Original Article

IMP3 expression is associated with epithelial-mesenchymal transition in breast cancer

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Abstract: IMP3 plays an important role in tumor invasion and metastasis, to which epithelial to mesenchymal transition (EMT) also contributes. The purpose of this study was to investigate whether IMP3 can regulate invasion and metastasis through EMT in breast cancers. The protein expression levels of IMP3 and EMT markers were analyzed by immunohistochemistry in 180 paraffin-embedded human breast tissue samples. There was an inverse correlation of IMP3 with E-cadherin protein expression ($P = 0.042$). IMP3 expression directly correlated with both Slug ($P = 0.004$) and vimentin ($P < 0.001$). Changes in E-cadherin, vimentin, and Slug mRNA and protein levels were examined by quantitative real-time reverse polymerase chain reaction (qRT-PCR) and western blotting. Overexpression of IMP3 reduced the expression of E-cadherin and upregulated Slug and vimentin in transfected cells. In contrast, knocking down IMP3 had the opposite expression of the three proteins. Ribo-immunoprecipitation qPCR revealed that IMP3 binds Slug mRNA directly. In a transwell assay, overexpression of Slug rescued the cell migration and invasion caused by silencing IMP3 in MDA-MB-231 cells. On the other hand, knockdown of Slug in T47D-IMP3 cells could also have the opposite change. Our results strengthen the association of IMP3 with the regulation of EMT. Slug is a functional target of IMP3. IMP3 could therefore promote invasion and migration through the EMT in breast cancer cells.

Keywords: Insulin-like growth factor II (IGF-II) mRNA binding protein 3 (IMP3), epithelial-mesenchymal transition, ribo-immunoprecipitation, metastasis, breast cancer

Introduction

IMP3 is a member of the human insulin-like growth factor II (IGF-II) mRNA binding protein (IMP) family that consists of IMP-1, IMP-2, and IMP-3. The three members contain six RNA binding motifs, including two RNA recognition motifs and four heterogeneous nuclear ribonucleoprotein K-homology (KH) domains [1]. They have important roles in RNA trafficking and stabilization, cell growth, and cell migration during the early stages of embryogenesis [2]. IMP3 is expressed in developing epithelium, muscle, and placenta during early stages of human and mouse embryogenesis but is expressed at low or undetectable concentrations in adult tissues [1, 2]. It is also known as KH domain-containing protein overexpressed in cancer (KOC) [3, 4] and is expressed in multiple malignant tumors. High IMP3 expression predicts metastasis formation and poor survival [5], suggesting it may be a promising cytological tumor marker in various cancers.

Epithelial to mesenchymal transition (EMT) also plays a role in tumor invasion and metastasis [6]. EMT was first recognized as an important process during normal embryonic development [7], but carcinoma cells are also capable of reactivating the EMT during tumor progression [8, 9]. In EMT, epithelial cancer cells lose cell-cell adhesion, apical-basal polarity, and epithelial markers, as well as acquire spindle cell shape and mesenchymal markers, all of which help them to metastasize. Several transcription factors, including Snail, Slug, and Twist, are aberrantly activated and/or overexpressed in human cancers and can induce EMT and metastasis by repressing E-cadherin transcription [10-13].
The exact association between IMP3 and EMT remain unknown. We are particularly interested in their relationship in breast cancer since IMP3 is expressed preferentially in triple-negative breast cancer [14]. Furthermore, EMT may be increased in triple-negative or basal-like breast cancers, although precise mechanisms (e.g. EMT-regulating transcription factors) are largely unknown [15]. In this study, we investigated whether IMP3 regulated metastasis through EMT.

Materials and methods

Patients and tissue samples

180 paraffin-embedded breast tissue samples were diagnosed as invasive ductal carcinoma at the Department of Pathology of Qilu Hospital, Shandong University, from 2007 to 2009. We obtained prior patient consent and approval from the Research Ethics Committee of Shandong University School of Medicine for the use of these clinical materials for research purposes.

