The Involvement of S100A14 in Cell Invasion by Affecting Expression and Function of Matrix Metalloproteinase (MMP)-2 via P53-dependent Transcriptional Regulation

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Running Title: S100A14 is involved in cell invasion by regulating MMP2

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Background: The role of S100A14 in tumorigenesis and the underlying mechanisms have not been fully understood. Results: S100A14 affects cell invasiveness by regulating MMP2 transcription in a p53-dependent manner. Conclusion: S100A14 acts as either an inducer or an inhibitor of cell invasion depending on the p53 status of cells. Significance: These studies significantly increase our understanding of how S100A14 regulates cell invasiveness.

SUMMARY

S100A14 has been implicated in tumorigenesis and metastasis. As a member of S100 proteins, the role of S100A14 in carcinogenesis has not been fully understood. Here, we show that ectopic overexpression of S100A14 promotes motility and invasiveness of esophageal squamous cell carcinoma (ESCC) cells. We investigate the underlying mechanisms and find that the expression of matrix metalloproteinase (MMP)-2 is obviously increased after S100A14 gene overexpression. Inhibition of MMP2 by a specific MMP2 inhibitor at least partly reverses the invasive phenotype of cells overexpressing S100A14. By serendipity, we find that S100A14 could affect p53 transactivity and stability. Thus, we further investigate whether the effect of MMP2 by S100A14 is dependent on p53. A series of biochemical assays show that S100A14 requires functional p53 to affect MMP2 transcription and p53 potently transrepresses the expression of MMP2. Finally, RT-qPCR analysis of human breast cancer specimens shows a significant correlation between S100A14 mRNA expression and MMP2 mRNA expression in cases with wild type p53 but not in cases with mutant p53. Collectively, our data strongly suggest that S100A14 promotes cell motility and invasiveness by regulating the expression and function of MMP2 in a p53-dependent manner.

Tumor metastasis remains to be a major problem in the management of cancer and is the main cause of death of cancer patients (1). Metastasis is an extremely complicated process that occurs by a series of steps including epithelial-mesenchymal transition (EMT), invasion, transportation through vessels, mesenchymal-epithelial transition (MET) and outgrowth of secondary tumors (2-4). Degradation of basement membranes and the stromal extracellular matrix (ECM) are critical steps for tumor invasion and metastasis. Matrix metalloproteinases (MMPs) belong to a large group of proteases responsible for degrading the multiple components of the ECM. They are implicated in tumorigenesis, cancer invasion, and metastasis (5). Among them, the gelatinases (MMP-2 and MMP9) were identified as the key MMPs involved in tumor invasion, metastasis and angiogenesis (6, 7).

The S100 protein family is one of the largest subfamily of EF-hand calcium
binding proteins that contribute to multiple key cellular and subcellular processes (8). Some S100 proteins have strong associations with some types of cancer. In particular, it is well known that some S100 proteins (S100A4, S100A8 and S100A9) are closely associated with tumor invasion and metastasis (9, 10), which involves in regulating expression and activity of matrix metalloproteinases (MMPs) (11,12). S100A14 is a member of S100 calcium-binding proteins, which is markedly up-regulated in several tumor tissues including ovarian, breast, and uterine tumor, but down-regulated in some tumors, such as kidney, colon, rectal and esophageal tumor (13). Recently, our study demonstrated that extracellular S100A14 affects esophageal cancer cell proliferation and apoptosis via binding to receptor for advanced glycation end products (RAGE) (14). In addition, S100A14 has also been reported to regulate oral squamous cell carcinoma (OSCC) cell proliferation and invasion (15,16). However, the role of S100A14 in tumorigenesis and progression and the underlying molecular mechanisms need to be further investigated.

P53 is a crucial tumor suppressor whose major function is inducing either growth arrest or apoptosis after cellular stress (17). Emerging evidence shows that p53 also contributes to the modulation of cell invasion (18, 19). The levels and activity of p53 are mainly regulated by posttranslational modifications, such as phosphorylation, acetylation, and ubiquitination (20). Several S100 proteins, including S100A2, S100A4 and S100B, have been reported to interact with p53 and have different effects on p53 transcriptional activity and biological functions (21-23). Although the interactive regulation between S100 proteins and p53 is particular interest, however, the mechanism requires to be further elucidated.

