Rearrangements and Amplification of IER3 (IEX-1) Represent a Novel and Recurrent Molecular Abnormality in Myelodysplastic Syndromes

David P. Steensma,1 Jessemey D. Neiger,1 Julie C. Porcher,1 J. Jonathan Keats,2 P. Leif Bergsagel,2 Thomas R. Dennis,1 Ryan A. Knudson,1 Robert B. Jenkins,1 Rafael Santana-Davila,1 Rajiv Kumar,1 and Rhett P. Ketterling1

1Mayo Clinic, Rochester, Minnesota; 2Mayo Clinic, Scottsdale, Arizona; and 3Translational Genomics Research Institute (TGen), Phoenix, Arizona

Abstract
IER3 (formerly IEX-1) encodes a 27-kDa glycoprotein that regulates death receptor–induced apoptosis, interacts with NF-κB pathways, and increases expression rapidly in response to cellular stresses such as irradiation. Animal models, gene expression microarray experiments, and functional studies in cell lines have suggested a potential role for IER3 in oncogenesis, but, to date, no abnormalities of IER3 at the DNA level have been reported in patients with neoplasia. Here, we describe breakpoint cloning of a t(6;9)(p21;q34) translocation from a patient with a myelodysplastic syndrome (MDS), facilitated by conversion technology and array-based comparative genomic hybridization, which revealed a rearrangement translocating the IER3 coding region away from critical flanking/regulatory elements and to a transcript-poor chromosome region, markedly decreasing expression. Using split-signal and locus-specific fluorescence in situ hybridization (FISH) probes, we analyzed 204 patients with diverse hematological malignancies accompanied by clonal chromosome 6p21 abnormalities, and found 8 additional patients with MDS with IER3 rearrangements (translocations or amplification). Although FISH studies on 157 additional samples from patients with MDS and a normal-karyotype were unrevealing, and sequencing the IER3 coding and proximal promoter regions of 74 MDS patients disclosed no point mutations, reverse transcription-PCR results suggested that dysregulated expression of IER3 is common in MDS (61% >4-fold increase or decrease in expression with decreased expression primarily in early MDS and increased expression primarily in later MDS progressing toward leukemia), consistent with findings in previous microarray experiments. These data support involvement of IER3 in the pathobiology of MDS. [Cancer Res 2009;69(19):7518–23]

Introduction
IER3 (immediate early response 3), formerly known as IEX-1 or p22/PRG1, was cloned from a squamous carcinoma cell line in 1996 and localized to chromosome 6p21.3 (1). IER3 is expressed in a broad range of human tissues, including hematopoietic cells, and encodes a 156 amino acid (27 kDa glycosylated) protein so named because intracellular levels increase rapidly following ionizing radiation exposure, with maximal expression at ~15 min postexposure (1, 2). Death receptor agonists [Fas ligand and tumor necrosis factor α (TNF-α)] also induce IER3 expression, as does the protein kinase C activator 12-O-tetradecanoylphorbol-13-acetate, retinoic acid, hydroxytamoxifen, or etoposide, and cellular stresses such as mechanical stretching or viral infection (3, 4).

The NF-κB pathway seems to be important in mediating the intracellular effects of IER3; blockade of NF-κB activation abolishes TNF-α–induced IER3 expression and augments TNF-α–induced cell death (5, 6). IER3 expression is under the control of tumor suppressor p53 and transcription factors Sp1 and c-Myc, which bind to the promoter region (7). Although IER3 has minimal homology to other factors, domains responsible for proapoptotic and antiapoptotic effects of IER3 under differing cellular stresses have been defined (8).

Several observations suggest that IER3 may play a role in neoplasia (3). Changes in IER3 expression alter apoptosis sensitivity (including susceptibility to death receptor ligation) and cell cycle progression or proliferation rates. In mice engineered to over-express IER3 in immune cells, a lupus-like syndrome resulted and T-cell lymphomas developed in the spleen and lymph nodes (9, 10). In contrast, IER3−/− mice have hypertension, thickened epidermis with an increased number of cellular layers, and cardiac hypertrophy without immunologic abnormalities (11). Global gene expression microarray experiments in diverse primary cancer cells (12, 13), CD34+ cells from patients with MDS (14, 15), and transformed cell lines [e.g., Colo320 colorectal (16) and MCF-7 breast (17) carcinoma cells] have identified IER3 as an expression outlier that may have prognostic importance (13, 15).

Despite these observations, no mutations or genomic rearrangements involving IER3 have been reported. Here, we describe IER3 rearrangements and amplification for the first time, in nine patients diagnosed with myelodysplastic syndromes (MDS).

