Alterations in Gemin5 Expression Contribute to Alternative mRNA Splicing Patterns and Tumor Cell Motility

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Abstract

The role of Gemin5 in alternative mRNA splicing, tumor cell motility, and proteomic instability was investigated. Isotope Capture Affinity Tag proteomic analysis was conducted on MDA-MB-435 tumor cells transfected with either a control vector (C-100) or the Nm23-H1 metastasis suppressor (H1-177). Ingenuity pathway analysis revealed that RNA posttranscriptional processing was the most prominent class of differentially expressed proteins. Within this category, overexpression of Acinus1, Poly(a) binding protein, HNRPA2B1, Bop1, and Gemin5 was confirmed in less metastatic H1-177 cells. Overexpression of the latter four proteins was also observed in the lower metastatic antisense Ezrin transfectant of a murine osteosarcoma model system, confirming the general relevance of the trends. Gemin5, a component of the splicesomal complex, was chosen for further study. Analysis of global mRNA splicing by SpliceArray chips revealed that 16 genes were differentially spliced in C-100 compared with H1-177 cells; transient transfection of gemin5 into C-100 cells restored the splice pattern to that of H1-177 cells. Alternative splicing patterns for the engulfment and cell motility 1 and thrombospondin 4 genes were confirmed by semiquantitative reverse transcription-PCR. Gemin5 overexpression coordinately reduced C-100 cell motility by 50%, and siRNA-mediated reduction of Gemin5 expression increased the motility of H1-177 cells by 2-fold (P < 0.004). The data provide the first demonstration that alterations in the expression of a splicesomal protein can affect both specific splicing events and tumor cell motility. The data also show that changes in mRNA splicing patterns accompany metastatic progression, which may contribute to proteome instability. [Cancer Res 2008;68(3):639–44]

Introduction

One of the hallmarks of metastatic tumor cells is their instability, measured in terms of genomic alterations, gene and protein expression patterns, and biological behaviors. Although a host of genes have been found to influence metastatic behavior in vivo, mechanistic explanations for the instability inherent in this process are few. We present evidence for the differential expression of Gemin5 in metastasis and its contribution to alternative mRNA splicing, a source of genomic variability.

A model system consisting of MDA-MB-435 tumor cells transfected with an empty vector (C-100) or the Nm23-H1 metastasis suppressor (H1-177) was used. Previous characterization of these lines indicated a significant suppression of metastasis to the regional lymph nodes and lungs with no significant effect on primary tumor size (1). Nm23-H1 is known to possess enzymatic activities and to bind multiple proteins. Recently, we reported a third contributor to the mechanism of action of Nm23-H1, the regulation of EDG2 expression (2). To continue our analysis of differential expression patterns downstream of Nm23-H1, control and Nm23-H1 transfectants of the human MDA-MB-435 tumor cell line were analyzed for protein expression differences by Isotope Capture Affinity Tag (ICAT). Surprisingly, the most prominent class of differentially expressed proteins was that controlling RNA posttranscriptional modifications. The data presented herein investigate a potential role for alternative mRNA splicing in tumor progression, demonstrating that a component of the splicesomal complex, Gemin5, links alternative mRNA splicing to tumor cell motility.

Materials and Methods

Cell lines and cell culture. The control (C-100) and Nm23-H1 (H1-177) transfectants of MDA-MB-435 tumor cells were described (1). Characterization and maintenance of the clonally related K7M2 (high metastasis and high Ezrin) and K7M2 AS1.46 (low metastasis and antisense ezrin) mouse osteosarcoma cell lines have been described (3).

Plasmid and transfection. The pcDNA3 plasmid containing V5-tagged gemin5 was kindly provided by Dr. Gideon Dreyfuss (University of Pennsylvania, Philadelphia, PA). Cells were transfected with V5-gemin5 or control vector using Effectene transfection reagent (Qiagen). Transient transfections were performed as previously described (2).

ICAT and ingenuity pathway analysis. ICAT was performed as described (4). Proteins from the C-100 and H1-177 transfectants or he K7M2 and K7M2 AS1.46 were labeled with light (ICAT-12C9) and heavy (ICAT-13C9) isotopic versions of the ICAT reagents, combined, and digested with trypsin. The ICAT-labeled peptides were isolated using avidin chromatography and analyzed using multidimensional chromatography coupled directly online with tandem mass spectrometry. Peptides were identified, and the relative protein quantitation was determined using BioWorks (ThermoElectron). The differentially expressed proteins list was uploaded to Ingenuity Pathway analysis (IPA; Ingenuity Systems), which was used for generating molecular and cellular functional analysis.

