Original Article

Thioredoxin-1 promotes colorectal cancer invasion and metastasis through crosstalk with S100P

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ABSTRACT

Thioredoxin-1 (Trx-1) is a small redox-regulating protein, which plays an important role in several cellular functions. Despite recent advances in understanding the biology of Trx-1, the role of Trx-1 and its underlying signaling mechanism in colorectal cancer (CRC) metastasis have not been extensively studied. In this study, we observed that Trx-1 expression is increased in CRC tissues compared to the paired non-cancerous tissues and is significantly correlated with clinical staging, lymph node metastasis and poor survival. Overexpression of Trx-1 enhanced CRC cell invasion and metastasis in vitro and in vivo. Conversely, suppression of Trx-1 expression decreased cell invasion and metastasis in vitro and in vivo. Moreover, Trx-1 activates S100P gene transcription. S100P, in turn, promotes Trx-1 expression and nuclear localization by upregulating p-ERK1/2 and downregulating TXNIP expression. Our finding provides new insight into the mechanism of Trx-1/S100P axis in the promotion of CRC metastasis, and suggests that the Trx-1/S100P axis and their related signaling pathways could be novel targets for the treatment of metastatic CRC.

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Introduction

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. In recent years, CRC incidence rates are rapidly increasing in developing countries that previously showed a decreased risk of CRC [1]. Despite great advances in diagnosis and treatment during the last few decades, the overall five-year survival rate for CRC remains 40 to 45 percent [2]. Metastasis is the leading reason for the resultant mortality of CRC patients, but the underlying molecular mechanisms of CRC metastasis are still not fully understood.

Oxidative stress has been demonstrated to be involved in the development and progression of several cancers, including CRC [3]. The thioredoxin (Trx) system is a major antioxidant system and plays an important role in various cellular functions, including maintaining cellular redox homeostasis and mediating growth, survival, and chemoresistance [4,5]. It contains thioredoxin-1 (Trx-1, TXN), a small 12-kDa protein with redox-activation present in the cytoplasm [6]. Overexpression of Trx-1 is associated with altered cellular redox status, growth promotion of cancer cells, anti-apoptotic and inflammation modulation by means of reducing equivalents and a transcriptional regulator [7–10]. Trx-1 expression was increased in several human tumor tissues, including liver [11], lung [12], pancreas [13], colorectal [14], uterine cervix [15], and gastric carcinoma [16]. Increased Trx-1 expression is associated with inhibition of apoptosis, enhanced proliferation of tumor cells, aggressive tumor growth, high reactive oxygen species generation and a poor survival rate [10]. Trx-1 overexpression increases both hypoxia-induced factor 1z (HIF-1z) levels and HIF-1z transcriptional activation in cancer cells, which in turn enhances tumor angiogenesis by increasing vascular endothelial growth factor (VEGF) production.
Table 1
Trx-1 expression and clinicopathological parameters in colorectal cancer specimens.

<table>
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<tr>
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<th>All cases</th>
<th>Trx-1 protein</th>
<th>Overexpression</th>
<th>P value</th>
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<td>47</td>
<td>19</td>
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<td>0.039*</td>
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<tr>
<td>Female</td>
<td>63</td>
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<td>49</td>
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<td><strong>Histologic grade(WHO)</strong></td>
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<td></td>
<td></td>
<td>0.762</td>
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<tr>
<td>Low</td>
<td>95</td>
<td>28</td>
<td>67</td>
<td></td>
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<tr>
<td>High</td>
<td>15</td>
<td>5</td>
<td>10</td>
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<tr>
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<tr>
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<td>22</td>
<td>35</td>
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<td>11</td>
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<tr>
<td>Yes</td>
<td>54</td>
<td>13</td>
<td>41</td>
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</tr>
</tbody>
</table>

Statistical analyses were performed by χ² test. *P < 0.05.

S100P is a member of the S100 family of proteins containing 2 EF hand calcium-binding motifs that is involved in the regulation of a number of cellular processes through its calcium-binding ability [18–20]. S100P expression is upregulated in various cancers and is associated with poor clinical outcomes [19,22–25]. Most studies have indicated that overexpression of S100P correlates with tumor growth and metastasis [22,23,26]. We previously reported that S100P expression is upregulated in CRC and correlates with clinical staging and lymph node metastasis and recurrence [22]. We have also demonstrated that overexpression of S100P promotes the migration and invasion of CRC cells, whereas silencing S100P expression by lentiviral vector mediated shRNA inhibits CRC invasion and metastasis in vitro and in vivo, suggesting that S100P plays an important role in CRC metastasis [22,27].

