MicroRNA-127 Is Downregulated by Tudor-SN Protein and Contributes to Metastasis and Proliferation in Breast Cancer Cell Line MDA-MB-231

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ABSTRACT

Tudor-SN is a multifunctional protein that is highly expressed in multiple cancers including breast cancer. Tudor-SN, as a component in RNA-induced splicing complex, was recently reported to regulate gene expression in a micro-RNA (miRNA)-dependent manner, such as let-7, miR-34a and miR-221. However, how Tudor-SN is associated with cancer development still remains largely elusive. In the present study, we explored the role of Tudor-SN in breast cancer. Stable knockdown of endogenous Tudor-SN, performed on the breast cancer cell line MDA-MB-231 by small hairpin RNA expression vectors, suppressed the in vitro migration and invasion ability of the metastatic breast cancer cell line. Interestingly, we found Tudor-SN as a miRNA regulator according to microarray analysis, and further identified that Tudor-SN negatively regulated the expression of miR-127, and consequently increased the expression of the proto-oncogene BCL6 which was a convincing target of miR-127. Moreover, overexpression of miR-127 reduced the in vitro migration and proliferation ability of breast cancer cell MDA-MB-231. Collectively, our results suggested a novel mechanism that Tudor-SN promoted metastasis and proliferation of breast cancer cells via downregulating the miR-127 expression.

Key words: breast cancer; Tudor-SN; miR-127; metastasis; proliferation

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INTRODUCTION

Tudor staphylococcal nuclease (Tudor-SN), also known as SND1 (staphylococcal nuclease domain containing 1) or p100, is a highly conserved and ubiquitously expressed multifunctional protein. Tudor-SN has been shown to function as the coactivator of a group of transcription factors, such as EBNA2 (Tong et al., 1995), Pim-1 (Leveryson et al., 1998), STAT6 (Valinova et al., 2005; Wang et al., 2010; Yang et al., 2002), and STAT5 (Paukku et al., 2007). It also takes part in snRNP assembly (Gao et al., 2012; Yang et al., 2007), and stress granules (SGs) formation (Gao et al., 2010). Recently, Tudor-SN has been identified as the major component of the RNA-induced silencing complex (RISC) which contributes to the RNA degradation at the post-transcriptional level (Caudy et al., 2003). Moreover, Tudor-SN could also promote the cleavage of hyper-edited double-stranded RNA containing multiple I.U and U.I pairs (Scadden, 2005; Scadden and Smith, 2001), which subsequently modulates microRNA (miRNA) processing and expression through RNA editing (Kawahara et al., 2007; Yang et al., 2005).

Although divergent roles of Tudor-SN have been discovered in cellular investigation, little information is available on the biological potential of Tudor-SN in carcinogenic process. Tudor-SN is upregulated in human colon cancers and overexpression of Tudor-SN in rat intestinal epithelial cells resulted in loss of contact inhibition and promoted cell proliferation via activation of the Wnt signaling pathway (Notterman et al., 2001; Tsuchiya et al., 2007). Tudor-SN overexpression has been detected in prostate cancer, and small interfering RNA (siRNA) inhibition of Tudor-SN inhibited viability of prostate cancer cells (Kuruma et al., 2009). Proteomic profiling identified high Tudor-SN expression in mouse metastatic breast cancer cells and also in tumor samples of metastatic breast cancer patients, and Tudor-SN as a metadherin (MTDH)-interacting protein could promote lung metastasis in breast cancer (Blanco et al., 2011; Ho et al., 2009). In human hepatocellular carcinoma, Tudor-SN is also overexpressed and the interaction between Tudor-SN and oncogene astrocyte-elevated gene 1 (AEG-1) in RISC increased RISC activity, which facilitated degradation of tumor suppressor messenger RNAs (mRNAs) by oncogenic miRNAs, such as miR-221 (Yoo et al., 2011).

Breast cancer is considered as a heterogeneous disease comprising various types of neoplasms. Since the miRNA deregulation in breast cancer was first reported earlier (Gao et al., 2005; Wang et al., 2010; Yang et al., 2002), pGenesil-Tudor-SN-small hairpin RNA (shRNA) was produced by GeneSil Biotechnology Co. (China). Tudor-SN siRNA and negative control oligomer were purchased from Invitrogen as reported earlier (Gao et al., 2012).

Generation of Stable Cell Lines

The plasmid pGenesil-Tudor-SN-shRNA or pGenesil-scramble-shRNA was transfected into MDA-MB-231 cells using FuGENE HD (Roche). After 48 hr incubation, the cells were cultured in the media containing 1,000 µg/mL G418 (geneticin) and lasted for 2 weeks. The knockdown of Tudor-SN was confirmed by Western blot analysis using anti-Tudor-SN antibody, and then cells further grown in culture medium supplemented with 500 µg/mL G418.