Cell extraction, fractionation, and Western blot analysis. Total cell lysates were prepared in radioimmunoprecipitation assay buffer. For nuclear and cytosol fractionation, cells were lysed in NE-PER extraction reagent (Pierce) according to the manufacturer’s protocol. Immunoblotting analysis was performed using anti-Nm23-H1 (BD Biosciences), anti-Acinus (BD Biosciences), anti-Gemin5 (Santa Cruz Biotechnology), anti–Poly(A)

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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"2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-2632"
binding protein (PABPC1; Novus Biologicals), anti-HNRPA2B1 (Abcam), anti–V5-HRP (Invitrogen), anti-Tubulin (Calbiochem), and anti–c-Jun (Cell Signaling Technology).

**SpliceArray chip preparation, labeling, hybridization, and analysis.** Splicing arrays were performed per manufacturer’s protocol (ExonHit Therapeutics, Inc.). The arrays were manufactured by Agilent Technologies on their custom 244K oligoarray format. Labeling, hybridization, and analysis of SpliceArray were performed per ExonHit Therapeutics website. To eliminate dye bias, a duplicate hybridization was performed with a dye swap. The arrays were scanned using Agilent’s Microarray Scanner (Agilent Technologies). For data extraction, the images were analyzed with the Feature Extraction software, version 9.1.3. The data analysis was performed with SpliceArray Visualization Tool (ExonHit Therapeutics, Inc.) and Partek Genomic Suite (Partek, Inc.). Data were quantile normalized across arrays, and an ANOVA analysis was performed to select significant probes.

**Cell motility assays.** Cell motility assays were performed as previously described, (2) and statistical significance were determined with a Student’s t test.

**Isolation of total cellular RNA, and semiquantitative and quantitative reverse transcription-PCR.** Total RNA was isolated from cells using TriZol (Invitrogen) following the manufacturer’s protocol. Semiquantitative reverse transcription-PCR (semi–qRT-PCR) and quantitative reverse transcription-PCR (qRT-PCR) were performed as previously described (ref. 2; Supplementary Table S1 lists the oligos sequences). qRT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bop1, and gemin5 was performed with an iQ5 Multicolor Real-time PCR Detection System (Bio-Rad). Diluted cDNA was combined with 15 μmol/L bop1 oligos and

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**Figure 1.** ICAT analysis of control (C-100) and Nm23-H1 metastasis suppressor (H1-177) transfectants of the MDA-MB-435 tumor cell line. A, expression of Nm23-H1 in C-100 and H1-177 cells was determined by Western blot analysis (top). Bottom, expression of tubulin was determined as a loading control. B, decreased migration of H1-177 cells to 1% FBS in Boyden chamber motility assays. Images shown at ×100 magnification. C, IPA of 129 differentially expressed proteins between C-100 and H1-177 cells, determined by ICAT analysis. Proteins in the top two classes of differentially expressed proteins are listed.

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4 http://portal.splicearray.com/PortalHome/
2 × SYBR Green Supermix (Bio-Rad). The relative gene expression value of each gene was calculated by the standard curve of GAPDH with cDNA from the C-100 cells.

**Gemin5 silencing.** H1-177 cells were transfected with small interfering RNA (siRNA) designed against the coding sequence of gemin5, available from Dharmacon, using HiPerFect (Qiagen). siCONTROL, a nontargeting siRNA pool, was used as a negative control. Transfection assays were performed as previously described (2).

**Results and Discussion**

ICAT analysis of control and Nm23-H1 metastasis suppressor transfectants reveals RNA posttranscriptional modification proteins. ICAT analysis of control (C-100) and less metastatic Nm23-H1 (H1-177) transfectants of the MDA-MB-435 cell line (1) was performed to identify quantitative differences in protein expression that may accompany metastasis. H1-177 cells overexpressed Nm23-H1 by ~10-fold compared with the C-100 cells and were significantly less motile in Boyden chamber assays to 1% fetal bovine serum (FBS) at the passages used herein (Fig. 1A–B).

