PCR products from two MDS patients confirmed methylation-specific PCR results. Of the 144 MDS patients evaluated, 51 patients (35.4%) showed ID4 methylation. The distribution of ID4 methylation among WHO subtypes is plotted in Fig. 2. Patients with advanced stage of WHO subtypes (RAEB-1 and RAEB-2) exhibited a significantly higher frequency of ID4 methylation (38/83) compared with those with early stage subtypes (RA, RARS, RCMD, RCMD-RS, 5q− syndrome, and MDS-U; 13/61, $P = 0.002$).

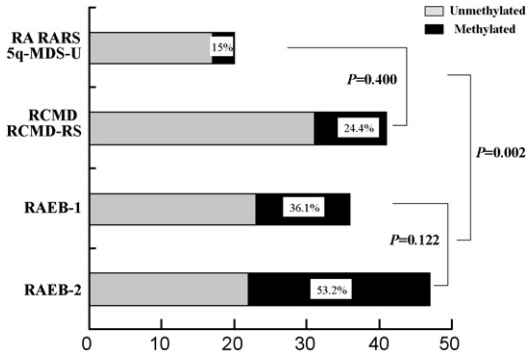

Fig. 2. The distribution of ID4 methylation among WHO subtypes.