In this study, we investigated the role of S100A14 in cell motility and invasiveness. The promotion of the invasiveness of cells by S100A14 showed a strong correlation with induction of MMP2. Importantly, our findings showed that both the promotion of cell invasiveness and the induction of MMP2 by S100A14 were dependent on p53 status in cell lines. Furthermore, we confirmed the transcriptional repression of MMP2 by p53. Convincingly, the overexpression of S100A14 was significantly correlated with the upregulation of MMP2 in clinical breast cancer samples with wild type p53.

**EXPERIMENTAL PROCEDURES**

**Tissue specimens.** Tissue specimens from 50 patients with breast cancer were analyzed. Patients were consecutively recruited at the Chinese Academy of Medical Sciences Cancer Hospital (Beijing). At recruitment, informed consent was obtained from each subject. This study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

**Cell culture.** Human colon carcinoma cell lines HCT116/p53+/+ and HCT116/p53−/− were kindly provided by Dr. Bert Vogelstein of Johns Hopkins University. Human ESCC cell line EC9706 was established in our own laboratory. MCF-7, HCT116/p53+/+ and HCT116/p53−/− cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), whereas H1299, HT1080 and EC9706 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml streptomycin, and 100 U/ml penicillin.

**Plasmids.** Full-length cDNA of human S100A14 was cloned into the mammalian expression vectors pcDNA3.1 and pcDEF. The promoter region of MMP2 (-1759~+25) was cloned into pGL3-basic vector. The resulting construct was verified by direct sequencing. PCB6 and pCB6-p53 plasmids were provided by Dr. Karen Vousden of NCI-FCRDC. The p53-Luc plasmid was purchased from Stratagene (La Jolla, CA).

**Transfection, generation of stable cell lines.** Transfection and establishment of stable cell lines were performed as previously described (24).

**Soft agar assay for colony formation.** Soft agar assay was performed as previously described (24).

**Immunofluorescence.** The experiment was performed as previously described (24).

**Invasion and migration assays.** These procedures were performed as described (25).

**siRNA transfection.** Cells were transfected with siRNAs (25nM) by
Hiperfect (Qiagen) and ON-TARGETplus pool siRNAs (25nM) by DharmaFECT 1 (Dharmacon) following the manufacturers’ protocol. The sequences for siRNAs were listed in Supplementary Table S1.

**RNA isolation and PCR analysis.** RNA purification and real-time RT-PCR were performed as previously described (26). Primers used are listed in Supplementary Table S1.

**Camptothecin treatment and adenovirus infection.** These experiments were performed as previously described (26).

**Chromatin immunoprecipitation (ChIP) assay.** ChIP was performed as previously described (27). Antibody used was anti-p53 (pAb421) from Oncogene Science (Cambridge, MA).

**Western blot analysis.** Western blots were performed as previously described (25). Antibodies used were anti-p53 (Sc-126, Santa Cruz, CA, USA), anti-S100A14 (gifts of Dr. Iver Petersen, University Hospital Charite, Berlin and Dr. Youyoung Lü, Beijing Cancer Hospital and Institute, Beijing) and anti-MMP2 (MAB13405, Millipore, Billerica, MA) and β-actin antibody (A5316, Sigma, St. Louis, MO).

**Luciferase assay.** Luciferase assay was performed as previously described (26).

**Statistical analysis.** We statistically evaluated experimental results using two-tailed paired Student’s t test, two-independent sample t test, Chi-square test. All tests of significance were set at p < 0.05.

**RESULTS**

**Altered S100A14 affects cell migration and invasion**—Our previous study has shown that extracellular S100A14 affected ESCC proliferation and apoptosis by interacting with RAGE (14). To characterize the role of intracellular S100A14 in the development of malignant phenotype, we established S100A14-overexpressed stable transfectants in EC9706 cells. Determination of S100A14 immunoreactivity using antibody against S100A14 indicated a high enrichment of S100A14 in stable transfectants (clone S12 and S13) compared to mock-transfected cells (Fig 1A). We analyzed the effect of S100A14 overexpression on cell proliferation of EC9706 cells using MTT, flow cytometry and soft agar assay. As shown in Fig 1B and Fig S1, no significant difference on cell proliferation was observed between S100A14-overexpressed cells and mock-transfected cells. Since cell shape controls some physiological processes such as cell growth, apoptosis, and motility and the actin cytoskeleton governs multiple cellular activities (28), we observed cell morphology under microscope images and actin cytoskeleton by phalloidin staining in stable transfectants. As shown in Fig 1C, S100A14 overexpressed cells appeared more spread and formed well-organized actin cytoskeleton compared to mock-transfected cells (clone P4). Since these processes are closely correlated with cell motility (29), we further investigated the effect of S100A14 overexpression on the cell migration and invasion, we conducted three-dimensional cell migration and invasion assays using transwell chambers and found that overexpression of S100A14 (S12 and S13) increased motility and invasiveness of EC9706 cells compared to mock-transfected cells (P4) (Fig 1D). Taken together, these results demonstrate that S100A14 plays an important role in cell motility and invasiveness.