Using ICAT, 3,262 peptides sequences were identified. Of these, 189 proteins were significantly down-regulated and 381 proteins were up-regulated in H1-177 cells ($\mu = 1.2154; \sigma = 0.6698$). Based on an IPA, the most prominent class of altered proteins was that of RNA posttranscriptional modification. Figure 1C lists the top differentially expressed proteins identified in this category; further information is provided in Supplementary Table S2. RNA posttranscriptional processing proteins have rarely been associated with tumor motility or metastasis. The second most common class of differentially expressed proteins, cellular assembly and organization, contained cell surface receptors and cytoskeletal proteins, which were previously identified by microarray analysis (2).

Several of the RNA posttranscriptional processing ICAT trends were confirmed using immunoblots. Nm23-H1 overexpressing H1-177 cells exhibited increased levels of Acinus1, which is...
Table 1. Selected genes exhibiting alternative mRNA splicing between low and high Nm23-H1–expressing cell lines, which is mediated by Gemin5 overexpression

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Probe</th>
<th>Reference accession</th>
<th>Variant accession</th>
<th>Splicing event</th>
<th>Fold change in mRNA expression</th>
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<tbody>
<tr>
<td>CAMK4</td>
<td>814.4.1_D</td>
<td>NM_001744</td>
<td>DB133605</td>
<td>Deletion of exon 4(^a)</td>
<td>1.85</td>
</tr>
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<td>CCND2</td>
<td>894.4.1_F</td>
<td>NM_001759</td>
<td>BP213650</td>
<td>Novel intron 5(^b)</td>
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<tr>
<td>CD68</td>
<td>968.2.1_B</td>
<td>NM_001251</td>
<td>BP330677</td>
<td>Novel intron 2</td>
<td>2.98</td>
</tr>
<tr>
<td>CTNNA1</td>
<td>827.4.2_F</td>
<td>NM_003798</td>
<td>AK123916</td>
<td>Intron retention 16–17(^c)</td>
<td>1.94</td>
</tr>
<tr>
<td>IL1R1</td>
<td>3554.2.1_B</td>
<td>NM_000877</td>
<td>BP278275</td>
<td>Novel exon before 1(^d)</td>
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</tr>
<tr>
<td>MAG</td>
<td>4099.7.2_B</td>
<td>NM_080600</td>
<td>B1603287</td>
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<tr>
<td>NSD1</td>
<td>64324.7.1_E</td>
<td>NM_022455</td>
<td>BP873335</td>
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<tr>
<td>STK32C</td>
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<tr>
<td>THBS4</td>
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<td>NM_003248</td>
<td>DA759337</td>
<td>Novel exon 3–4</td>
<td>1.95</td>
</tr>
</tbody>
</table>

NOTE: MDA-MB-435 tumor cells were transfected with a vector (C-100) or Nm23-H1 (H1-177). C-100 cells were then transiently transfected with the same vector or Gemin5, and expression were verified by Western blots. RNA extracted from each line was hybridized to Agilent Technologies 244K Slice/Array chips, using a dye swap. Fold differences in the expression of mRNA splice events was quantified.

\(^a\)Deletion of an exon.
\(^b\)Retention of a splice sequence in the translated mRNA.
\(^c\)Insertion of a novel exon between the indicated exons.
\(^d\)Donor site for indicated exon is altered leading to exon extension or shortening.

Gemin5 modulation of mRNA splicing and tumor cell motility. Alternative mRNA splicing, in which identical pre-mRNA molecules are spliced to form distinct mature mRNAs, may contribute to proteome diversity in both tumorigenesis and progression. Alternative splicing is regulated in part by canonical splicing signals defining intron-exon boundaries and a complex set of exonic splicing enhancers and splicing silencers that preferentially recruit the splicingosomal complex. The splicingosomal complex consists of small nuclear ribonrotein particles, which contain small nuclear RNA (snRNA), a core structure composed of seven Sm proteins, and other proteins. The Sm core structure consists of the SMN protein and Gemins 2 to 7 (reviewed in refs. 5–8). Gemin5 plays a critical role in this process. Gemin5 functions as the snRNA binding protein of the SMN complex (9). Gemin5 knockdown in HeLa cells lead to reduced binding of the SMN complex to snRNAs and reduced assembly of Sm cores (9). A role for Gemin5 protein levels in alternative mRNA splicing and tumor progression has not been reported.

