The cancer-testis antigen NXF2 is activated by the hypomethylating agent decitabine in acute leukemia cells in vitro and in vivo

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Abstract. Cancer-testis antigens (CTAs) are a group of tumor-associated antigens restricted to male germ cells under normal physiological conditions. CTAs are expressed in certain types of tumors and thus are a novel target for immunotherapy. Nuclear RNA export factor 2 (NXF2) is a CTA of which the expression pattern, regulation and clinical significance are unclear. In the present study, following treatment with a demethylating agent, decitabine, NXF2 expression was detected in the majority of the NXF2-negative acute leukemia cell lines, but not in healthy donor samples. This finding was confirmed by western blot analysis. Eight primary acute leukemia bone marrow samples were treated with decitabine in vitro, and results showed that NXF2 expression was significantly upregulated. In another nine acute myeloid leukemia or myelodysplastic syndrome patients, it was noted that the expression of NXF2 was upregulated in all patients following the first cycle of decitabine, which suggested that NXF2 was activated by decitabine treatment in vivo. Furthermore, NXF2 expression in acute leukemia cells was demonstrated to be regulated by CpG island hypermethylation. To the best of our knowledge, this is the first study to demonstrate that NXF2 is activated by demethylation in acute leukemia cells in vitro and in vivo. NXF2 may therefore serve as a novel target for immunotherapy against acute leukemia.

Introduction

Cancer-testis antigens (CTAs) are a group of antigens that are exclusively expressed in germline cells, such as testis and placental cells under normal physiological conditions. CTAs are also expressed in numerous human tumor cells of various histological origins (1). The first CTA identified in 1991 is termed melanoma-associated antigen 1 (MAGE-A1) (2). In 1994, Weber et al (3) demonstrated that decitabine, a demethylation agent, upregulates MAGE-A1 expression in melanoma cell lines. In 1996, the activation of MAGE-A1 in tumor cells was correlated with genome-wide demethylation (4). Subsequent to this, more CTAs were identified, including PRAME (5), NY-ESO-1 (6) and SSX family antigens (7). Numerous studies concerning the immunogenicity of CTAs and the association between CTAs and demethylation have been conducted (8-11). For example, in acute myeloid leukemia (AML) cell lines, NY-ESO-1 was upregulated by decitabine and thus decitabine-treated AML cells become susceptible to NY-ESO-1-specific T-cell cytotoxicity (8). Those results have suggested CTA to be a potential target for tumor immunotherapy. However, the majority of studies concerning CTAs and decitabine were limited to in vitro investigations and few CTAs have been demonstrated to be activated by decitabine in vivo by clinical decitabine treatment.

The nuclear RNA export factor 2 (NXF2) human gene was first identified in spermatogonia (12). Early studies on NXF2 were focused on its function as an mRNA exporter (13) and its involvement in male infertility (14-16). NXF2 has been found to be positive in 1.8% of invasive ductal carcinomas of the breast (17). Dubovsky et al (18) observed its involvement in chronic lymphocytic leukemia (CLL). Following screening of 22 CLL patients, two were observed to be positive for NXF2-specific IgG antibodies. The presence of these antibodies supported the hypothesis that NXF2 exhibits high enough immunogenicity to induce tumor-specific immune responses (18). However, no studies have been conducted concerning the expression pattern, regulation mechanism and clinical significance of NXF2 in acute leukemia. The aim of the current study was to investigate the expression and epigenetic regulation mechanism of NXF2 in acute leukemia cells, in vitro and in vivo.

Materials and methods

Cell lines and patient samples. Bone marrow and peripheral blood samples were collected from healthy donors and
leukemia patients in the Hematology Department of the Chinese People’s Liberation Army General Hospital, (Beijing, China). Samples of tests were provided by the laboratory of the Urology Department, Peking University Third Hospital (Beijing, China). Written informed consent was obtained from all donors and patients. All experiments were approved by the ethics committee of the Chinese People’s Liberation Army General Hospital. Raji, Z-138, Hut-78, Jurkat, Molt-4, Kasumi-1, NB4, THP-1, U937 and K562 acute leukemia cell lines were obtained from the cell culture center of Peking Union Medical College (Beijing, China). Primary cells were separated from bone marrow or peripheral blood samples using the Ficoll-Paque method (19). Cell lines and primary acute leukemia cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glucose. Cultures were maintained in a 5% CO2-humidified incubator at 37°C. Decitabine (5-aza-2-deoxycytidine, Dacogen, DAC) was purchased from Xi’an Janssen Pharmaceutical, Ltd. (Xi’an, China). For demethylation treatment, decitabine was added to the in vitro culture system at a concentration of 1 µmol/l for 3 days for leukemia cell lines and 5 µmol/l for 3 days for primary leukemia samples (18). For clinical treatment, decitabine was administered alone at a dose of 20 mg/m2/day for 5 consecutive days (20).