**S100A14 modulates cell migration and invasion by regulating MMP2 expression**—To explore the molecular mechanism of S100A14 promoting cell migration and invasion, we characterized the expression of some metastatic related genes by RT-PCR analysis (Fig 2A). Interestingly, among the MMPs that were detected, MMP2 expression was dramatically induced in the S100A14-overexpressed cells (S12 and S13). In contrast, expression of MT1-MMP (MMP14), a major activator of MMP2, and TIMP2, an endogenous inhibitor of MMP2 were not significantly altered (30, 31). RT-qPCR, Western blot analysis further confirmed the dramatic increase of MMP2 expression, as well as the secretion and activation of MMP2 in S100A14 stable transfectants (Fig 2B-D). To define the role of MMP2 in S100A14-enhanced cell invasion, we pretreated cells with a potent
specific MMP2 inhibitor OA-Hy (10µM, Chemicon, Temecula, CA) and evaluated the effect on cell invasion. We found that pretreatment with OA-Hy significantly attenuated S100A14-enhanced cell invasion (Fig 2E). Taken together, these data demonstrate that S100A14 controls invasive potential through regulation of MMP2 expression.

**Ectopic overexpression of S100A14 inhibits endogenous and exogenous p53 protein expression and transcriptional activity of p53**— By serendipity, we found that the protein expression of p53 was drastically reduced after S100A14 overexpression in EC9706 cells, whereas no effect on p53 mRNA levels (Fig 3A). Next, we detected the expression of some p53 target genes: p21, PCNA and Bax. Unexpectedly, we didn’t find any significant effect on the expression of these genes accompanied by downregulation of p53 upon S100A14 overexpression in EC9706 cells (Fig S2). Several possibilities may cause this: (1) S100A14 overexpression might lead to changes of multiple molecules or a number of signaling pathways, the combinatorial interactions of multiple factors ultimately remain p53 target genes such as p21, PCNA and Bax stable level. As a result, the balance of expression of multiple genes regulates the biological behavior and determines the phenotype of cells. (2) S100A14 may influence p53 promoter selectivity, thereby affecting the transcription of p53 specific target genes (32-34). (3) The p53 levels, the specific p53 response element (p53RE) sequences, and cell types may be responsible for S100A14-mediated p53 target gene selectivity (33). Since p53 status in EC9706 cells has not been reported yet, we performed p53 coding region sequencing and found two point mutations resided at 230 and 1126 codons. To ascertain whether p53 is functional in EC9706 cells, DNA damage experiments were carried out. EC9706 cells were treated with 2µM camptothecin (CPT) and harvested at 0, 3, 6, 9, 12 and 24h. Western Blot and RT-qPCR analyses showed that p53 target genes (CDKN1A, Bax, and MDM2) were promptly induced within 6h in parallel with the induction of p53 at 6h, indicating that p53 is functional in EC9706 cells (Fig S3). In order to further confirm the effect of S100A14 on p53 in cell lines with wild type p53, we determined the effect of S100A14 on p53 in HT1080 and MCF7 cells by transient transfection. The results showed that ectopic overexpression of S100A14 resulted in a reduction of endogenous p53 in HT1080 and MCF-7 cells with WT p53 (Fig 3B). Moreover, overexpression of S100A14 significantly induced reductions of promoter transactivation of p53-luc (a reporter plasmid expressing firefly luciferase under the control of p53 response element) (Fig 3C). As p53 is degraded by the ubiquitin-proteasome pathway, we examined whether S100A14 influenced p53 ubiquitination per se. The stability (half-life) of endogenous and exogenous p53 was decreased in cycloheximide (CHX)-treated S100A14-overexpressed cells compared with control cells (Fig S4), indicating that decreased expression occurs through its stabilization. However, the level of ubiquitination of exogenous p53 in H1299 cells did not show any significant changes (data not shown), suggesting that other mechanisms may be responsible for S100A14-decreased p53 levels (35).

