Identification of RHOXF2 (PEPP2) as a cancer-promoting gene by expression cloning

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Abstract. Multiple mutations contribute to establish cancers. We have searched for potential oncogenes by screening cDNA libraries derived from gastric cancer cell lines, pancreatic cancer cell lines and glioma cell lines, using retrovirus-mediated expression cloning. Two types of interleukin-3 (IL-3)-dependent cell lines, Ba/F3 and HF6, were transduced with the cDNA libraries and several genes that render these cells factor-independent were identified including PIM-1, PIM-2, PIM-3, GADD45B and reproductive homeobox genes on the X chromosome gene F2 (RHOXF2). Although no mutation in these genes was found, these molecules were highly expressed in cancer cell lines and they may play important roles in cell transformation. Among them, we focused on a transcriptional repressor RHOXF2. Transduction of RHOXF2 rendered HF6 cells factor-independent, while knockdown of RHOXF2 inhibited growth of the HGC27 gastric cancer cell line which highly expresses RHOXF2. In addition, RHOXF2-transduced HF6 cells quickly induced leukemia when transplanted into sublethally irradiated mice. Moreover, RHOXF2 is highly expressed in some leukemia cell lines and a variety of human cancer samples including colon and lung cancers. Thus, these results indicate that RHOXF2 is involved in carcinogenesis.

Introduction

Cancer develops through a multistep process involving a variety of gene alterations and epigenetic changes (1,2). Genes that are commonly altered include Rb, p16INK4a, VHL, Ras, p53 and APC (2,3). Fusion genes resulting from chromosomal translocations are established hallmarks of hematopoietic malignancies and include BCR-ABL, MLL-fusions and AML1-ETO (6). More recently, fusion genes have also been identified in solid tumors, for example EML4-ALK in lung cancer (4), EWS-FLI-1 in Ewing's sarcoma (5) and ETV6-NTRK3 in fibrosarcoma and breast cancer (6). Oncogenic mutations were originally identified using transfected NIH3T3 cells (7) in the focus-forming assay. A combination of the focus-forming assay with inoculation of NIH3T3 cells in vivo later increased the sensitivity to detect oncogenic potential (8). Recently, Soda et al identified a fusion gene EML4-ALK in non-small cell lung cancer by retrovirus-mediated expression cloning using transformation of the NIH3T3 cells as an assay (4). EML4-ALK is now a promising molecular therapeutic target; ALK inhibitors proved to be effective in the therapy for non-small cell lung cancer patients bearing this fusion gene (9). Detection of oncogenes has largely depended on the classical focus-forming assay with NIH3T3, which mainly detects activated mutations of Ras and gene alterations that activate the Ras pathway (7). To detect different classes of oncogenic mutations, different assays are likely to be required.

In this study, two hematopoietic cell lines, Ba/F3 (10) and HF6 (11), were used to search for potential oncogenes in several cancer types. Both cell lines are interleukin-3 (IL-3)-dependent early myeloid cells, but to become factor-independent, Ba/F3 requires JAK-STAT signaling and HF-6 requires Ras activation (11-13). Genes were tested for the ability to render these cell lines factor-independent. Using this strategy, several genes from cDNA libraries of gastric cancer cell lines, pancreatic cancer cell lines and glioma cell lines were identified including PIM-1, PIM-2, PIM-3, GADD45B and RHOXF2. Although mutations in these genes were not found, overexpression of PIM-1, PIM-2, PIM-3, GADD45B and RHOXF2 rendered HF6 cells factor-independent, confirming the validity of the functional screening. Of the genes identified, we have focused on a homeobox protein, reproductive homeobox genes on the X chromosome F2 (RHOXF2), also known as PEPP2. Homeobox proteins share a DNA-binding homeodomain motif of 60 amino acids, and are transcription factors that regulate development. The Rhox genes belong to a recently discovered homeobox family whose members are clustered on the X chromosome (14-16). Whereas the human RHOX family consists of three members, RHOXF1, RHOXF2 and RHOXF2B, the murine
Rhox family consists of >30 members (14-16). Rhox proteins are expressed in germ cells, embryonic cells and somatic cells of reproductive tissue, and play important roles in embryonic, postnatal and adult development, especially of the male and female reproductive systems. Recently, human and mouse Rhox protein expression in some cancer cells including breast and colon cancer was reported (16), and expression of a mouse Rhox family member, Rhox5, was found in 50-65% of cancer cells (17,18). Interestingly, Rhox protein expression is regulated by epigenetic mechanisms, and the treatment with DNA methyltransferase inhibitors, such as decitabine, induces Rhox expression (16). These findings indicate that Rhox proteins are involved in carcinogenesis. In the present study, it was found that knockdown of RHOXF2 attenuated the growth of a gastric cancer cell line HGC27 and overexpression of RHOXF2 in HF6 cells rapidly induced leukemia in transplanted mice. These results support a role for RHOXF2 in cell transformation.