Materials and Methods

Propositus. G-banded karyotyping of sequential marrow samples from a 70-y-old man with refractory cytopenia with multilineage dysplasia (RCMD) showed persistent t(6;9)(p21.3q34), in ~18 of 20 metaphases (Fig. 1A). Testing with fluorescent in situ hybridization (FISH) probes for genes previously implicated in myeloid neoplasia localized to the 6p21 and 9q34 regions (i.e., DEK, NUP214/CAN, and ABL1 oncogenes) disclosed no rearrangements. Analysis of nonhematopoietic tissue confirmed the translocation was
Figure 1. A, ideogram of t(6;9)(p23.1;q34), shown to rearrange IER3, in a patient with MDS. Left, normal chromosomes 6 and 9; right, derivative chromosomes seen in the propositus. B, aCGH demonstrating change in DNA copy number for chromosome 6p, centered on IER3, in a patient with MDS. Green, sample DNA, representing intensity of fluorescein signal (proportional to DNA copy number); red, control DNA, representing intensity of Texas red signal. The IER3 gene is the closest to the apparent breakpoint on chromosome 6p. aCGH narrowed the breakpoint to within an 800-bp region on chromosome 6p (which included the IER3 transcript initiation site) and 13 kbp on chromosome 9q. C and D, sequencing chromatogram of breakpoints on derivative chromosome 6p (C) and derivative chromosome 9q (D). Sequencing-defined breakpoints were at bp 137,350,819 on chromosome 9 and 30,820,344 on chromosome 6 (National Center for Biotechnology Information build 36.3), with 1 bp ambiguity due to a repeated nucleotide. Sequencing was performed in both directions. Southern blotting confirmed that this was a simple, balanced reciprocal translocation.

IER3 in MDS
clonally restricted, not constitutional. Because isolated translocations are uncommon in MDS (interstitial deletions and numerical chromosomal abnormalities are more typical), this case seemed likely to be molecularly informative, so we cloned the breakpoint and sought similar rearrangements in other patients. The study protocol was approved by the Institutional Review Board of Mayo Clinic, and patients consented to sample collection and molecular analysis.

**Somatic hybrid cell lines and chromosome isolation.** Using an established conversion technology (18), somatic mouse/human hybrid cell lines were generated for each of the derivative chromosomes [i.e., der(6) and der(9)] and normal chromosomes 6 and 9 from the propositus. Briefly, after electrofusion of propositus leukocytes with E2 murine cells, mouse/human hybrid colonies were grown in selection medium (86% DMEM, 10% fetal bovine serum, 1% hypoxanthine-aminopterin-thymidine supplement 1% G418, 1% penicillin-streptomycin, and 1% minimal essential medium with nonessential amino acids; Invitrogen), selected at day 19 for initial expansion and genotyping, and subcultured. The presence of the derivative chromosomes in clones was confirmed with microsatellite markers [D6S1610, D6S441, D6S1581, and D6S264 and D9S1838 for der(6); D9S157, D9S1817, D9S283, D9S1677, D6S309, D6S1574 for der(9)]. Clones bearing >40% cells of interest, defined by these markers, were cryopreserved. Derivative and normal comparator chromosomes were then individually isolated by laser capture microdissection.

**Array-based comparative genomic hybridization.** High-resolution array-based comparative genomic hybridization (aCGH) was performed with a custom 4×44K Human Genome CGH Microarray kit (for derivative chromosomes 6p and 9q) and an off-the-shelf 244A kit (whole genome; Agilent Technologies), following the protocol suggested by the manufacturer. Briefly, 800 ng of sample and control DNA, amplified from microdissected chromosomes using degenerate oligonucleotide primers, were labeled with Cy5 or Cy3 (BioPrime Array CGH Genomic Labeling Module, Invitrogen) and purified using Vivaspin 500 columns (Sartorius Stedim Biotech). Equal amounts of test and reference DNA were hybridized to the microarray at 65°C for 40 h in a rotation oven at 20 rpm. Slides were washed and then scanned with the G2505B DNA microarray scanner (Agilent). The microarray images were analyzed using Feature Extraction software V8.1 (Agilent), and Log2-transformed ratio data were analyzed with GeneSpring GX V7.3.1 and CGH Analytics V3.4.27 (Agilent).

![Figure 2. FISH using breakapart probe set for IER3 in MDS samples. A, interphase preparation from patient with t(6;9)(p23.1;q34) demonstrating separation of red signal and green signal. Only a single fusion signal is present from the normal chromosome 6. B, metaphase cells from the same patient. C and D, two other MDS patients in whom IER3 signal was amplified, indicating increased copy number.](image-url)
Southern blotting. To determine whether the rearrangement in the propositus was a simple translocation of IER3 or more complex, DNA extracted from der(6) and der(9) somatic hybrid cells, propositus-derived whole marrow cells, and control marrow cells were digested with BamHI and EcoRI restriction endonucleases (New England Biolabs), subject to agar gel electrophoresis for 24 h, blotted overnight on a nylon membrane, cross-linked with UV radiation, hybridized with a radiolabeled IER3 probe and salmon sperm blocking DNA, washed, and visualized by autoradiography. The IER3 probe was generated via PCR with primers 5′-TCC GGG CTT CCA AAG ATA CAC T-3′ and 5′-GGG GGG CAA AAA TAC CAT A-3′, cloned into MaxEfficiency DH5α competent cells (Invitrogen), labeled with P32-dCTP using a TOPO PCR Cloning kit (Invitrogen), and purified before hybridization.