In the present study, we observed that the expression of Trx-1 is increased in human CRC tissues compared to the paired noncancerous tissues and high Trx-1 expression is associated with advanced clinical stages, lymph node metastasis and poor clinical outcomes. We also found a novel positive feedback loop between Trx-1 and S100P, which promotes CRC invasion and metastasis. Trx-1 regulates S100P gene transcription through interaction with SP1. S100P in turn promotes Trx-1 expression and nuclear localization by upregulating p-ERK1/2 and downregulating Thioredoxin Interacting Protein (TXNIP) expression. Our findings provide new insight into the mechanism of Trx-1/S100P axis in the promotion of CRC metastasis, and suggests that the crosstalk between Trx-1 and S100P and their related signaling pathways could be significant potential therapeutic targets for the treatment of CRC metastasis.

Materials and methods

Cell culture and chemicals

Human CRC cell lines SW480, SW620 and DLD-1, and HEK293T cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. SW480 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). SW620, DLD-1 and HEK293T cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS. Cells were cultured in a humidified 37°C incubator with 5% CO2.

Lentiviral vector construction and transduction

See Supplementary Materials and Methods for details.

Cell proliferation, migration and invasion assays, real-time Polymerase Chain Reaction (PCR) and Western blot analysis

See Supplementary Materials and Methods for details.

siRNA transfection

siRNAs were purchased from GenePharma (GenePharma Co., Ltd., Shanghai, China). The sequence of selected regions to be targeted by siRNAs for TXNIP was 5'-CAU UCC UGC AGU UGA AUA UTT-3'. Cells were transfected with 50 nM scramble siRNA (Negative control, NC), or TXNIP-siRNA by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.
In this study, 112 cases of CRC were retrospectively selected from the surgical pathological database of the First Affiliated Hospital of Wenzhou Medical University, China, between 2003 and 2007. These CRC cases included 49 male and 63 female patients with a mean age of 61.54 years (34–81 years). All the patients underwent colectomy and their clinical characteristics are shown in Table 1. None of the patients received radiotherapy or chemotherapy prior to surgery. Patients who died of causes other than CRC were excluded from the study. The paraffin embedded blocks were then cut into 4 μm sections for immunostaining. IHC analysis is described in the Supplementary Materials and Methods. In addition, 45 pairs of fresh CRC samples and their adjacent non-tumor tissues were obtained from surgical specimens after informed consent. The tissues were immediately snap-frozen in liquid nitrogen after collection and stored at −80 °C till extracted for RNA. Tumor grades were defined according to the criteria of WHO (2010). The pathological Tumor-Node-Metastasis (TNM) status was assessed according to the criteria of the sixth edition of the TNM classification of the International Union Against Cancer (2002). This study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Immunofluorescence (IF) staining

Cells were grown on a coverslip and fixed in 4% paraformaldehyde at 4 °C for 20 min. After blocking with 10% goat serum in PBS with 0.3% Triton X-100 solution, cells were incubated with a primary antibody overnight at 4 °C. After thorough washing, cells were incubated with a fluorescence-conjugated secondary antibody.
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Luciferase reporter gene assay

The S100P promoter (−236 to +58 relative to the transcription start site) was inserted into the pGL3-basic luciferase reporter vector (Promega, Madison, WI) using primers (S100P promoter-F-Kpn I: 5′-TCC GGT ACC TCA CTG AGT GCG CCG AGA CA-3′, S100P promoter-R-Bgl II: 5′-GAA CAT GTG CTG GTA CAT GCA CAC C-3′). Deletion of the SPI binding site (nt−96 to −76) by PCR from the corresponding S100P promoter construct was performed by overlap extension PCR. The deletion mutation was verified by sequencing. HEK293T and SW480 cells were seeded at a density of 5 × 10^4 cells/well in 24-well plates and co-transfected with the pGL3-promoter reporter vector, a renilla plasmid (RL-SV40), along with a Trx-1–expressed vector or GFP-expressed vector. Cell extracts were harvested 48 h post-transfection and Dual-Luciferase Reporter Assay System (Promega) was used to detect reporter gene activities according to the manufacturer’s instructions. All reporter gene assays were performed in triplicates and repeated twice.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using a Chip assay kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Purified DNA was then analyzed by PCR using primers (forward: 5′-CGA CAC GAC AGT GCA CA GAG C-3′; reverse: 5′-CGA GAA AAT GTC CCA CTG GC-3′) that ampliﬁed the 236 bp S100P promoter around the indicated region or primers (forward: 5′-ATT GGG CCT ACT GGG GTT CT-3′; reverse: 5′-TCC AGA GCA TGA GGA CA-3′) that were located approximately 6 kb upstream of the GAPDH promoter (negative control). Further experimental details are described in Supplementary Materials and Methods.