Materials and methods

Cells. Human gastric cancer cell lines (HGC27, GCIY, KATOIII, MKN45, OCUM-1, AGS, MKN1, MKN7, MKN45, MKN74, NUGC3, SNU719, TMK-1), human pancreatic cancer cell lines (Bx-PC-3, AsPC-1, capan1) and human glioma cell lines (U87MG, T98G, U251) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Ecotropic and amphotropic retrovirus packaging cell lines, PLAT-E and PLAT-A respectively, were maintained in DMEM supplemented with 10% FCS, 1 µg/ml puromycin and 10 µg/ml blasticidin (19). A murine pro-B cell line Ba/F3 was maintained in RPMI-1640 with 10% FCS and 1 ng/ml mouse IL-3 (mIL-3), and a murine myelomonocytic cell line HF6 was maintained in RPMI-1640 with 20% FCS and 10 ng/ml mIL-3 (11).

Plasmids and primers. The coding region of RHOXF2 was amplified from HGC27 cDNA by PCR using Phusion polymerase (Finnzymes, Oy, Finland), then subcloned into pMXs-puro or pMXs-IG vectors (19,20) and the sequence was confirmed. Primers for RHOXF2 used in RT-PCR were designed using the siRNA Target Finder (Ambion Inc., Austin, USA) and 1 µg/ml puromycin and 10 µg/ml blasticidin (19). A murine pro-B cell line Ba/F3 was maintained in RPMI-1640 with 10% FCS and 1 ng/ml mouse IL-3 (mIL-3), and a murine myelomonocytic cell line HF6 was maintained in RPMI-1640 with 20% FCS and 10 ng/ml mIL-3 (11).

To generate shRNA vectors, first two oligonucleotides were designed using the siRNA Target Finder (Ambion Inc., Austin, USA) and were synthesized as follows; RHOXF2-1 (sense: ggcacagcacaaggagaat, antisense: ttcttcgtctgtagctgc) and RHOXF2-2 (sense: gcagcacaatggagagc, antisense: ttcttcgtctgtagctgc). These oligonucleotides were annealed and ligated to the pReps vector, which was kindly provided by T. Hara (21). Control shLuc vector targeting firefly luciferase was constructed in the same way.

Generation of anti-RHOXF2 antibody. Anti-RHOXF2 polyclonal antibody was generated from the serum of rabbits immunized with the purified GST-RHOXF2 fusion protein (Scrum Inc., Tokyo, Japan).

Retroviral gene transduction. Retroviral transduction was performed as described (19,20). Briefly, ecotropic and amphotropic retroviruses were generated by the packaging cell lines Plat-E and Plat-A, respectively, and then transiently transfected with a pMXs-based construct using FuGENE 6 reagent (Roche Applied Science, Basel, Switzerland). Two days after transfection, ecotropic and amphotropic retroviruses were collected and used to infect mouse HF6 cells or human HGC27 cells, respectively.

Screening of cDNA libraries. pMXs-based cDNA libraries were generated from gastric cancer cell lines, pancreatic cancer cell lines and glioma cell lines, as previously described (13). Each cDNA library was retrovirally introduced into two IL-3-dependent cell lines, Ba/F3 and HF6. Two days after transduction, the cells were seeded into 96-well plates in the absence of IL-3 in order to select factor-independent clones. To identify the cDNA that conferred Ba/F3 or HF6 cells with factor-independency, the integrated cDNAs were isolated from factor-independent clones by genomic PCR and were sequenced using primers specific to the retroviral vector sequence.