RNA isolation, reverse transcription and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from samples or cell lines with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. Total RNA was reverse transcribed to complementary DNA (cDNA) as previously described (21). Reverse transcription and semi-quantitative RT-PCR were conducted on a Veriti® Thermal Cycler (Applied Biosystems, Inc., Foster City, CA, USA). Primers used for semi-quantitative RT-PCR were: Forward: 5’-TGA AAC CCT GCA AGG AAA AC-3’ and reverse: 5’-GCA CTG AGG GAG TCC ACA AT-3’. PCR amplifications were conducted in 20 µl reaction volume with Taq polymerase, and the cycling conditions were as follows: 40 cycles of denaturation for 15 sec at 95°C and annealing for 60 sec at 60°C. NXF2 mRNA expression was determined by 2-ΔΔCT relative to GAPDH.

**Western blot analysis.** Protein extracts from U937 and Raji cells were isolated using immunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA) and quantified to ensure equivalent protein loading. Polyacrylamide gel electrophoresis, tank-based transfer to Immobilon Hybond-C membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and immunodetection were performed as described (22). β-actin antibody was purchased from Abcam (Cambridge, MA, USA) and NXF2 antibody was purchased from Sigma-Aldrich. Signals were visualized using Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore Corporation, Billerica, MA, USA) by exposure to films (Kodak, Rochester, NY, USA).

**Firefly luciferase reporter constructs.** Five promoter regions of the wild-type human NXF2 gene were amplified from one testis sample by PCR using specific primers (Fig. 3A). Primers were designed to contain KpnI and HindIII restriction enzyme sites. Sense primers used were: 5’-CGGGGTACCATGGTGGGCTGAGGGTTG GA -3’, 251 bp to +28 bp for P1; 5’-CGGGGAATCAGGTGGGCTGAGGGTTG GA -3’, 744 bp to +28 bp for P2; 5’-GGGGTACAAGTTGACTTGATAGTG -3’, -1,000 to +28 bp for P3; 5’-GGGGTACCATGGTGGGCTGAGGGTTG GA -3’, -144 bp to +28 bp for P4; and the antisense primer was 5’-CCCAAGCTTCAAGCTTGAGGGGAGGAGCC -3’. The amplified promoter regions were cloned downstream of the firefly luciferase-coding region between KpnI and HindIII of a modified pGL3-control plasmid. Successful construction was confirmed by sequencing.

**Transfection and luciferase assays.** 293T cells were plated in 24-well plates at 5x104 cells/well and grown overnight. Firefly luciferase reporter vector (500 ng) containing different promoter regions of NXF2 and 10 ng control vector pRL-TK (Promega Corporation) containing Renilla luciferase were cotransfected to 293 T cells in a final volume of 0.35 ml using SuperFect (Qiagen, Hilden, German). Cells were collected 48 h following transfection and luciferase activity was measured using a dual-reporter luciferase assay system (Promega Corporation).

**Bisulfite modification and genomic sequencing.** Genomic DNA was extracted from cells using the Wizard Genomic DNA Purification kit (Promega Corporation). DNA (1 µg) was modified with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen). Primers for bisulfite-sequencing analysis were designed by MethPrimer software (San Francisco, CA, USA) (23) using the bisulfite-treated DNA as a template (Forward: 5’-GAGTTTTTAATGTTTGTGTGAG-3’ and reverse: 5’-GGTAGAGGTTTTGATGAGATG-3’). PCR products were gel-purified and cloned into a pGEM-T vector (Promega Corporation). The inserted PCR fragments of individual clones were sequenced using an ABI PRISM DNA sequencing (Applied Biosystems, Inc.).
Results