Immunohistochemistry

The immunohistochemical method to study altered protein expression was performed as previously described [16]. In brief, paraffin-embedded specimens were cut into 4 μm sections and incubated at 60°C for 60 min. The sections were deparaffinized with xylenes and rehydrated. Sections were submerged in antigenic retrieval buffer (citrate, 0.01 mol/L; pH6.0 for IMP3, E-cadherin, vimentin, and Slug), microwaved for antigenic retrieval, and then cooled at room temperature for 30 min. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with normal serum to block nonspecific binding. All the markers were incubated with the sections overnight at 4°C. Monoclonal antibodies (Mab) were used at the following dilutions: mouse anti-human IMP3 clone 69.1 (Dako, Carpinteria, CA, USA) 1:100, rabbit Mab anti-E-Cadherin (24E10) (Cell Signaling Technology, Boston, MA, USA) 1:400, rabbit Mab anti-Vimentin (D21H3) XP™ (Cell Signaling Technology) 1:100, rabbit Mab anti-Slug (C19G7) (Cell Signaling Technology) 1:50. The secondary Ab was from PV9000 IHC reagent kit (Zhongshan Biotechnology Company, Beijing, China). Sections were stained with diaminobenzidine (DAB) and counterstained with hematoxylin. For negative controls, the Abs were replaced with PBS.

Evaluation of immunohistochemical staining

The stained slides were evaluated independently by two observers blinded to the patients' information. Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow-brown), and 3 (strong staining = brown). Tumors were regarded as immune-positive if > 10% of tumor cells showed immunoreactivity. We considered any cytoplasmic staining to be positive for IMP3 and vimentin, any nuclear staining positive for Slug, and any membranous staining positive for E-cadherin.

Cell lines and reagents

The breast cancer cell lines MDA-MB-231 and T47D were obtained from American Type Culture Collection (Manassas, VA, USA). IMP3-specific siRNA and Slug-specific siRNA were purchased from Shanghai GenePharma Co., Ltd. Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) was used for regular siRNA transfection. MDA-MB-231 cells were transfected with IMP3-specific siRNA (231-siRNA-IMP3) with target sequence GCUGGAGCUUCAAUUAAGATT. We also established negative control (231-nc) and mock (231-mock) transfected cells. The target sequence for Slug was UCCGAAUAUGCAUCUCAUGGCGCCCA [17]. IMP3 expression vector pEGFP-C1-IMP3 was a gift from Dr. Yung-Ming Jeng, Department of Pathology, National Taiwan University Hospital. The human Slug cloning vector was purchased from OriGene Technologies (SC118205, Rockville, MD, USA). The target gene was subcloned into a mammalian expression vector, pcDNA3.1 (+) (Life Technologies, NY, USA), and a negative control vector was generated as previously described [18]. Thermo Scientific TurboFect Transfection Reagent (Thermo Scientific Fermentas, Vilnius, Lithuania) was used for DNA transfection. T47D cells were transfected with the pEGFP-C1-IMP3 vector (T47D-IMP3). The empty pEGFP-C1 vector served as a control (T47D-vector). 231-siRNA-IMP3 cells transfec-
ed with Slug vectors are referred as 231-siRNA-IMP3/vector and 231-siRNA-IMP3/Slug, and T47D-IMP3 cells transfected with Slug-specific siRNA are referred as T47D-IMP3/nc and T47D-IMP3/siRNA-Slug.

Cell culture

MDA-MB-231 cells were routinely cultured in Leibovitz’s L-15 (Gibco, Grand Island, NY, USA) Medium containing 10% fetal bovine serum. T47D cells were maintained in Dulbecco’s modified Eagle’s (Gibco) medium containing 0.01 mg/ml bovine insulin and 10% fetal bovine serum. Both were at 37°C in a humidified incubator supplied with 5% CO₂.