Results

Immunochemistry study of Trx-1 in colorectal tissues and clinicopathological signiﬁcance of Trx-1 expression in CRC patients

To investigate the expression status of Trx-1 in CRC, 112 pairs comprising CRC specimens and corresponding normal colorectal mucosal tissues were analyzed for Trx-1 expression using immunohistochemistry (Fig. 1A–C). Fig. 1D shows that Trx-1 expression in CRC tissues was signiﬁcantly higher than that of the matched adjacent normal tissues (149.10 ± 41.52 vs. 123.20 ± 60.86, P < 0.001). Significant upregulation of Trx-1 protein expression was also revealed in CRC with lymph node metastasis, relative to CRC without lymph node metastasis (160.3 ± 37.03 vs. 136.4 ± 43.06, P = 0.0044) (Fig. 1E). Overexpression of Trx-1 was seen in 77 out of 110 (70%) primary CRC samples compared to only 48 out of 110 (43.64%) normal mucosal tissues (P < 0.01). Correlation analysis demonstrated that the overexpression of Trx-1 was signiﬁcantly correlated with sex, clinical staging and lymph node metastasis (Table 1).

Ectopic expression of Trx-1 enhances CRC SW480 cell migration and invasion in vitro and liver metastatic potential in vivo

We analyzed Trx-1 expression in CRC cell lines SW480, SW620 and DLD-1 by Western blotting. As the expression levels of Trx-1 in various CRC cell lines (Fig. 51), we used SW620 and DLD-1 cells with high levels of Trx-1 for knockdown experiments and SW480 cells with low levels of Trx-1 for overexpression experiments. A stable cell line with Trx-1 overexpression (SW480-Trx-1) was constructed by lentivirus–mediated gene transfer and increased mRNA and protein levels of Trx-1 in SW480 cells transfected with lentivirus–Trx-1 were veriﬁed by quantitative RT-PCR and Western blotting, respectively (Fig. 2A and B). The MT and plate colony formation assays revealed that overexpression of Trx-1 did not signiﬁcantly change the ability of SW480’s for cell growth (Fig. S2A and B).

Knockdown of Trx-1 by lentiviral mediated shRNA inhibited CRC cell migration and invasion in vitro and liver metastatic potential in vivo

To further study the biological role of Trx-1 in CRC tumor progression, we constructed lentiviral vectors carrying two different shRNAs targeting Trx-1 (Lenti-shTrx-1-1 and Lenti-shTrx-1-2), and infected DLD-1 and SW620 cells with these two shRNA lentiviruses. Quantitative RT-PCR demonstrated that both lenti-shTrx-1-1 and lenti-shTrx-1-2 had inhibitory effects on Trx-1 mRNA expression (Fig. 3A). Lenti-shTrx-1-1 reduced Trx-1 mRNA expression by 92.8% and 91% in DLD-1 and SW620 cells, respectively. Lenti-shTrx-1-2 reduced Trx-1 mRNA expression by 57.6% and 67.3% in DLD-1 and SW620 cells, respectively. Moreover, decreased Trx-1 protein levels in DLD-1 and SW620 cells transduced with lenti-shTrx-1-1 or lenti-shTrx-1-2 were observed by Western blotting (Fig. 3B). Lenti-shTrx-1-1 showed a much stronger effect for inhibition of Trx-1 mRNA and protein expression than lenti-shTrx-1-2. Although the

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MTT and plate colony formation assays revealed that knockdown of Trx-1 had no significant effect on CRC cell growth in vitro (Fig. S3A-D) and mice subcutaneous tumor growth in vivo (Fig. S3E). Knockdown of Trx-1 by lenti-shTrx-1-1 or lenti-shTrx-1-2 significantly reduced the cell migratory and invasive abilities of DLD-1 and SW620 cells (Fig. 3C and D). Specifically, CRC cells transduced with lenti-shTrx-1-1 showed a stronger inhibition of cell migratory and invasive abilities than cells transduced with lenti-shTrx-1-2. Eight weeks after injection with DLD-1-shLuc cells and DLD-1-shTrx-1-1 cells in the spleen, macroscopic nodules on the liver surface were found in seven out of 10 mice in the DLD-1-shLuc cells group, while only three out of 10 mice injected with DLD-1-shTrx-1-1 cells showed macroscopic nodules on the liver surface (Fig. 3E). Knockdown of Trx-1 in DLD-1 cells showed a significant decrease in the average number of macroscopic nodules in the livers of mice compared to the controls (P < 0.01, Fig. 3F).