Invasion Assay

Different MDA-MB-231 stable cells were first starved overnight. 1 × 10⁴ cells were resuspended in the serum-free Dulbecco’s modified eagle medium containing 0.1% bovine serum albumin (Sigma), and placed in the top chambers (8 µm membrane) coated with Matrigel (BD Biosciences) in triplicate. The lower chamber was filled with 10% fetal bovine serum as the chemoattractant. Keep the chambers on 24-well plates at 37°C for 18 hr. The invaded cells on the other side of membrane were fixed and stained in 0.1% crystal violet, and quantified by counting the numbers in five randomly chosen microscopic fields and photographed under a light microscope at 100× magnification.

Wound Healing Assay

The parental or different transfected MDA-MB-231 cells (5 × 10⁵/well) were seeded in six-well, and grown to reach confluence, the cell layer was scratched with a sterile plastic pipette tip. The distance of cell migration was examined dynamically and recorded using an OLYMPUS CKX41 microscope system (Japan) at 0 hr, 24 hr, or 48 hr to quantify the wound healing assay. The average wound width was measured with Image J software and distance of cell migration was calculated. Each experiment was done in triplicate.

Cell Proliferation Assay

Cells were plated in 96-well plates at a density of 2.5 × 10³ per well after transfected with the pre-miR-127 plasmid using FuGENE HD (Roche) and incubated at 37°C in a humidified 5% CO₂ for 5 days. At each time
miRNA Microarray

Total RNA (50–100.0 µg) samples were extracted from HeLa-Tudor-SN-shRNA and HeLa-scramble-shRNA stable cell line which was constructed previously in our laboratory, and were sent to Capital Bio Corp (Peking, China) for miRNAs microarray assay. All probe sequences in the array were based on Sanger miRBase Release 8.2, which represents the updated validated homo miRNAs sequences. RNA samples were labeled with fluorescence dye, followed by hybridization with the probe-containing Chip. The signals were presented after background subtraction, normalization and detection evaluation. The ratio of the two sets of detected signals and P values of the t test were calculated; differentially detected signals were those with a P value <0.05.

Western Blot Analysis

Total cell lysate of MDA-MB-231 was collected by ice-cold RIPA lysis buffer. Proteins were subject to 8% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) and analyzed by Western blotting with mouse monoclonal antibody anti-Tudor-SN, anti-Flag (Sigma), and monoclonal antibody anti-β-actin (Sigma). The mouse monoclonal antibody anti-Tudor-SN antibody was generated against SN4 domain (amino acids 507–674) of Tudor-SN in Dr. Silvennoinen’s laboratory. The results were visualized by using chemiluminescent substrate kit (Super Signal West Pico Trial kit; Pierce Biochemicals).

Quantitative Reverse Transcription Polymerase Chain Analysis of miRNA

Total RNA was extracted from cells using the mirVana miRNA Iso Kit (Ambion, Austin, TX) according to the manufacturer’s instruction. The quality of the total RNA was evaluated using smart spec Plus (BioRad, Hercules, CA). The primers used for miR-127 and snRNA U6, and stem-loop real-time polymerase chain analysis (RT-PCR) for mature miR-127 was performed as previously described (Saito et al., 2006). The Taqman MicroRNA Assays were obtained from Applied Biosystems (Foster City, CA). PCR products were analyzed on 3% agarose gels with an internal control snRNA U6 RNA. All reactions were run in triplicate.

Real-Time PCR for mRNA

Total RNA was isolated from cells as mentioned above. Total RNA was used for cDNA synthesis with M-MLV (TaKaRa Biotechnology). Quantitative Real-time PCR reactions were run on an ABI 7500 apparatus and results were analyzed with SDS software (Applied Biosystems) by the way of ΔΔCt. Amplification patterns were normalized on the β-actin housekeeping gene.

Flow Cytometry

Cells were harvested by trypsinization, washed and resuspended in PBS for cytometric analysis. All samples were performed on the Guava easyCyte Flow Cytometer (Merck Millipore). Forward scatter and side scatter data were used to identify viable cells, and gates were set to exclude cellular debris. Green fluorescence (FL1) was measured using a 525/30 nm band pass filter, and the results were analyzed using GuavaSoft 2.1 software (Merck Millipore).

Statistical Analysis

Results were reported as mean ± standard error (SE), Independent-Samples Student’s t test or one-way analysis of variance were performed to analyze the data, and the changes were considered significant if P < 0.05.