RNA isolation and real-time PCR analysis

Total RNA was isolated from cultured cells using RNAiso Plus (Takara, Dalian, China), and the complementary DNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). We quantified mRNA by real-time PCR analysis using UltraSYBR Mixture with ROX (Beijing CoWin Biotech Co., Ltd., China) and ΔΔCt method. β-actin was used as reference gene. The following primer pairs were used for real-time PCR analysis: IMP3 forward primer 5’-CCGCAGTTTGAGCAATCAGAA-3’, IMP3 reverse primer 5’-CGAGAAAGCTGCTTGATGTGC-3’ [19]; IGF-II forward primer 5’-CCGAAACAGGCTACTCTCCT-3’, IGF-II reverse primer 5’-AGGGTGTTTAAAGCCAATCG-3’ [20]; ESR2 forward primer 5’-ATGGATATAAAAAACTCACCA-3’, ESR2 reverse primer 5’-CGCATTTCCCCTCATCC-3’; E-cadherin forward primer 5’-GGTGCTCTTGGAACCTC-3’, E-cadherin reverse primer 5’-GGAAACTCTCTGCGTCAGC-3’; Vimentin forward primer 5’-CGAAAACACCCTGCAATCTT-3’, Vimentin reverse primer 5’-GTTTTAACCCCTGGACGTC-3’; Slug forward primer 5’-AAAGAGGAGAGAGGCCATTGGGTA-3’ [21]; β-actin forward primer 5’-CTCCATCTCTGCGCTGTCC-3’, β-actin reverse primer 5’-GCTGTCACCTTACCGTTCC-3’.

Western blot analysis

Cellular protein extracts were prepared as described previously [18], 20 μg of proteins were separated by electrophoresis on 10% SDS–PAGE, and then blotted onto polyvinylidene fluoride membranes (PVDF) (Millipore, Billerica, MA, USA). Transferred blots were blocked with 5% fat-free milk powder in TBST at room temperature for 2 hr. Blots were then incubated overnight at 4°C with the relevant primary Ab, washed, and probed again with species-specific secondary Ab coupled with horseradish peroxidase. Immunoreactivity was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Ribo-immunoprecipitation (RIP) assay

The interaction between IMP3 protein and Slug mRNA was determined using a ribo-immunoprecipitation qPCR assay as described previously [20]. Briefly, MDA-MB-231 cells were extracted and centrifuged. 20 μg of non-immune rabbit IgG (Beyotime) was added to the supernatant and kept on ice for 45 min and then incubated with 50 μl of protein A/G-Sepharose beads (Beyotime) for 3 hr at 4°C with rotation. For immunoprecipitation, the pre-cleared extract was incubated with beads pre-coated with the same amount of either non-immune rabbit IgG or anti-human IMP3 Ab in 800 μl of NT-2 buffer containing RNase inhibitor and protease inhibitors overnight at 4°C with rotation. Beads were washed, digested with 20 units of RNase-free DNase I (Promega, Madison, WI, USA) for 20 min at 30°C, washed with NT-2 buffer, and further digested with 0.5 mg/ml protease K (BioTeke Corporation, Beijing, China) in 100 μl of NT-2 buffer containing 0.1% SDS at 55°C for 30 min. RNA was extracted with TRlzol (Invitrogen). Glycogen (Beyotime) was added to facilitate precipitation of RNA. Real-time PCR was performed on equivalent amounts of sample to quantify protein-bound mRNAs.