**Altered S100A14 expression differently affects MMP2 expression and cell invasiveness dependent on p53 status**— It has been previously shown that p53 positively or negatively regulates the expression of MMP2 (36, 37), we then suspect whether there is a correlation between S100A14-induced MMP2 elevation and S100A14-induced p53 decrease. We subsequently examine whether the regulation of MMP2 by S100A14 is dependent on p53. We selected several cell lines with low levels of S100A14 expression and different p53 status: HT1080 (p53+/+), MCF7 (p53+/+) and H1299 (p53−/−) cells and transfected these cells with pcDNA3.1-S100A14 and pcDNA3.1 vectors to establish pooled neomycin (G418)-resistant stable transfectants. The forced expression of S100A14 in HT1080, MCF7 and H1299 cells obviously increased the expression of S100A14, accordingly,
overexpression of S100A14 in HT1080 and MCF7 cells significantly increased the mRNA and protein levels of MMP2, whereas an opposite effect was observed in H1299 cells (Fig 4A left and middle panels). To further investigate whether altered S100A14 expression has a direct effect on human MMP2 promoter activity, MMP2 luciferase reporter construct was transfected into stable transfectants. Remarkably, MMP2 promoter activity was significantly induced in S100A14 overexpressing transfectants in HT1080 and MCF7 cells compared to mock-transfected cells. In contrast, the effect was not observed in H1299 cells (Fig 4A, right panel). These results imply that the induction of MMP2 by S100A14 is dependent on p53 status.

Because the regulation of MMP2 by S100A14 is dependent on p53 status, we next analyzed whether the effect of S100A14 on cell invasiveness is correlated with its p53 status. In vitro invasion assays showed that the forced expression of S100A14 increased the invasiveness of HT1080 and MCF7 cells, but decreased the invasiveness of H1299 cells (Fig 4C). Meanwhile, we also created stable silencing of S100A14 HCT116 cells using two different shRNA target sequences which have already been verified to highly reduce S100A14 expression by siRNAs transfection, S100A14 expression was effectively suppressed in stable transfectants (S1-7, S2-8) compared to control cells (pGC-1). In line with S100A14 ablation, p53 expression was significantly increased, MMP2 expression was dramatically decreased (Fig 4D, left panel). Taken together, these data further support that S100A14 affects MMP2 expression and cell invasiveness in a p53 dependent manner.

MMP2 is repressed at transcriptional level following the increased expression of p53—To define the effect of p53 on MMP2 expression, we treated p53 wild-type cells (HCT116 p53+/+) and p53 null cells (HCT116 p53−/−) with 2µM CPT. After 6h and 12h, MMP2 mRNA levels were significantly decreased in parallel with increased p53 accumulation in HCT116p53+/+ cells (Fig 5A), however, no effect was observed in HCT116p53−/− cells, confirming that MMP2 downregulation is dependent on p53. We also compared mRNA expression of MMP2 in HCT116p53+/+ cells with HCT116p53−/− cells. As expected, MMP2 mRNA level was significantly greater in HCT116p53+/+ cells versus HCT116p53−/− cells. To rule out the possibility that decreased MMP2 was the result of p53-induced apoptosis, HCT116p53+/+ cells were treated with CPT in combination with Z-VAD-FMK (50µM), and RT-qPCR analysis was performed. As shown in Fig 5B left panel, Z-VAD-FMK treatment did not prevent the inhibition of MMP2, indicating that p53 indeed contributes to the regulation of MMP2. To further investigate whether CPT-mediated decrease in MMP2 mRNA expression is dependent on CPT-induced cell cycle arrest, we synchronized HCT116p53+/+ cells to G1/S phase by using double-thymidine block (38) and examined the effect of CPT on MMP2 mRNA level in synchronized HCT116p53+/+ cells. Double thymidine block caused a complete G1/S arrest, which was not further enhanced by CPT treatment (Fig 5B right panel). Significantly, treatment of HCT116p53+/+ cells with CPT decreased MMP2 mRNA level in HCT116p53+/+ cells already arrested in G1/S phase (Fig 5B right panel). These results indicate that CPT can directly decrease MMP2 mRNA expression in a cell cycle arrest-independent manner. Collectively, these data strongly suggest that activated endogenous p53 inhibited MMP2 mRNA expression. To further investigate the effect on MMP2 mRNA expression by exogenous p53 overexpression, several cell lines were infected with 40 multiplicity of
infection (MOI) of adenovirus expressing wild-type p53, the levels of MMP2 mRNA expression were significantly repressed by exogenous p53 at 48h post infection (Fig 5C). We next analyzed p53 response elements (p53REs) in the promoter region of the MMP2 gene (from -3000 to +1 site) by Jaspar database (39) (Sup Table 2) and identified a potential p53RE located at position -1649~1630 (Fig 5D). This p53RE has been previously reported, however, the interaction between p53 and the p53RE-containing DNA region has not been examined yet. Therefore, we performed chromatin immunoprecipitation (ChIP) assay to test the functional interaction between p53 and the potential p53RE in the MMP2 regulatory regions. Enriched p53 binding to the MMP2 promoter was observed compared to IgG control in MCF7 and HCT116p53+/+ cells with WT p53. Moreover, the treatment with CPT significantly increased the recruitment of p53 to the MMP2 promoter in HCT116p53+/+ cells. In contrast, the recruitment of p53 to the MMP2 promoter was similar compared to IgG control in HCT116p53−/− cells with null p53. The WAF1/p21 promoter region was used a positive control for p53 binding (40) (Fig 5E). These results strongly suggest that p53 regulates MMP2 transcription by binding to the p53RE located in the MMP2 promoter. Moreover, p53 binding was significantly decreased in line with the decrease of p53 protein level in S100A14-overexpressed cells (Fig S6). To further explore the role of p53 in regulating MMP2 transcription, we examined whether p53 regulates MMP2 promoter activity. We cotransfected the MMP2 promoter-luciferase construct and p53 expression or control vector into HT1080, MCF7, H1299, and HCT116/p53−/− cells, and p53 expression significantly inhibited the luciferase activity of MMP2 promoter in HT1080, MCF7, and H1299 cells. Nevertheless, p53 induced slight activation of MMP2 promoter activity in HCT116/p53−/− cells (Fig 5F). Taken together, these data clearly show that the repression of MMP2 by p53 occurs at the transcriptional level.