RESULTS

The Expression of Tudor-SN in Human Breast Cancer Cell Lines

To investigate the potential role of Tudor-SN in breast cancer, we tested the expression profile of Tudor-SN in three human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 which had different potential malignancy. The MDA-MD-231 cell line, which had highly invasive and metastatic ability, demonstrated a significantly higher expression of Tudor-SN (Fig. 1A) compared with the poorly invasive/nonmetastatic breast cancer cell lines MCF-7 and T47D. So, in this study, we chose human MDA-MB-231 cell line as the in vitro research model to perform further experiments.

The stable MDA-MB-231 cell line with knockdown of the endogenous Tudor-SN protein was established by transfecting the cells with the plasmid containing Tudor-SN-shRNA (pGenesil-Tudor-SN-shRNA). On the other hand, MDA-MB-231 cells were stably transfected with the plasmid containing the scramble shRNA (pGenesil-scr-shRNA) as negative control (Scr-cont). As shown in Fig. 1B, the results of western blot assay showed that the endogenous Tudor-SN protein was efficiently decreased in Tudor-SN-shRNA MDA-MB-231 cells (Lane...
2) compared with Scr-cont (Lane 3) or parental (Lane 1) cells. Thereafter, the established stable MDA-MB-231 cells with knockdown of endogenous Tudor-SN protein (Tudor-SN-shRNA) were used to carry out the relative experiments to investigate the function of Tudor-SN in the development of breast cancer.

High Expression of Tudor-SN Correlates With Metastatic Ability of Breast Cancer Cells

Migration and invasion are two key components of the metastatic cascade. We therefore performed wound healing assay and in vitro invasion assay to evaluate the migration and invasive abilities of the Tudor-SN in MDA-MB-231 cells. The Tudor-SN-shRNA stable cells, the Scr-cont cells or the parental MDA-MB-231 cells were cultured to confluence and scratched in six-well plates. As shown in Fig. 2A, the parental and Scr-cont MDA-MB-231 cells migrated more rapidly into the wounded area, while knockdown of endogenous Tudor-SN protein (Tudor-SN-shRNA) in the cells resulted in the retardation of the wound healing. The results were quantified by image J software and demonstrated in Fig. 2B. Statistical analysis demonstrated that compared with Scr-cont and parental MDA-MB-231 cells, the migration distance of Tudor-SN-shRNA MDA-MB-231 cells reduced by 71.4% and 67.0% at 24 hr, and 66.7% and 68.7% at 48 hr, respectively ($P < 0.05$). This data suggested that knockdown of Tudor-SN decreased the migration ability of human breast cancer cells.

The influence of Tudor-SN on the invasive ability of breast cancer cells was examined by in vitro invasion assay. The number of cells that invaded the Matrigel layer and attached to the lower side of the filter was counted. As shown in Fig. 2C and D, knockdown of Tudor-SN in MDA-MB-231 (Tudor-SN-shRNA) cells led to an average of 68% and 62% decreased invasiveness, compared with Scr-cont or parental 231 cells, respectively ($P < 0.05$). These results demonstrated that Tudor-SN was associated with the migration and invasion ability of breast cancer cells in vitro, indicating the potential role of Tudor-SN in breast cancer metastasis.

Tudor-SN Regulates the Expression of miR-127

Tudor-SN is one of the essential components of RISC, which is involved in gene silencing (Caudy et al., 2003), and it may take part in regulating miRNA expression as well (Li et al., 2008). Using microarray analysis, we examined the miRNA expression pattern in HeLa cells with knockdown of endogenous Tudor-SN protein previously, and found that a group of miRNAs were upregulated or downregulated. We further verified the expression of several miRNAs in HeLa cells as presented in Table 1, and miR-127 was one of the downregulated miRNAs by Tudor-SN. To further validate the results of microarray in breast cancer cells, we examined the effects of Tudor-SN on miR-127 expression on the basis of introducing exogenous miR-127 into the MDA-MB-231 cells. The pre-miR127 plasmid (GFP-pre-miR-127) was first transfected into MDA-MB-231 cells. After 36 hr
incubation, the cells were divided into four different plates and then transfected with pSG5-Tudor-SN-Flag, Tudor-SN siRNA, scramble control (Scr) or pSG5 plasmid as control, respectively. Total cell lysate or RNA was collected 48 hr post-transfection and analyzed from different transfected cells. As shown in Fig. 3A, the protein level of endogenous β-actin from different lysates were comparable, the ectopically expressed Tudor-SN was detected by blotting with anti-Tudor-SN antibody (upper panel, Lane 2). On the other hand, the endogenous Tudor-SN was efficiently depleted (upper panel, Lane 4) by siRNA. As shown in Fig. 3B, upregulation of Tudor-SN inhibited the expression of miR-127 (about half of the control), while depletion of Tudor-SN enhanced the expression of miR-127 to 1.5-fold higher than the control group. As BCL6 mRNA is the target for miR-127, we therefore detected the expression of BCL6 by Real-time PCR. As shown in Fig. 3D, the mRNA level of BCL6 was reverse to miR-127 expression. Meanwhile, we also detected the protein level of BCL6 (Fig. 3C), which was consistent with the mRNA level in different samples. These data further indicated that Tudor-SN could negatively regulate the expression of miR-127, and consequently upregulated the expression of BCL6.