Analyses of cell morphology, migration, and invasion

Selected cell clones were studied morphologically by phase contrast microscopy. For the migration assay, cells were seeded on the upper side of the chamber of the transwell (Corning Costar, NY, USA); the lower chamber was filled with 10% serum in L-15 or DMEM medium. After 24 hr, cells were fixed, stained and counted. The invasion assay was similar to the migration assay described above, except that the upper side of the membranes was coated with the matrigel (BD Biosciences, Bedford, MA, USA).
IMP3 and epithelial-mesenchymal transition in breast cancer

Analyses were performed using the statistics software package SPSS 18.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The chi-square test or Fisher's exact test was used to evaluate the correlation between IMP3 expression and the EMT markers, if appropriate. Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients. All real-time PCR, migration, and Matrigel experiments were performed with a minimum of three replicates, and results were depicted as mean ± SD. Significance was assessed by the Student's t-test. Differences were considered statistically significant for \( P \) values < 0.05.

**Results**

**IMP3 expression correlated with EMT markers in human breast cancer**

In the analysis of a 180-member tissue microarray (TMA), IMP3 expression was seen in 23 cases (12.8%); 157 tumors (87.2%) did not express IMP3. To explore the correlation, we stained for the expression of three genes (E-cadherin, vimentin, and Slug) which have tight relation with EMT (Figure 1). E-cadherin belonged to markers of epithelial tissues. Vimentin and Slug were always treated as markers of mesenchymal tissues. The immunohistochemical expression of IMP3 inversely correlated with E-cadherin (\( P = 0.042 \)), which was further confirmed by Spearman correlation analysis (\( r = -0.163, P = 0.029 \)) (Table 1). IMP3 expression directly correlated with both Slug (\( P = 0.004 \)) and vimentin (\( P < 0.001 \)), and its Spearman correlations were 0.27 (\( P < 0.001 \)) and 0.366 (\( P < 0.001 \)), respectively. The over-expression of IMP3 therefore contributed to the EMT in breast cancer progression.

### Table 1. Correlation among IMP3 expression and EMT markers (E-cadherin, Slug, vimentin) in human breast cancer

<table>
<thead>
<tr>
<th>EMT markers</th>
<th>n</th>
<th>IMP3 expression</th>
<th>( P ) value</th>
<th>Spearman correlation*</th>
<th>( r ) value</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>negative</td>
<td>33</td>
<td>25</td>
<td>8</td>
<td>0.042</td>
<td>-0.163</td>
<td>0.029</td>
</tr>
<tr>
<td>positive</td>
<td>147</td>
<td>132</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slug</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>170</td>
<td>152</td>
<td>18</td>
<td>0.004</td>
<td>0.27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>positive</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>vimentin</td>
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<td></td>
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<tr>
<td>negative</td>
<td>165</td>
<td>150</td>
<td>15</td>
<td>&lt; 0.001</td>
<td>0.366</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>positive</td>
<td>15</td>
<td>7</td>
<td>8</td>
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</tbody>
</table>

*The Spearman correlation was used to compare the degrees of correlation. Positive numbers reflected direct correlation, and negative numbers reflected inverse correlation.

**Statistical analysis**

The expression of IMP3 and EMT markers in human breast cancer. Representative fields of view from the TMA cores show examples of positive IMP3, negative E-cadherin, positive Slug and vimentin (upper row, 200×) and negative IMP3, positive E-cadherin, negative Slug and vimentin (lower row, 200×). IMP3 and vimentin exhibited cytoplasm immunoreactivity, Slug nuclear immunoreactivity, and E-cadherin membrane immunoreactivity. Correlations among these markers from these human cases were analyzed algorithmically and are depicted in Table 1.
Overexpression of IMP3 changed the mRNA and protein levels of EMT markers in T47D cells.

To further explore the mechanism and effect of IMP3 on the expression of E-cadherin, Slug, and vimentin, we performed transient transfections of full-length IMP3 into IMP3-negative T47D cells (i.e. T47D-IMP3 cells). The results confirmed that IMP3 was overexpressed in T47D-IMP3 cells but not in the empty (control) vector-transfected cells (T47D-vector) (Figure 2).
The expression of three genes was also measured by real-time PCR. Expression of all the genes changed dramatically. The expression of E-cadherin decreased \((P < 0.05)\) accompanied by an upregulation of Slug and vimentin \((P < 0.01)\) in T47D-IMP3 cells (Figure 2A). We obtained similar results for protein expression of E-cadherin, Slug, and vimentin by western blot (Figure 2D, 2F).