Trx-1 regulates S100P gene transcription in CRC cells

We conducted further investigation of the downstream effector genes of the Trx-1-mediated invasion and metastasis of CRC cells.

Table 2

<table>
<thead>
<tr>
<th>Cases</th>
<th>S100P</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal expression</td>
<td>overexpression</td>
</tr>
<tr>
<td>Trx-1</td>
<td>25</td>
<td>66</td>
</tr>
<tr>
<td>Normal</td>
<td>57</td>
<td>34</td>
</tr>
<tr>
<td>expression</td>
<td>20 (35.1%)</td>
<td>5 (14.7%)</td>
</tr>
<tr>
<td>overexpression</td>
<td>37 (64.9%)</td>
<td>29 (85.3%)</td>
</tr>
</tbody>
</table>

Our previously study showed that PX-12, an irreversible and competitive inhibitor of Trx-1, could inhibit S100P mRNA expression in DLD-1 cells [28]. We used various concentrations PX-12 (10, 20, 30 μM) to treat DLD-1 and SW620 cells and found that the mRNA expression levels of S100P were reduced in a dose-dependent manner (Fig. S4). Moreover, we found it interesting that overexpression of Trx-1 increased S100P mRNA and protein levels (Fig. 4A) whereas silencing Trx-1 decreased the S100P mRNA and protein levels (Fig. 4B) detected using real-time PCR and Western blotting, respectively. Luciferase reporter gene assays demonstrated that Trx-1 could increase the promoter activity of S100P in HEK293T (with a 5.5-fold increase) and SW480 cells (with a 2.4-fold increase) (Fig. 4C). An SP1 binding site is located between –96 and –85 bp relative to the translational start site (+1) of the S100P gene [29]. As demonstrated in Fig. 4C, SP1 site deletion in the S100P promoter significantly reduced the promoter activity in both cell lines compared to the respective wild-type S100P promoters, indicating that the SP1 binding site may be required for Trx-1-mediated S100P transcription. Immunoprecipitation assay confirmed the formation of a complex between Trx-1 and SP1 in S100P overexpressing SW480 cells (Fig. 4D).
S100P promotes Trx-1 expression and nuclear localization by upregulating p-ERK1/2 and downregulating TXNIP expression in human CRC cells

Western blotting showed that knockdown of S100P by shRNA resulted in decreased expression of Trx-1 in DLD-1 and SW620 cells (Fig. 5A). Furthermore, overexpression of S100P in SW480 cells resulted in increased expressions of Trx-1 and p-ERK1/2 and decreased expression of TXNIP (Fig. 5B). To investigate the localization of Trx-1 in S100P-overexpressing SW480 cells, nuclear and cytosolic extracts were used to detect Trx-1 expression by Western blotting. Indirect immunofluorescent staining was used to confirm the Western blot results. As expected, both Western blotting and immunofluorescent staining revealed that overexpression of S100P induced Trx-1 nuclear translocation in SW480 cells (Fig. 5C and D).

To understand the nuclear translocation mechanism of Trx-1, phosphorylated ERK1/2 MAP kinase was inhibited by incubation of S100P-overexpressing SW480 cells with MEK inhibitor PD98059 for 48 h. Fig. 6A shows that S100P-induced Trx-1 expression and TXNIP downregulation were prevented by PD98059 treatment. Moreover, PD98059 treatment prevented S100P-induced Trx-1 nuclear translocation (Fig. 6A and B) and cell migration and invasion (Fig. 6C). Overexpression of S100P induces SW480 cell Trx-1 expression and nuclear Trx-1 localization (Fig. 6B-D), and migration and invasion [22]. Similar findings were also observed with knockdown of TXNIP by siRNA in SW480 cells. Knockdown of TXNIP induced SW480-cell Trx-1 expression and nuclear Trx-1 localization (Fig. 6D and E), and migration and invasion (Fig. 6F).