To investigate a potential role for differential Gemin5 expression in alternative mRNA splicing, global mRNA splicing patterns were determined using custom 244K Slice/Array chips. Supplementary Table S3 lists splice variants between the control C-100 cells and the Nm23-H1 overexpressing H1-177 cells. Genes with known functions in proliferation, motility and invasion, and apoptosis are represented. To determine which of these mRNA splicing events were caused by Gemin5 overexpression in the H1-177 cells, C-100 cells were transiently transfected with another vector or gemin5 and the SpliceArrays repeated. Table 1 lists genes that were differentially spliced >1.5-fold in replicate arrays of both the (a) control C-100 cells compared with Nm23-H1 expressing H1-177 cells and (b) C-100 cells transiently transfected with another vector compared with gemin5. In general, these genes do not represent involved in chromosome condensation and spliceosome function; Gemin5, part of the survival of motor neurons (SMN) complex involved in mRNA splicing; HNRPA2B1, which has diverse roles in mRNA splicing and RNA trafficking; and PABPC1, which functions in both the initiation and termination of translation (Fig. 2A). Antibodies were unavailable to Bop1, which is involved in pre-rRNA processing. Figure 2B shows its overexpression in H1-177 cells at the mRNA level using qRT-PCR.

We also asked whether similar trends were observed in another set of metastasis-related cell lines. The cytoskeletal organizer Ezrin is required for both motility and in vivo metastasis in a mouse osteosarcoma model (3). ICAT analysis of K7M2 (high metastasis and high Ezrin) and K7M2 AS1.46 (low metastasis and antisense ezrin) transfectants included 1,791 sequenced peptides and revealed ~300 differentially expressed proteins (data not shown). For four of the five differentially expressed proteins, overexpression was also observed in the lower metastatic potential K7M2 AS1.46 line (Fig. 2C, columns below the line). These data indicate that alterations in RNA posttranscriptional processing proteins may be a general property of metastasis. Gemin5, which exhibited the largest fold difference between two sets of transfectants, was chosen for further study.

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the classic genes involved in the mechanics of motility, invasion, or metastasis (actin, Fak, Src, etc.), but many have reported regulatory roles in these processes. For instance, Catenin α-like 1 (CTNNAL1) is part of the Rho signaling complex (10), whereas myelin-associated glycoprotein (MAG) lies upstream of Rho in neuronal signaling and participates in their invasion/outgrowth (11); engulfment and cell motility 1 (ELMO1) is part of the Rac signaling complex (12). Two receptors, FGFR1 and IL-1R, have been reported to regulate motility (13, 14). Calcium/calmodulin-dependent protein kinase IV (CAMK4) regulates sperm motility (15), whereas thrombospondin 4 (THBS4) regulates neurite outgrowth (16). Tie-1 is expressed on both carcinoma and endothelial cells, in the latter where it regulates angiogenesis through interactions with Tie-2 (17). These patterns seemed specific, affecting particular but not all possible splice forms of each gene. Almost all occurred within the coding sequences of genes and most involved either the deletion of an exon or the insertion of a novel exon compared with the wild-type transcript, suggestive of altered biochemical functions.

To confirm the SpliceArray data, semi–qRT-PCR was conducted for two splicing events. C-100 and Nm23-H1 overexpressing H1-177 cells were transiently transfected with an empty vector (C-100/V and H1-177/V), and C-100 cells were transiently transfected with gemin5 (Fig. 3A). Figure 3B shows the effect of Gemin5 overexpression on the splicing patterns of two genes: THBS4 and ELMO1. For THBS4, the most abundant splice form contains exons 3 to 4, whereas an alternative form inserts a novel exon in between.