**Knockdown of IMP3 changed the mRNA and protein levels of EMT markers in MDA-MB-231 cells**

In addition to examining the effects of IMP3 overexpression, we knocked down IMP3 in a strong IMP3-expressing line, MDA-MB-231, which expresses endogenous Slug and vimentin. Initially, transient knockdown experiments were carried out with IMP3 siRNA. We observed a dramatic decrease in Slug and vimentin mRNA by real-time PCR when IMP3 was knocked down \((P < 0.05)\). In contrast, E-cadherin showed a significant increase in expression \((P < 0.05)\) (Figure 2B). Strikingly, the E-cadherin, Slug, and vimentin protein expression levels have the same trend as mRNA \((P < 0.05)\). This situation was more physiologically relevant than the situation of overexpression. Notably, we did not detect any E-cadherin expression in MDA-MB-231, but we detected a slight upregulation of E-cadherin in 231-siRNA cells (Figure 2E, 2G).

**Overexpression or knockdown of IMP3 induced alterations in morphology, migration, and invasion**

We next explored the specific biological characteristics influenced by IMP3 in our two cell lines. T47D-IMP3 cells exhibited a slight alteration in morphology, growing in elongated individual cells rather than tight aggregates (Figure 3B). The 231-siRNA-IMP3 cells grew in more tightly-organized rather than irregular sheets (Figure 3A). We also evaluated both cell migration and invasion by a transwell assay. Consistent with previous data [19], the MDA-MB-231 line was highly invasive, but 231-siRNA-IMP3 cells exhibited reduced migration and invasion compared to the control group. T47D cells were poorly invasive, but T47D-IMP3 cells showed moderately high invasion and migration. The changes in morphology, migration, and invasion during IMP3 overexpression or knockdown also suggested a reversal or induction of EMT, respectively.

**IMP3 promotes EMT by binding to Slug mRNA and regulating its expression**

An important consideration based on the above findings is whether IMP3 interacts directly with...
Slug or regulates its expression indirectly. We therefore performed ribo-immunoprecipitation qPCR, which detects specific protein-RNA interactions. IGF II was used as a positive control, and estrogen receptor β (ESR2) was a negative control [20]. Indeed, IMP3 bound to Slug mRNA at a level comparable with its binding to IGF II mRNA (Figure 2C). These data demonstrate that IMP3 bound to Slug mRNA and, as a consequence, regulated its expression.

**Slug rescues IMP3 induced alterations in EMT, cell morphology, migration, and invasion in T47D-IMP3 cells and 231-siRNA-IMP3 cells**

To further determine whether IMP3 exerts its function through regulation of Slug, we per-
formed gain-of-function analyses by overexpressing Slug in 231-siRNA-IMP3 cells and loss-of-function analyses by knocking down Slug in T47D-IMP3 cells. As expected, overexpression of Slug rescued the morphological change (Figure 3A), cell migration and invasion caused by silencing IMP3 in MDA-MB-231 cells (Figure 4C, 4E). The upregulation of vimentin and the downregulation of E-cadherin were also observed (Figure 4A). On the other hand, knockdown of Slug in T47D-IMP3 cells could also have the opposite change in EMT, morphology, migration and invasion (Figure 4B, 4D, 4F). Taken together, these results suggest that Slug is a functional target of IMP3.