DNA sequencing. After narrowing of the propositus’ chromosome 6p and 9q breakpoints via aCGH, both breakpoint regions were amplified directly by long-range PCR, using a series of primer pairs spaced 0.5- to 1-kb apart. Amplicons detected by ethidium-bromide-impregnated agarose gel electrophoresis were then sequenced using fluorescent dye chemistry sequencing to explicitly define the rearrangement (ABI Prism 3730 DNA Analyzer, Applied Biosystems), with chromatograms compared with NC_000009.10, NC_000006.10 and NM_003987 (IER3) using Sequencher 4.5 software (Gene Codes).

To assess for point mutations in the propositus and other patients with MDS with and without 6p rearrangements, genomic DNA purified with QIAamp DNA Blood Mini kit (QIagen) was amplified by PCR on a PTC-200 Peltier thermocycler (MJ Research) and sequenced as for breakpoint cloning. Reagents included GeneAmp PCR Buffer II (Applied Biosystems), 1.5 mmol/L MgCl2 (Applied Biosystems), 200 μmol/L deoxonucleotide triphosphates (Roche), forward and reverse oligonucleotide primers (see Supplementary Table S1), 100 ng of template DNA, and AmpliTaq Gold DNA polymerase (total, 1 U; Applied Biosystems).

Murine hematopoietic colonies. Animal protocols were approved by an Institutional Animal Care and Use Committee. Five IER3+/− mice and five IER3++ C57/BL6 mice were sacrificed and marrow extracted from femoral heads. Next, 300-μl aliquots of density centrifugation-separated marrow mononuclear cells were plated in 3 mL Methocult GF M3434 murine methylcellulose medium with cytokines (StemCell Technologies). Duplicate aliquots were plated in 35-mm gridded plates and incubated at 37°C in 5% CO2 for 10 d as specified by the media manufacturer, at which time hematopoietic progenitor colonies [blast-forming unit (erythroid; BFU-E), granulocyte-macrophage colony-forming unit (CFU-GM), colony forming...
Assays were performed in duplicate, and expression ratios calculated using glyceraldehyde-3-phosphate dehydrogenase and IER3 FAM multiplex primer-probe set (interrogates exons 1–2), and SuperScript III RTS First-Strand cDNA Synthesis kit (Invitrogen). With an Agilent 2100 Bioanalyzer (Agilent), and generated cDNA using a and controls, using an RNEasy Mini kit (QIAgen). We confirmed RNA quality and marrow mononuclear cells, and from equivalent cells in MDS patients and controls.

Results

Rearrangement of IER3 in the propositus. In the propositus, somatic hybrid aCGH results narrowed the translocation breakpoint to within an 800-bp region on chromosome 6p that included the transcription initiation site of IER3, and a 13-kbp region on chromosome 9q (Fig. 1B). PCR and sequencing of these regions showed that the t(6;9)(p21;q34) rearrangement resulted in separation of the IER3 gene at 6p21.33 from its upstream regulatory elements, and translocation to a transcript-poor region of 9q, along with just 40 bp of 5′ flanking region adjacent to the IER3 transcript initiation site (Fig. 1C), containing only a TATA box, which is inadequate to drive expression. Previous systematic examination of the IER3 promoter had indicated that at least 279 bp of flanking region are critical for full expression (7). Both Southern blotting and DNA sequencing results indicated a simple reciprocal translocation. By RT-PCR, expression of IER3 in peripheral blood mononuclear cells (PBMC) and whole marrow of the propositus was <5% of the mean for healthy controls.

FISH results for other patients. Split-signal and locus-specific probes were incubated with archival cell pellets from 204 patients with diverse hematologic malignancies where G-banded karyotyping had detected deletions, translocations, or other rearrangements of chromosome 6p21. An additional eight patients with abnormalities of IER3 were identified (Fig. 2). Including the propositus, there were four split signals and six amplifications (one patient had both abnormalities). All nine patients with IER3 breakapart or amplification had forms of MDS, with the 6p21 rearrangements part of a more complex karyotype in 8 of 9 cases (Table 1). There were not enough patients with IER3 rearrangements or amplification to perform detailed prognostic modeling.