**miR-127 Retards the Migration and Proliferation of MDA-MB-231 Cells**

We performed wound healing assay to investigate whether the effect of miR-127 on breast cancer metastasis was correlated with Tudor-SN. MDA-MB-231 cells were first transfected with GFP-pre-miR-127 plasmids. The autofluorescence of wild-type cells was measured by the flow cytometer 48 hr post-transfection, immediately following the fluorescent intensity measurement of the GFP-transfected cells. The transfection efficiency was found to be 67.4%, as shown in Fig. 4A. Meanwhile, Fig. 4B indicated the expression of miR-127 in the MDA-MB-231 cells (upper panel), and U6 was used as control (lower panel). Otherwise, the cells were plated for wound healing assay. As shown in Fig. 4C, overexpression of miR-127 reduced the migration ability of the MDA-MB-231 cells, which was in line with the knockdown of Tudor-SN protein. The results were quantified by image J software and demonstrated in Fig. 4D. The overexpression of miR-127 led to an average of 30.0% decreased in vitro migration ability compared with the control group. We then used the MTS assay to determine the effect of miR-127 overexpression on cell growth, and found that ectopic expression of miR-127 inhibited cell proliferation compared with control MDA-MB-231 cells (Fig. 4E). Western blot analysis indicated that overexpression of miR-127 downregulated the expression of BCL6 (Fig. 4F, upper panel). These data indicated that miR-127 was sufficient to influence the tumorigenic properties of the MDA-MB-231 cells. Therefore, Tudor-SN protein could modulate the breast cancer progress via regulating the expression of miR-127.

**TABLE 1. List of miRNAs regulated by Tudor-SN**

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-204</td>
<td>−2.13</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-127</td>
<td>−2.09</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-101</td>
<td>−2.03</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-376a</td>
<td>−2.14</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-181a</td>
<td>+2.03</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-191</td>
<td>+2.13</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-let7e</td>
<td>+2.04</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td>+1.55</td>
<td>0.01</td>
</tr>
</tbody>
</table>

![Fig. 3. Tudor-SN downregulates the miR-127 expression in MDA-MB-231 cells.](image-url)
DISCUSSION

The recent intriguing findings suggested that Tudor-SN played essential roles in the progress of different cancers. For example, it was involved in early stages of colon carcinogenesis through its novel function as a post-transcriptional regulator (Tsuchiya et al., 2007). In addition, Tudor-SN contributed to hepatocarcinogenesis via two mechanisms: one was the overexpression of AEG-1 and Tudor-SN leading to increased RISC activity (Yoo et al., 2011), and the other one was through the activation of NF-κB (Santhekadur et al., 2012). Tudor-SN has also been reported to overexpress in breast cancer, and Tudor-SN as a metadherin (MTDH)-interacting protein, could promote lung metastasis in breast cancer (Blanco et al., 2011). In the present study, we demonstrated that the expression level of Tudor-SN was higher in the invasive/metastatic breast cancer cell line (MDA-MB-231) than the poorly invasive/nonmetastatic breast cancer cell lines (MCF-7 and T47D), which implied that Tudor-SN took part in the regulation of migration and invasion in the breast cancer. Supportively, Kuruma et al. (2009) reported that Tudor-SN expression is increased with increasing grade and aggressiveness of prostate cancer, and might play an important role in distinguishing the presence of a more aggressive and clinically significant phenotype of prostate cancer.