Discussion

A role for IMP3 in invasion was foreshadowed by the finding that IMP1 and IMP3 induce invadopodia formation by stabilizing CD44 mRNA in HeLa cells [22]. IMP3 is expressed in a variety of malignant neoplasms, including endometrial, renal clear cell, hepatocellular carcinomas, prostate, and breast cancers, and is emerging as a useful indicator of the progression and outcome of several cancers. Specifically, high IMP3 expression predicts a propensity for metastasis in renal-cell carcinoma patients [5]. IMP3 also plays an important role in tumor invasion and metastasis and is a strong prognostic factor for patients with hepatocellular carcinoma [23]. IMP3 is an important factor in the migration of HeLa cells [24] and in the aggressive behavior (including invasion) of glioblastoma cells [25]. A recent study shows that IMP3 is an independent predictor of patients’ poor survival in muscle-invasive bladder cancer [26]. In the present study, our data provide insight into the mechanisms and functions of IMP3 in migration and invasion.

We focused on the EMT, since understanding its molecular mechanisms is crucial for developing new therapeutic strategies against breast cancer invasiveness and metastatic dissemination of carcinoma cells. EMT is believed to be a key step during embryonic morphogenesis, heart development, chronic degenerative fibrosis, and cancer metastasis. In the progression of malignancy, EMT also enables carcinoma cells to lose their epithelial adherence, undergo cytoskeleton remodeling, facilitate their detaching from the tumor mass, migrate to distant tissue sites, and eventually form metastatic tumor masses. IMP3 overexpression is associated with a more aggressive phenotype and decreased overall survival and contributes to migration and invasion through binding with CD164 and MMP9 mRNAs in breast cancer [14, 19]. We therefore hypothesized that IMP3 and EMT are associated.

In the present study, we presented several lines of evidence showing that IMP3 is involved in EMT. Our immunohistochemical results demonstrated that breast cancer with high IMP3 expression displayed the enhanced expression of the mesenchymal markers Slug and vimentin and decreased expression of the epithelial markers E-cadherin, suggesting an EMT during IMP3-regulated breast cancer development. We transfected full-length IMP3 into IMP3-negative T47D cells to further explore the mechanism and investigate the effect of IMP3 on EMT. Consistent with the morphological change, E-cadherin was downregulated while vimentin and Slug were upregulated in transfected cells on both mRNA and protein levels. In contrast, knockdown of IMP3 in the strong IMP3-expressing MDA-MB-231 cell line upregulated E-cadherin expression and downregulated Slug and vimentin expression. The change of E-cadherin expression was the same as previously reported [23]. In addition, we showed that IMP3 could significantly enhance the invasion and migration ability of breast cancer cells in vitro in transwell assays. These findings are also consistent with previous data [19]. The acquisition of EMT characteristics may give these breast cancer cells a higher aggressive potential, resulting in the invasive and metastatic behavior. Thus, our findings shed new light on the role of IMP3 in the invasion and metastasis of breast cancer cells through EMT.

Given that IMP3 is an mRNA-binding protein, it should regulate the expression of specific mRNA that is critical for migration and invasion. To find the elaborate mechanism of interaction between IMP3 and EMT marker genes, we performed ribo-immunoprecipitation qPCR and found that IMP3 could bind to Slug mRNA directly. Slug is a ces-1-related zinc finger transcription factor that belongs to the Snail superfamily. Slug is thought to repress E-cadherin expression, leading to EMT, and to facilitate the invasion and metastasis of cancers, including
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breast [27], ovarian [28], and esophageal cancers [29]. We also found overexpression or knockdown of Slug could rescue IMP3-induced alterations in morphology, migration, and invasion. In this study, we confirmed that Slug was a novel, direct and functional target of IMP3.

In summary, IMP3 could promote invasion and migration through EMT in breast cancer cells. To the best of our knowledge, we are the first to provide evidence that IMP3 could induce EMT through binding to Slug mRNA in breast cancer. Further studies are underway to identify the special pathway involved in IMP3-mediated EMT. Thus, exploring the roles and underlying mechanisms of IMP3 in progression may provide a promising target for developing a novel treatment strategy for breast cancer.

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Disclosure of conflict of interest
None.

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