Soft agar colony formation. One thousand cells were suspended in 0.6% agar supplemented with complete culture medium. This suspension was layered over 1.2% agar-medium bottom layer in 35-mm dishes. After 14 days, colonies were counted.

Mouse bone marrow transplantation (BMT). One million HF6 cells transduced with empty (mock) or RHOXF2-containing retroviral vector were injected into the tail vein of sub-lethally irradiated (5.25 Gy) C57BL/6 (Ly-5.1) 8-week-old mice. Overall survival of the transplanted mice was analyzed using the Kaplan-Meier method. Animal studies were conducted in accordance with the guidelines of the Animal Care Committee at the Institute of Medical Science, University of Tokyo.

Generation of tissue microarray. Tissue microarrays (TMA) were generated from 1150 cases of 14 common cancer types as previously described (22). In addition, TMA composed of 280 non-neoplastic adjacent tissues from the same patients were made using identical methods. Each sample of TMA was cut in 4-µm section for immunohistochemical analysis. Tissue microarray methods in the present study were approved by the Ethics Committee of Toyama University (no. 19-12).

Immunohistochemical analysis of tissue microarrays. Immunohistochemical detection of RHOXF2, and statistical analyses of results were carried out as previously described (22). In short, after deparaffinization and rehydration, sections were processed for heat-induced antigen retrieval at 125˚C and incubated with diluted antibody (1:500) for 30 min at room temperature. Antibody staining was visualized using the Envision+ system (Dako, Kyoto, Japan) and diaminobenzidine.

Nuclear staining was scored according to 4 grades (0, none; 1, mild; 2, moderate; 3, marked). Scores 0 and 1 were considered negative while scores 2 and 3 were considered positive. RHOXF2 staining of sections of an embedded pellet of HF6 cell line and colonic mucosa were used as positive and negative staining controls, respectively (data not shown).

Results

Expression cloning of potential oncogenes from cancer cell lines. cDNA was generated from gastric cancer cell lines,
pancreatic cancer cell lines and glioma cell lines as described, and a cDNA library was constructed from the mixed cDNAs from each cancer in a retrovirus vector pMXs (20) (Table I). The resulting library was introduced into IL-3-dependent Ba/F3 cells and HF6 cells via retrovirus infection, and the infected cells were cultured in the absence of IL-3 to identify potential oncogenes that render the cells factor-independent. Several factor-independent clones were established from HF6 transfectants and PIM-1, PIM-2, PIM-3, GADD45B and RHOXF2 were isolated from these clones by genomic PCR (Table II). These genes were introduced into HF6 cells and the ability of these genes to confer HF6 cells with autonomous growth was confirmed (data not shown). However, no mutation was found in these genes, indicating that overexpression of these genes alone could transform HF6 cells.

Expression of RHOXF1 and F2 in a variety of cell lines. The Rhox family consists of 30 members in mice and 3 members in humans, RHOXF1, F2 and F2B. Interestingly, expression of RHOXF1 and F2 was almost mutually exclusive in gastric cancer cells examined (Fig. 1A). HGC27 (undifferentiated carcinoma), GCIY (poorly differentiated adenocarcinoma) and TMK-1 (poorly differentiated adenocarcinoma) and TMK-1 (poorly differentiated adenocarcinoma) cell lines express RHOXF2 but not RHOXF1. On the other hand, GCIY (scirrhous), MKN1 (adenoc-squamous), MKN7 (well differentiated adenocarcinoma), MKN74 (moderately differentiated adenocarcinoma) and SNU719 (well differentiated adenocarcinoma) cell lines express RHOXF1 but not RHOXF2. These results suggested complementary roles for RHOXF1 and F2.

Table I. Characteristics of c-DNA libraries.