Discussion

Trx-1 is a small, ubiquitously expressed redox-active protein, that has numerous important functions including protein disulfide reduction, DNA synthesis, regulation of transcription factors, such as AP-1, Refl, and NF-KB, reduction of H$_2$O$_2$, and protection from apoptosis by binding to ASK-1 [6,30–32]. Trx-1 expression in human cancer is associated with cell proliferation and tumor metastatic progression [18]. Despite having made advances in understanding the biology of Trx-1, its role and its underlying mechanism of action in CRC metastasis is still poorly understood. Our clinical data indicated that Trx-1 was overexpressed in CRC tissues and it was significantly correlated with clinical staging and lymph node metastasis. Notably, high expression levels of Trx-1 are associated with poor survival. Furthermore, overexpression of Trx-1 enhanced CRC cell migration and invasions in vitro and liver metastasis in vivo. In contrast to the Trx-1 overexpression...
experiments, shRNA-mediated Trx-1 knockdown decreased the CRC cell migration and invasion abilities in vitro and in vivo [27]. On the other hand, overexpression of S100P in CRC SW480 cells promotes migration and invasion [22]. In this study, we observed that Trx-1 could regulate S100P expression. The S100P promoter contains an SP1 binding site that is critical for the activation of S100P gene transcription in cancer cells [29]. Trx-1 stimulated MMP-9 expression at the transcriptional level through NF-κB, AP-1 and SP1 elements [36]. Trx-1 can bind and activate a number of transcription factors including: NF-κB, glucocorticoid receptor, AP-1, p53 and SP1 [37]. SP1 is a redox-sensitive transcription factor.}

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regulated transcription factor, and it has been reported that Trx-1 could increase the DNA binding capacity of the SP1 transcription factor and this enhancement could regulate SP1 mediated gene expression [36,38,39]. Thus, we speculated that Trx-1 might regulate the expression of S100P at the transcriptional level through interaction with SP1 that bind to SP1 site. Our results support this hypothesis. Immunoprecipitation assay confirmed the Trx-1/SP1 complex formation. Furthermore, a significant positive correlation between Trx-1 and S100P expression was observed in CRC tissues. These results, collectively suggest that Trx-1 might regulate S100P expression by affecting the transcriptional activity of the S100P promoter in CRC cells.

Our previous studies using proteomic analysis demonstrated that Trx-1 expression is downregulated in DLD-1 cell with knockdown of S100P [27], however regulatory mechanism remains unclear. In this study, we found that S100P could regulate Trx-1 expression and nuclear localization by upregulating p-ERK1/2 and downregulating TXNIP expression. Trx-1 is predominantly localized in the cytoplasm of human cells, however, it can also translocate into the nucleus upon stimulation [40,41]. Ogata et al. have demonstrated that Trx-1 nuclear translocation was induced under nitrosative/oxidative stress conditions through the activation of the ERK1/2 MAP Kinases and downregulation of TXNIP [42]. TXNIP is an important Trx binding protein that directly interacts with Trx-1 in the pathogenesis of various diseases such as cancer and autoimmune diseases [10,43,44]. In this study, we observed that p-ERK1/2 expressions were increased and TXNIP expression was decreased in S100P-overexpressing SW480 cells. Inhibition of phospho-ERK1/2 by the MEK inhibitor PD98059abolished the S100P-induced Trx-1 expression and nuclear translocation, TXNIP downregulation, and CRC cell migration and invasion. On the other hand, silencing TXNIP by siRNA could upregulate Trx-1 expression, facilitate Trx-1 nuclear translocation and promote migration and invasion of SW480 cells. These results suggest that S100P-induced Trx-1 expression and nuclear translocation, and migration and invasion are dependent on the expression levels of TXNIP, which are regulated by the activation of ERK1/2 MAP kinases.

In the present study, we demonstrate, for the first time, a novel positive feedback loop between Trx-1 and S100P, which promotes CRC invasion and metastasis. Trx-1 regulates S100P gene transcription by activating the S100P promoter, S100P in turn promotes Trx-1 expression and nuclear localization by upregulating p-ERK1/2 and downregulating TXNIP expression (Fig. 7). High Trx-1 expression was associated with CRC progression and prognosis, and Trx-1 enhanced invasion and metastasis of CRC cells. These findings strongly suggest that the Trx-1/S100P axis and associated signaling pathways may lead to new therapeutic strategies for the treatment of CRC metastasis.

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**Conflicts of interest**

None.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2017.04.036.

**References**