To assess the frequency of karyotypically occult IER3 genomic rearrangements, 157 normal-karyotype patients representing all forms of WHO-defined MDS [including 90 patients with International Prognostic Scoring System (IPSS; ref. 19) Intermediate-2 or High-risk disease, and 67 with IPSS Low or Intermediate-1 risk disease] were studied by FISH. No rearrangements or amplifications were detected in this group.

Sequencing for mutations or polymorphisms. Sequencing of the IER3 coding and promotor regions in 148 alleles from MDS patients without 6p rearrangements revealed no point mutations. An apparently novel polymorphism, a 4-bp deletion in the 3′ untranslated region of IER3 (not a known or predicted area of microRNA binding), was present in 12% of MDS patients and 11% of healthy controls (200 control alleles examined; P value not significant). The prevalence of nonsynonymous single nucleotide polymorphism rs3094124 (p.A127P) was somewhat higher than expected in patients (variant in 14.6% of MDS alleles versus 8.6% of 116 HAP-CEU controls, P = 0.01). Other single nucleotide polymorphisms did not differ in frequency between patients and controls.

RT-PCR. RT-PCR for IER3 in PBMCs (n = 46 MDS patients without 6p rearrangements, n = 21 healthy controls) and whole marrow (n = 23 patients, n = 13 controls; Fig. 3) showed both down-regulation and up-regulation of IER3 in subsets of MDS samples compared with the mean for healthy controls, consistent with results from prior microarray experiments using MDS CD34+ cells (14, 15). Marrow and blood expression of IER3 correlated highly when both types of samples were available. IER3 expression distribution in PBMCs was greater in MDS patients compared with controls, with 16 of 46 patients (35%) and 11 of 16 had high expression.
higher risk MDS) demonstrating >4-fold increase in expression compared with mean for healthy controls, and 12 of 46 (26%; 7 of the 12 with lower risk MDS) with >4-fold decrease in expression compared with controls.

Murine hematopoietic colony growth. We noted no significant differences in BFU-E, CFU-E, or CFU-GM growth under unstressed conditions between IER3−/− mice and IER3 wild-type mice.

Discussion

These results show that IER3 is rearranged or amplified in a subset of patients with MDS, and dysregulated expression of IER3 is common in MDS. To our knowledge, this is the first demonstration of translocations and amplification involving IER3 in a human disease. IER3 is a plausible candidate for involvement in MDS because of its known role in regulating death receptor-induced apoptosis (3, 4), interaction with NF-κB pathways (5, 6), regulation by hematopoietic growth factors including thrombopoietin (20), and importance in immediate response to genotoxic stresses such as ionizing irradiation (1).

Although the molecular mechanisms of MDS remain largely obscure, one commonly observed abnormality is dysregulation of apoptosis. Excessive intramedullary apoptosis in early stages of the disease is followed by diminished apoptosis later, as immature blast cells accumulate and the disease progresses toward acute myeloid leukemia (AML). Increased apoptosis of hematopoietic progenitor cells in early MDS may explain the typical “MDS paradox”: a hypercellular bone marrow, coupled with peripheral blood cytopenias. Conversely, the diminished ability of cells to undergo apoptosis late in the course of the disease has been postulated to contribute to the poor treatment response of the forms of leukemia that evolve in the setting of prior MDS.

The dichotomous IER3 RNA expression results reported here are consistent with those reported in two global cDNA expression microarray studies with CD34+ cells from MDS patients, and the proposed role of apoptosis in MDS pathobiology—i.e., decreased expression of IER3 early on in lower risk disease when intramedullary apoptosis is excessive, and increased expression later as MDS progresses toward AML and a frankly neoplastic clone emerges (14, 15).

It seems likely that IER3 rearrangements or amplification are a secondary, rather than primary, abnormality in MDS. IER3−/− mice are not anemic and do not have dysplastic changes in the marrow (although mice >1 year old have not been systematically studied). We observed normal hematopoietic progenitor colony growth from IER3−/− mice, at least in the absence of specific cellular stressors. In addition, for eight of the nine MDS patients in whom IER3 rearrangements were confirmed, 6p21 abnormalities were present as part of a more complex karyotype.

aCGH is increasingly used to investigate copy number variation in neoplasia, but unless individual chromosomes are sorted in some way before array hybridization, aCGH is not useful for investigation of balanced translocations. Here, we facilitated aCGH mapping of rearranged chromosomal boundaries with somatic hybridization technology, which allowed refinement of translocation breakpoints to within a few kbp—well within the range of long-range PCR—without using a labor-intensive “shotgun” approach requiring dozens of BAC clones. This technique could be applied to other translocations, including those associated with MDS, to discover new neoplasia-associated genes.

Conclusion. IER3 translocations and amplification represent novel, clonally restricted recurrent genetic abnormalities in a subset of patients with MDS. Additionally, altered IER3 expression is common in MDS, even in patients without 6p rearrangements. Clarification of the role of IER3 in MDS is likely to yield new insights into MDS pathobiology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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