The results from other studies showed that Tudor-SN transcriptionally regulated 48 genes related to lung metastasis in the process of breast-to-lung metastasis, and knockdown of Tudor-SN in the breast cancer

Fig. 4. Ectopic expression of miR-127 inhibits the cell migration ability and proliferation of MDA-MB-231 cells. (A) The MDA-MB-231 cells were transfected with plasmid GFP-pre-miR-127 as indicated. The transfection efficiency of miR-127 was calculated by flow cytometric analysis of GFP-expression. (B) The expression level of miR-127 was detected by stem-loop RT-PCR (upper panel), U6 RNA as control (lower panel). (C) Representative photomicrographs showing the response of different MDA-MB-231 cells as indicated in the wound healing assays. (D) Represent the quantification of wound healing assays in (C). Mean ± SE of three independent field measurement is shown. *P < 0.05, compared with control group. (E) Ectopic expression of miR-127 inhibits the cell proliferation. Values are presented as the mean ± SE of three independent experiments with six samples. **P < 0.01. (E) The protein level of BCL6 (upper panel) or β-actin (lower panel) in different cell lysates.
reduced the lung metastasis in the nude mice (Blanco et al., 2011). In line with their data, we found that knockdown of Tudor-SN suppressed the in vitro migration and invasion ability of the metastatic breast cancer cell line MDA-MB-231, which indicated that Tudor-SN might be an important determinant of breast cancer progression. Based on the knowledge, we considered that Tudor-SN might be implicated in carcinogenesis via different molecular mechanisms.

Accumulating evidences indicated that miRNAs played important roles in the cancer development via post-transcriptional regulation. Tudor-SN has been described as an essential component of RISC (Caudy et al., 2003). It could regulate gene transcription via the association of RNA polymerase II and other basal transcription proteins (Yang et al., 2002). Additionally, Tudor-SN has been identified as a ribonuclease specific for inosine-containing primary transcripts of miRNA. It modulates miRNA processing and expression by degrading the primary transcripts edited by ADAR enzymes (Yang et al., 2005). Therefore, it is possible that Tudor-SN also took part in the regulation of miRNA expression. We thus explored the functional link of Tudor-SN, miRNAs and the breast cancer development.

miR-127 is one of the miRNAs regulated by Tudor-SN protein in our study. Human miR-127, embedded in a CpG island, is a potential tumor suppressor whose expression decreased or silenced in cancer cells and in sets of primary tumors such as prostate cancer and bladder cancer (Saito et al., 2006). miR-127 has also been demonstrated to be downregulated in BRAF V600E mutated papillary thyroid carcinoma cell lines (Cahill et al., 2007). Subsequently, Lee et al. (2008) showed that increased miR-127 expression was significantly associated with lymph node metastasis ($P = 0.006$), suggesting the ability of miR-127 in promoting metastasis of cancer cells. However, its importance has yet to be explored in breast cancer. The proto-oncogene BCL6 has been verified as a target gene of miR-127 (Saito et al., 2006), which acted as a tumor promoter in breast cancer and played differentiation-suppressive role in mammary epithelial (Bos et al., 2003; Logarajah et al., 2003). Consistently, our findings indicated that Tudor-SN downregulated the expression of miR-127 in the breast cancer, meanwhile, the expression of BCL6 was upregulated. On the other hand, the ectopic expression of miR-127 downregulated the expression of BCL6, and retarded the migration and proliferation of breast cancer cells, which presented the same effect as the knockdown of endogenous Tudor-SN. Our observation was the same as reported by Chirstian et al that miR-127 could trigger its target mRNA degradation in vivo (Matranga et al., 2005). The present study demonstrated the novel finding that breast cancer metastasis and growth was regulated by high expression of Tudor-SN protein, which could downregulate the miR-127 level and increase the BCL6 expression which is the target of miR-127.

In fact, miRNAs as a mediator regulated by Tudor-SN protein in tumorigenesis has been previously reported. For instance, the suppressive effect of Tudor-SN on rat sarcoma (Ras) expression occurred in a let-7-deendent manner, which was implicated as a tumor suppressor in lung tissue (Johnson et al., 2005). In addition, Tudor-SN has been demonstrated to mediate the E2F5 (E2F transcription factor 5) gene suppression via upregulating the expression of miR-34a, which played important role in colon carcinogenesis (Tsuchiya and Nakagama, 2010). As we reported earlier, Tudor-SN protein participated in the assembly of small nuclear ribonucleoprotein (snRNPs) and regulated the in vitro pre-mRNA splicing and splicingosome assembly (Gao et al., 2012; Yang et al., 2007). On the other hand, siRNAs and miRNAs has been shown to function as components of two ribonucleoprotein complexes, RISCs and miRNPs, respectively, while Tudor-SN is also one of the components consistently found in RISCs and miRNPs (Filipowicz et al., 2005). Therefore, it is possible that Tudor-SN regulates the expression of miRNAs via the involvement of miRNP assembly. However, it is unclear why Tudor-SN negatively regulates the expression of miR-127, and whether it is direct or indirect regulation. Further studies need to be conducted to unravel these open questions.

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LITERATURE CITED