The overexpression of S100A14 is correlated with upregulation of MMP2 in clinical samples with wild type p53—To verify cell studies (MCF-7) in clinical samples, we performed p53 sequence analyses and RT-qPCR in fifty pairs of breast cancer tissues and their adjacent normal tissues. Sequencing analyses revealed that alterations of p53 included point mutation and deletion/insertion and the mutation rate of p53 gene in breast cancer is 40%. Of 50 cases, 1 case was excluded since p53 mutation was also observed in its adjacent normal tissues. Next, we examined the mRNA expressions of S100A14 and MMP2 simultaneously in 49 cases. The term -Δ Ct (Ctβ-actin-CtS100A14 or CtMMP2) was used to describe the expression of S100A14 and MMP2. The results showed that both S100A14 and MMP2 mRNA were highly expressed in breast cancers (paired t-test; P=0.012 for S100A14; P=0.001 for MMP2) with wild type p53 versus their surrounding normal tissues. In contrast, the overexpression of MMP2 was not observed (paired t-test; P=0.132) although S100A14 was also highly expressed (paired t-test; P=0.001) in cases with mutant p53. Moreover, we did find a significant correlation between high expression of S100A14 and overexpression of MMP2 in breast cancer samples with wild-type p53 (Table 1, p=0.035). However, the correlation is not observed in samples carrying mutant p53 (Table 1, p=0.154). Collectively, these results further supported the regulation of MMP2 by S100A14 is dependent on p53 status in clinical samples.

DISCUSSION

Increasing evidence showed the importance of S100 family in cell migration, invasion and cancer metastasis. Among them, S100A4 has been identified as a well-known metastasis marker (9). In addition, S100A2 has also been reported as a strong metastasis inducer in non-small cell lung cancer (NSCLC) (41). S100A8/S100A9 has been implicated in both myeloid cell recruitment and tumor-cell invasion in lung cancer (10). Recently, it has been reported that S100A14 low-expression in combination with
S100A4 high-expression was correlated with high colorectal cancer metastatic potential (42). S100A14 has also been identified as an invasion suppressor in oral squamous cell carcinoma (OSCC) cells (13). In this study, we further demonstrated that S100A14 may act as either an inducer or an inhibitor of cell invasion depending on the p53 status of cells.