<table>
<thead>
<tr>
<th></th>
<th>Gastric Cancer</th>
<th>Pancreatic cancer</th>
<th>Glioma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td>HGC-27, OCUM-1, MKN45, GCIY, KATOIII</td>
<td>BxPC-3, AsPC-1, capan1</td>
<td>U87MG, T98G, U251</td>
</tr>
<tr>
<td>Library size</td>
<td>4.5x10⁶</td>
<td>3x10⁶</td>
<td>3x10⁶</td>
</tr>
<tr>
<td>Average length of cDNAs (kb)</td>
<td>1.2</td>
<td>1.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The name of the cell lines used for making each library (cell lines), the numbers of independent cDNA clones of each library (library size), and the average length of the cDNAs included in each library (average length of cDNAs) are described.

Table II. Details of the isolated clones from screening.a

<table>
<thead>
<tr>
<th></th>
<th>Gastric</th>
<th>Pancreatic</th>
<th>Glioma</th>
</tr>
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<tbody>
<tr>
<td>Total isolated clone</td>
<td>5</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>The clones with full ORF</td>
<td>PIM-1,3, RNF67, RHOXF2</td>
<td>PIM-1, GADD45B</td>
<td>PIM-2,3, CSF3, GADD45B</td>
</tr>
<tr>
<td>The clones with partial ORF</td>
<td>TENC1, RAF1</td>
<td>FOXM1, ATXN1</td>
<td></td>
</tr>
<tr>
<td>The candidate oncogenes</td>
<td>PIM-1,3, RHOXF2</td>
<td>PIM-1, GADD45B</td>
<td>PIM-2,3, GADD45B</td>
</tr>
</tbody>
</table>

aThe clones isolated from 3 different c-DNA libraries are shown. The clones consist of two categories, the clones with full length of open reading frame (ORF) of cDNAs (the clones with full ORF) and the clones with partial sequence of ORF (the clones with partial ORF). The names of the genes which the clones of each category from 3 kinds of libraries contain, are shown in the table. Among the genes from the clones with full length ORF, some genes rendered HF6 factor-independent and the names of the genes are indicated in the table (the candidate oncogenes).

Expression of RHOXF2 in cancer cell lines. Expression of RHOXF2 was analyzed by RT-PCR. The expression in gastric cancer cell lines (A) and cell lines derived from hematological malignancies (B) are shown.
The undifferentiated to poorly differentiated gastric cancers tended to express RHOXF2, while the adenocarcinoma cell line AGS appeared to express both RHOXF1 and F2.

RHOXF2 was also highly expressed in the human immature leukemic cell lines including TF-1 and K562 (Fig. 1B). It was also expressed in the other human hematopoietic cell lines, MOLM13, UT-7, HL60, MHC-1 and Jurkat (Fig. 1B).

Effects of overexpression of RHOXF2 in HF6 cells. HF6 cells that were manipulated to express RHOXF2 proliferated autonomously in vitro in the absence of IL-3 (data not shown). Next the effects of RHOXF2 overexpression in vivo were examined using a mouse BMT model. While unmanipulated HF6 cells did not induce disease in 5 months after the transplantation into sub-lethally irradiated mice, HF6 cells expressing RHOXF2 rapidly induced leukemia-like disease (Fig. 2A). In these mice, the leukemic cells infiltrated to the liver and spleen, causing hepato-splenomegaly (Fig. 2B).

Effects of RHOXF2 knockdown in HGC27. Next, the effects of RHOXF2 knockdown were evaluated. To achieve this, shRNA specific for RHOXF2 was retrovirally introduced into HGC27 gastric cancer cells which express RHOXF2 at high levels (Fig. 3A). While the control shLuciferase did not inhibit the expression of RHOXF2, two RHOXF2 sh-RNA constructs efficiently suppressed the expression of RHOXF2 in HGC27 cells (Fig. 3A). Knockdown of RHOXF2 inhibited the colony forming ability of HGC27 cells in soft agar (Fig. 3B) and the growth rate in a liquid culture (Fig. 3C), implicating RHOXF2 in transformation and cell growth.