Figure 3. Gemin5 expression level mediates alternative mRNA splicing and tumor cell motility. A, C-100 cells were transfected with a vector (C-100/V) or gemin5 tagged with the V5 epitope (C-100/Gemin5). A Western blot shows Gemin5 expression. B, semi–qRT-PCR analysis of THBS4 and ELMO1 mRNA splicing in C-100/V and C-100/Gemin5 cells, compared with a vector-retransfected H1-177 cell line (Nm23-H1 overexpressing). For each gene, a diagram shows the wild-type transcript and a splicing event identified on SpliceArray analysis. Forward (F) and reverse (R) oligos indicate the reference splicing pattern. The splice event detected by SpliceArray analysis is diagrammed beneath. Semi–qRT-PCR determined the relative expression of each splice variant in each cell line. No RTase is shown as a negative control. For each gene, transfection of gemin5 into C-100 cells altered the relative expression of the splice event similar to that of H1-177 cells. Ref, reference mRNA; event, mRNA-splicing variant. C, Gemin5 overexpression in C-100 cells decreases in vitro motility. The C-100/V and C-100/ Gemin5 transfectants and a vector-transfected H1-177 transfectant (H1-177/V) were assayed for motility to 1% FBS in Boyden chamber assays. Top, quantification of migrating tumor cells from three representative experiments. Comparison of C-100/V and C-100/Gemin5, P < 0.0001. Bottom, micrographs of tumor cell motility (× 100). D, siRNA to gemin5 stimulates the motility of Nm23-H1 overexpressing H1-177 cells. H1-177 cells were transiently transfected with siRNA to gemin5 or a scrambled siRNA, and cell motility was tested to 1% FBS. Top, quantification of migrating tumor cells from two representative experiments (P < 0.004); bottom, micrographs of tumor cell motility.
This splicing event is demonstrable in C-100/V cells but virtually gone in H1-177/V and C-100/Gemin5 cells. Insertion of a novel exon in between exons 1 and 2 of ELMO1 is more apparent in H1-177/V cells than in C-100/V cells; transient transfection of C-100 cells with Gemin5 increased the expression of this variant splice form to levels approaching that of H1-177/V cells. Loading controls for the semi–qRT-PCR are shown in Supplementary Fig. S1.

Finally, we asked if Gemin5 overexpression and the coordinate alternate mRNA splicing patterns created in the C-100/Gemin5 cells exerted a phenotypic effect. No difference was observed in the in vitro proliferation of C-100/V, H1-177/V, and C-100/Gemin5 cells (data not shown). One of the hallmarks of Nm23-H1 function is its diminution of in vitro tumor cell motility to multiple chemotaxants (2). Also, many of the differentially spliced genes have reported functions in the regulation of motility. We therefore assessed the effect of Gemin5 overexpression on C-100 cell motility. As positive and negative control, H1-177/V cells inhibited motility by 90% comparing with C-100/V cells. Surprisingly, expression of Gemin5 partially recapitulated the motility suppressive effect of Nm23-H1, inhibiting motility by 50% over three replicate experiments (P < 0.0001; Fig. 3C). Alternatively, transient transfection of Nm23-H1 overexpressing H1-177 cells with siRNA to gemin5 reduced gemin5 mRNA levels (Supplementary Fig. S2) and increased motility 2-fold (P < 0.004; Fig. 3D). The data indicate a role for Gemin5 and alternative mRNA splicing in the regulation of tumor cell motility.

The contribution of alternative mRNA splicing to cancer represents an emerging area of research. It is estimated that 74% of human genes encode transcripts that undergo splicing, and that 15% of human genetic diseases are associated with a mutation in either splice junctions or the spliceosomal apparatus (18). Examples include BRCA1 mutations in splice sites, and functional differences in alternatively spliced forms of p73, survivin, caspases, and vascular endothelial growth factor (reviewed in ref. 5). Only one previous report linked alternative mRNA splicing with tumor cell motility. This study revealed that differential expression of exonic splicing enhancer SF2/ASF in Ron mediated motility (18). Our data extend this trend to indicate that expression of criticalspliceosome machinery also effects alternative splicing and tumor cell motility. Furthermore, the data suggest the hypothesis that alternative mRNA splicing, which can regulate transcript diversity, is modulated in metastasis and may contribute to metastatic instability.

Translational approaches to alternative mRNA splicing are being reported, including targeted antisense oligos to exonic splicing factors and histone deacetylase inhibitor augmentation of SMN protein levels (19, 20). These efforts indicate that alternative mRNA splicing may be a worthwhile molecular target for cancer.

Acknowledgments

Received 7/11/2007; revised 11/19/2007; accepted 12/14/2007.

Grant support: The intramural research program of the Center for Cancer Research, National Cancer Institute.

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We thank Dr. Gideon Dreyfuss, University of Pennsylvania, for the Gemin5 construct and Dr. David Goldstein, Center for Cancer Research, National Cancer Institute for assistance with the SpliceArray technology.

References

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