Importantly, S100 proteins are reported to affect the cell migration and invasion through regulating the expression of MMPs (11, 12, 15). Our study also showed that S100A14 affected cell invasion by regulating the expression of MMP2, a key proteolytic enzyme in the process of cell invasion (7), and MMP2 is an important mediator of S100A14 promotion of EC9706 cell invasion. To our surprise, in line with up-regulation of MMP2, the protein level of p53 is dramatically reduced in S100A14-overexpressed EC9706 cells. Furthermore, we confirmed the effect of S100A14 on p53 expression, DNA binding, and transactivity in HT1080 and MCF7 cells expressing wild type p53. Consistent with previous observations that several S100 proteins interact with p53 to perform different effects on p53 activity (21-23), our present study showed that S100A14 is a critical negative regulator of p53. In addition, our previous study demonstrated that S100A14 is a target gene of p53 pathway (26). Therefore, a p53-S100A14 negative feedback loop was formed. Although under our experimental conditions, the interaction between S100A14 and p53 was not found, our data demonstrated that p53 regulation by S100A14 is likely mediated by affecting p53 stability. It is well documented that some E3 ubiquitin ligases such as MDM2, COP1, and Pirh2 regulate p53 stability (43), and we also found that MDM2 was slightly induced by overexpression of S100A14 in HT1080 cells. However, we didn't detect the change of p53 ubiquitination by Co-IP assay (data not shown). Thus, it is possible that S100A14 promotes p53 degradation and limits its activity via the effect on other molecules to regulate p53 stability. The identification of the interactive protein of S100A14 will further improve our standing of the mechanism underlying regulating p53 by S100A14.

Our study further showed that the regulation of MMP2 by S100A14 is dependent on the p53 status of cells. In the presence of p53, S100A14 enhanced MMP2 expression by decreasing p53 protein levels. However, in the absence of p53, S100A14 inhibited MMP2 expression, indicating that other molecules are responsible for mediating inhibition of MMP2. The unknown mechanism needs to be further investigated in future studies. Collectively, our data strongly suggest that S100A14 plays a dual role in regulating the expression of MMP2 and cell invasion in a p53-dependent manner.

Previous studies showed that p53 is involved in cancer progression by specifically regulating cancer invasion (18, 19, 44). Moreover, several reports have demonstrated that p53 transrepresses the expression of distinct MMPs, including MMP1, MMP9, and MMP13 (45-47). However, p53 has dual effect on regulation of MMP2. Capogrossi and colleagues previously demonstrated that p53 inhibited transcription of MMP2 in cells with mutant p53 (36). In contrast, there is report that p53 plays a dual role in the regulation of MMP2 promoter activity (37). We examined the transcriptional regulation on MMP2 by p53 under our experimental conditions. Luciferase reporter studies showed the introduction of wild type p53 suppressed
MMP2 promoter activity in several cells except for HCT116/p53-/- cells. The disparity of the regulation of MMP2 by p53 may reflect the diverse characteristics of the experimental systems, including cell types. We further investigated the regulation of MMP2 by p53. The results showed that MMP2 mRNA level was significantly decreased in HCT116/p53+/+ but not HCT116/p53-/- cells with CPT treatment, demonstrating that decreased MMP2 expression is dependent on p53 status. In accordance with these results, exogenous p53 overexpression significantly inhibited the MMP2 mRNA levels. Previous studies showed that p53 functioned as a transactivator as well as a “trans-enhancer” to activate MMP2 transcription, and p53RE located at position -1649~-1630 in the promoter of MMP2 gene is crucial to activate MMP2 transactivity (37). We searched for potential p53 binding sites in the promoter of MMP2 by Jaspar database. In agreement with previous reports, we acquired the same p53RE located at the promoter of MMP2. Subsequently, we confirmed that p53 can directly bind to the regulatory regions of MMP2 containing p53RE by ChIP assay. Moreover, p53 binding was significantly decreased in line with the decrease of p53 protein level in S100A14-overexpressed HT1080 cells (Fig S6). Although the mechanism of p53-mediated transrepression has not been fully elucidated, a number of studies showed that p53 can transrepress target gene transcription by directly repressing the basal transcriptional machinery, interfering with the functions of coactivators or recruiting corepressors (48). Previous investigators also speculated that p53 may transrepress MMP2 expression by the element(s) other than p53RE or other transcription factors (37). It will be of great importance to identify and characterize the potential p53 negative regulatory elements or cofactors in the regulatory regions of MMP2 in future studies.

Through its action to p53 and MMP2, S100A14 could function as a metastasis inducer or inhibitor. Moreover, S100A14 overexpression was significantly correlated with MMP2 overexpression in breast cancer tissue samples. In future studies, it will be interesting to examine the correlations among S100A14, p53 status, and MMP2 expression with clinicopathologic features including tumor metastasis, survival, prognosis in many types of cancers. Moreover, the effect on invasiveness of S100A14 using xenograft mouse models after overexpression of S100A14 will be determined in future.