Activation of NXF2 expression following decitabine treatment in acute leukemia cell lines. NXF2 expression was screened in two normal bone marrow (NBM) samples, two normal peripheral blood samples and two normal testis samples. The results showed that NXF2 was only positively expressed in the testis samples (Fig. 1A). NXF2 expression was screened in 10 acute leukemia cell lines, including five acute myeloid leukemia cell lines (Kasumi-1, NB4, THP-1, U937 and K562) and five acute lymphocytic leukemia cell lines (Raji, Z-138, HuT-78, Jurkat and Molt-4) prior to and following 1 µM decitabine treatment for 3 days in vitro. NXF2 expression was positive in K562 cells and was activated in the majority of other acute leukemia cell lines except NB4 following decitabine treatment. In Raji and U937 cells, upregulation of NXF2 expression following decitabine treatment was confirmed by western blot analysis. β-actin was used as the positive control. In Raji cells, NXF2 activation was decitabine dose-dependent and its expression lasted ≥28 days following the cessation of decitabine treatment. DAC, decitabine-treated cells; NFX2, nuclear RNA export factor 2.

Activation of NXF2 expression following decitabine treatment in primary acute leukemia cells in vitro and in vivo. Primary cells from 11 bone marrow samples, including four AML patients, four ALL patients and three healthy donors were collected. The primary cells were treated with 5 µM decitabine for 3 days in vitro. Relative NXF2 expression (Fig. 2A) was low in the majority of the samples. Following decitabine treatment, NXF2 mRNA expression in all acute leukemia samples was significantly upregulated. However, this upregulation was not observed in healthy donor samples. The results suggested that decitabine treatment activated NXF2 expression in primary acute leukemia cells in vitro.

In addition, another nine patients with AML or myelodysplastic syndrome, who received decitabine treatment clinically were selected. The samples were collected prior to and following the first cycle of decitabine treatment. The
characteristics of these patients are listed in Table I. Samples were obtained from the bone marrow, with the exception of patient 6, which was a peripheral blood sample as the patient had hyperleukocytosis.

As the nine patients did not respond to the first cycle of decitabine, leukemia cells were observed in all of the samples. qPCR results showed that NXF2 expression was upregulated to varying degrees in all nine patients following decitabine treatment (Fig. 2B). This result suggests that decitabine treatment activated NXF2 expression in primary acute leukemia cells in vivo.

NXF2 expression correlates with CpG island hypomethylation in its promoter region. There was a typical CpG island located in the NXF2 promoter region spanning 282 bp and containing 14 CpG sites (Fig. 3A and C). By luciferase reporter construction, it was demonstrated that the promoter region containing the CpG island was essential for NXF2 expression (Fig. 3B). The methylation status of this CpG island in two NBM samples without NXF2 expression in one testis sample and in K562 cells with NXF2 expression was detected. The CpG island was densely-methylated in the two NBM samples (>90%), but only partially methylated in the testis sample and in the K562 cells (Fig. 3D). Raji and U937 cell lines were originally NXF2-negative and were analyzed for the methylation status of the CpG island prior to and following decitabine treatment. The methylation level of the CpG island decreased following decitabine treatment, concurrent with NXF2 activation (Fig. 3E). These results suggest that NXF2 expression was silenced by CpG island hypermethylation; thus, decitabine activated NXF2 expression by the demethylation mechanism.

**Discussion**

NXF2 is a CTA gene, however, there is limited information available concerning its expression pattern and the mechanism of NXF2 regulation. The present study showed that NXF2 expression was activated by a demethylating agent, decitabine, in acute leukemia cell lines and primary acute leukemia samples in vitro and in vivo. This study also showed that NXF2 expression is regulated by CpG island hypermethylation in its promoter region. To the best of our knowledge, this is the first study to demonstrate the mechanism of NXF2 regulation in acute leukemia cells.

Currently, certain CTAs have been demonstrated to be potential targets for immunotherapy against cancer due
to their tumor specificity and strong immunogenicity. For example, Hunder et al (9) expanded autologous CD4+ T-cell clones specific for NY-ESO-1 (a CTA). Specific T cells were infused into a patient with refractory metastatic melanoma and a durable clinical remission was observed. In another study, Quintarelli et al (24) showed that cytotoxic T lymphocytes specific for PRAME, another type of CTA, target leukemic and leukemic-precursor cells. It was also demonstrated that NXF2 exhibits a high enough level of immunogenicity to induce immune responses in CLL patients (18). However, the application of CTA-specific immunotherapy is limited due to a relatively low expression in acute leukemia. It was suggested that this limitation was able to be overcome by demethylation treatment. Yan et al (25) demonstrated that, following demethylation treatment, the expression of PRAME in the ALL cell line (Raji cells) was upregulated. These Raji cells with greater PRAME expression showed increased sensitivity to killing by formerly low avidity PRAME-specific T-cell clones. However, the majority of similar studies are performed in vitro and few studies have observed that CTAs were activated in malignant