Expression of RHOXF2 in a multi-cancerous tissue microarray. Expression of RHOXF2 in a variety of cancers and non-neoplastic tissues was evaluated using two TMA blocks (Fig. 4). RHOXF2 was not highly expressed in any of the normal tissues tested except for thyroid. RHOXF2 was highly expressed in some of the cancers including lung squamous cell carcinoma, thyroid tumors, colon, breast, gastric, prostate, ovarian and uterine corpus cancers.
Discussion

In the present work, a transcriptional repressor RHOXF2 has been identified as a potential oncogene by functional expression cloning. Knockdown of RHOXF2 inhibited the growth and colony forming ability of the gastric cancer cell line HGC27. On the other hand, overexpression of RHOXF2 conferred IL-3-dependent HF6 cells with autonomous growth and leukemogenicity in vivo. In addition, RHOXF2 expression was found in a variety of cancer tissues. Recently, several studies have reported that expression of RHOXF2 is regulated by some epigenetic mechanisms, especially in cancer tissues (16). These findings suggest that RHOXF2 plays a role in tumorigenesis and that it can be controlled epigenetically. To elucidate the molecular mechanisms, further studies using gene chips and bioinformatics are required to identify the responsive genes downstream of RHOXF2. HF6 is a mouse bone marrow-derived IL-3-dependent cell line immortalized by the fusion gene MLL-SEPT6, and activation of the Ras pathway in these cells can lead to transformation (11,13). Ras activation frequently plays an important role in carcinogenesis and it is possible that genes repressed or enhanced by RHOXF2 are involved in this pathway.

It is now recognized that as in solid tumors, multiple mutations are involved in the development of hematopoietic malignancies. These mutations are classified into two groups; class I mutations include activating mutations of tyrosine kinases and oncogenes, and inactivating mutations of tumor suppressor genes such as p53, NF1, while class II mutations include dominant-negative mutations of transcription factors and chromatin modification enzymes (23-25). We have previously demonstrated that a class II mutation MLL-SEPT6 could immortalize mouse bone marrow cells by blocking the differentiation of hematopoietic progenitors (11-13). However, these immortalized cells still require interleukin 3 (IL-3) for their growth. Interestingly, class I mutations such as those that activate Ras signal or constitutively active receptors tyrosine kinase mutants, like FLT3-ITD, were able to fully transform the IL-3-dependent HF6 cells immortalized by class II mutations (13).

The assay system using NIH3T3 cells has been the gold standard for screening of oncogenes, however this system mainly detects activators of the Ras pathway (7). For this reason, different cell types have been needed to be tested in the search for the oncogenic mutations. In the present study, HF6 cells and Ba/F3 cells were used. HF6 cells and Ba/F3 cells require activation of the Ras pathway and the JAK-STAT pathway, respectively, for the full transformation. In addition, we have established a cell line HF8 cells by introducing Hes1 to bone marrow progenitors. Neither Ras nor JAK/STAT activation could confer HF8 cells with autonomous growth (unpublished observation), indicating that these IL-3-dependent cell lines examined have distinct signaling profiles. The use of different cell lines could therefore, identify different classes of mutations. In the present study, PIM-1, PIM-2, PIM-3, GADD45B and RHOXF2 were shown to render HF6 cells factor-independent, demonstrating the potential for using cells of hematopoietic origin to identify oncogenic mutations. It is possible that different types of mutations and translocations could be identified, using distinct types of IL-3-dependent cell lines.

In conclusion, the transcriptional repressor RHOXF2 is expressed in a variety of cancers, and plays a critical role in tumorigenesis. The use of IL-3-dependent cell lines for screening cDNA libraries has potential as a strategy in the search for oncogenes.

Acknowledgements

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References


Figure 4. Tissue microarray analysis. Immunohistochemical analysis of RHOXF2 expression in multi-tissue array is shown. (A and B) The array contains 280 non-neoplastic tissue samples and 1150 tumor tissue samples from 14 major types of cancers. Each column represents each case and the number in the column indicates the score corresponding to the level of RHOXF2 expression.

A Normal Tissue Microarray

B Cancer tissue Microarray

Lung Lung Kidney Thyroid Colon Stomach
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Lung Lung Kidney Thyroid Colon Stomach
Lung Lung Kidney Thyroid Colon Stomach
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