In summary, we demonstrated that S100A14 controls cell invasion at least in part through the regulation of MMP2, and the regulation of MMP2 by S100A14 is dependent on p53. We suggest that S100A14 might be a potential biomarker as well as a new therapeutic target in cancer treatment, which could be realized through development of specific S100A14 inhibitors or use of a gene therapy approach.

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REFERENCES

FIGURE LEGENDS

Figure 1. Stable overexpression of S100A14 in EC9706 cells promotes cell motility and invasiveness. (A) Western blot using S100A14 polyclonal antibody determined S100A14 expression in stable transfectants, P4: mock-transfected clone; S12 and S13: S100A14-transfected clones, β-actin served as a loading control. (B) Soft-agar colony formation assay was performed with EC9706 transfectants (P4, S12 and S13). Left panel showed the representative pictures, and right panel showed the quantification of colonies. (C) Cells were cultured for 24h, and then optical images were acquired (top). Cells were cultured for 16h, and then were fixed and stained by FITC labelled-phalloidin, fluorescent images were visualized with fluorescent microscope (bottom). (D) Left panel: Representative pictures of invading (top) and migrating (bottom) cells (P4, S12, and S13). Right panel: Relative numbers of migrating and invading cells. Cells were counted in 4 randomly selected fields. Error bars represent the SEM of triplicate experiments. *p < 0.05, **p < 0.01 two tailed student’s t test.

Figure 2. Overexpression of S100A14 promoted cell invasiveness by regulating MMP2. (A) The expression of some metastasis-related genes was examined by RT-PCR in EC9706 transfectants. (B) MMP2 mRNA expression levels were further confirmed by RT-qPCR. Western blot analysis showed the expression in cell lysates (C), secretion and activation of MMP2 in conditioned medium in EC9706 transfectants (D). (E) EC9706 cells (P4, S12, and S13) were pretreated with 10µM OA-Hy or solvent (DMSO) for 24h and suspended in 150µl serum-free medium containing OA-Hy or solvent. Cells were then seeded in the upper chamber of the matrigel plate for the invasion assay. Left panel: Representative pictures of invading cells. Right panel: Relative numbers of invading cells. Cells were counted in 4 randomly selected fields. Error bars represent the SEM of triplicate experiments. *p < 0.05, **p < 0.01 two tailed student’s t test.

Figure 3. S100A14 reduces p53 protein expression and transcriptional activity. (A) Western blot and RT-qPCR analyses showed the expression of p53 protein (left) and mRNA (right) in EC9706 transfectants (P4, S12, and S13). (B) MCF-7 and HT1080 cells were transfected with the indicated constructs, 48 hours later, cell lysates were blotted with the indicated antibodies. (C) P53-luc construct was cotransfected with the indicated constructs into HT1080 and MCF7 cells, reporter activity was then determined.

Figure 4. Effects of altered S100A14 expression on MMP2 expression and the promoter activity of MMP2 are dependent on p53 status. (A) H1299, HT1080 and MCF-7 cells were transfected with the indicated constructs and pooled neomycin (G418)-resistant colonies were established as stable transfectants. The protein expression of S100A14, p53, and MMP2 was determined by Western blot, β-actin served as a loading control (left panel); the mRNA expression of MMP2 was determined by RT-qPCR (middle panel); and MMP2 transcriptional activity was detected by MMP2-luc reporter assay (right panel). (B) siRNAs targeting S100A14 (25nM) (ON-TARGETplus SMARTpool) or control siRNAs were transfected into HCT116p53+/+ and HCT116p53-/- cells, 72 hours later, proteins and mRNA were extracted and subjected to Western blot or RT-qPCR. (C) Cell invasion assay was performed in
S100A14-overexpressed H1299, HT1080, and MCF7 cells. Representative pictures (left panel) and relative numbers of invading cells (right panel) are shown. Cells were counted in 4 randomly selected fields. Error bars represent the SEM of triplicate experiments. *p < 0.05, **p < 0.01 two tailed student’s t test. (D) Stable silenced-S100A14 HCT116 cells was obtained, proteins and RNA were extracted and subjected to Western blot or RT-qPCR (left panel). pGC-1: control shRNA-transfected clone; S1-7 and S2-8: shRNA targeting S100A14-transfected clones. Cell invasion assay was performed in stable silenced-S100A14 HCT116 cells (right panel).