Table I. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Chromosome</th>
<th>Gene abnormalities</th>
<th>Diagnosis</th>
<th>Response</th>
<th>DAC cycles</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>M</td>
<td>46,XY</td>
<td>-</td>
<td>MDS-RAEB</td>
<td>Failure</td>
<td>3</td>
<td>Progressed to AML after 3 cycles</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>M</td>
<td>46,XY,12p- inc[10]/, hypodiploid(43-45)</td>
<td>NUP98/ HOXA9(+)</td>
<td>AML (MDS transformed)</td>
<td>SD</td>
<td>8</td>
<td>Still in DAC maintenance</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>M</td>
<td>46,XY</td>
<td>-</td>
<td>AML (MDS transformed)</td>
<td>SD</td>
<td>5</td>
<td>Quit DAC treatment after the 5th cycle</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>F</td>
<td>46,XX</td>
<td>-</td>
<td>AML (MDS transformed)</td>
<td>PD</td>
<td>3</td>
<td>Quit DAC treatment after the 3rd cycle</td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>F</td>
<td>46,XX</td>
<td>GIT2/ PDGFRB(+)</td>
<td>AML (CMML transformed)</td>
<td>PD</td>
<td>3</td>
<td>This patient received TKI treatment first. DAC was added in combination with TKI when her CMML progressed to AML. She quit DAC treatment after the 3rd cycle and received allo-HSCT</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>AML-M6</td>
<td>SD</td>
<td>7</td>
<td>Quit DAC treatment and received allo-HSCT after the 7th cycle</td>
</tr>
<tr>
<td>7</td>
<td>59</td>
<td>F</td>
<td>46,XY</td>
<td>-</td>
<td>AML (MDS transformed)</td>
<td>CR</td>
<td>14</td>
<td>CR after the 7th cycle, but relapsed after the 14th cycle, quit DAC treatment after relapse</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>M</td>
<td>46,XY</td>
<td>P15 hyper-methylated</td>
<td>AML (MDS transformed)</td>
<td>CR</td>
<td>12</td>
<td>CR after the 5th cycle, but relapsed after the 10th cycle, quit DAC treatment after relapse.</td>
</tr>
<tr>
<td>9</td>
<td>82</td>
<td>F</td>
<td>46,XX</td>
<td>-</td>
<td>AML (MDS transformed)</td>
<td>PR</td>
<td>10</td>
<td>PR after the 4th cycle, but turned to AML after the 7th cycle, another 3 cycles of DAC after progression did not work</td>
</tr>
</tbody>
</table>

All samples were bone marrow samples except for patient 6. The samples collected from patient 6 were peripheral blood samples because the patient had hyperleukocytosis. M, male; F, female DAC, decitabine ; MDS, myelodysplastic syndrome; RAEB, refractory anemia with excess blasts; AML, acute myeloid leukemia; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; CMML, chronic myelomonocytic leukemia; TKI, tyrosine-kinase inhibitor; allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML-M6, acute erythroid leukemia.
cells \textit{in vivo} by clinical decitabine treatment. In the present study it was demonstrated that NXF2 was activated in acute leukemia cells not only \textit{in vitro}, but also \textit{in vivo} by clinical decitabine treatment. It was hypothesized that NXF2 served as a novel target for immunotherapy against acute leukemia following clinical demethylation treatment. However, as no epitope sequence of NXF2 has yet been identified, it was difficult to determine the details of NXF2-specific immune responses following decitabine treatment. Thus, future studies are required to investigate the epitope of NXF2.

In conclusion, to the best of our knowledge, this was the first study to demonstrate that NXF2 is activated by decitabine in acute leukemia cells \textit{in vitro} and \textit{in vivo}; this activation was due to demethylation of the CpG island in the NXF2 promoter region. According to these results, it was hypothesized that NXF2 may serve as a novel clinical target for immunotherapy against acute leukemia.

Acknowledgements

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Increased PRAME: A multicenter phase II + De novo duplication of PRAME, a gene
Inactivation of Nxf2 causes - -
- Treatment of metastatic A gene -

References