Figure 5. P53 transrepresses the expression of MMP2. (A) HCT116p53+/+ and HCT116p53−/− cells were exposed to CPT, then protein and RNA were extracted and analyzed by Western blot or RT-qPCR following treatment at the indicated time points. Fold reduction of MMP2 mRNA level was measured by the percentage of HCT116p53+/+ cells (taken as 1). (B) Left panel: Cells were treated with 50µM Z-VAD-FMK for 1h prior to the addition of CPT, then RNA was extracted and analyzed by RT-qPCR; Right panel: Cells were synchronized to G1/S phase by using double-thymidine block and were treated with 2µM CPT for 6 h prior to the cell harvest, the mRNA level of MMP2 was examined. (C) H1299, MCF7 and HCT116 cells were infected with Ad-p53 of 40 MOI, 48 hours later, cells were harvested. Protein and RNA were extracted and analyzed by Western blot or RT-qPCR. (D) p53 binds to the promoter of MMP2 gene. The p53 consensus sequences are shown in capitals and potential p53 response element (p53 RE) are located at -1649~1630 of MMP2 promoter. (E) ChIP assay demonstrated that p53 bound to the promoter region of MMP2 gene. MCF7 and HCT116/p53−/− cells were harvested and HCT116/p53+/− cells were collected with or without CPT treatment. ChIP assay was performed with p53 antibody, anti-Mouse IgG antibody was used as a negative control. (F) MMP2-luc was cotransfected with the indicated constructs into H1299, HT1080, MCF7, and HCT116/p53−/− cells, reporter assay was then performed. Error bars represent the SEM of triplicate experiments. *p < 0.05, **p < 0.01 two tailed student’s t test.

Figure 6. The expression of S100A14 and MMP2 in clinical samples with different p53 status. RT-qPCR analysis was performed in 49 breast cancer tissues and paired adjacent normal tissues. The term -Δ Ct (Cβ-actin-CtS100A14 or CtMMP2) was used to describe the expression level of S100A14 and MMP2. The expression of S100A14 and MMP2 in breast cancers and their matched surrounding normal tissues with wild type p53 (A & B) and mutant p53 (C & D).
Table 1. The correlation between S100A14 overexpression and MMP2 overexpression in paired breast cancer tissues with different p53 status

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>S100A14 expression</th>
<th>Case number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-overexpressed*</td>
<td>Overexpressed*</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>MMP2 expression in cases with wild type p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-overexpressed*, n (%)</td>
<td>3 (75.0)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Overexpressed*, n (%)</td>
<td>6 (23.1)</td>
<td>20 (66.9)</td>
</tr>
<tr>
<td>MMP2 expression in cases with mutant p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-overexpressed*, n (%)</td>
<td>2 (33.3)</td>
<td>4 (66.6)</td>
</tr>
<tr>
<td>Overexpressed*, n (%)</td>
<td>1 (76.9)</td>
<td>12 (23.1)</td>
</tr>
</tbody>
</table>

NOTE: These results were analyzed by the Pearson $X^2$ test. $P$ values with significance are shown as superscripts.

*For S100A14 and MMP2 mRNA expression levels, the matched cancer/normal ratio $>1$ was taken as overexpressed group, and the ratio $\leq 1$ was taken as non-overexpressed group.
Chen et al. Figure 2

A

- E-cadherin
- N-cadherin
- Fibronectin
- Vimentin
- MMP2
- MMP9
- MMP14
- TIMP1
- TIMP2
- SLUG
- TWIST
- β-actin

B

RT-PCR (MMP2)

C

- P4
- S12
- S13

D

- P4
- S12
- S13

E

- DMSO
- OA-Hy (5μM)

Relative mRNA level

Pro-MMP2
active MMP2

Relative cell number

DMSO
OA-Hy

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Chen et al. Figure 3

A

\[
\begin{align*}
\text{P4} & \quad \text{S12} & \quad \text{S13} \\
\text{S100A14} & \quad p53 & \quad \beta\text{-actin}
\end{align*}
\]

B

\[
\begin{align*}
\text{vector} & \quad \text{S100A14} \\
\text{p53} & \quad \text{MDM2} & \quad \text{S100A14} & \quad \beta\text{-actin}
\end{align*}
\]

\[\text{HT1080} \quad \text{MCF7}\]

C

RT-PCR (S100A14 and p53)

\[
\begin{align*}
\text{S100A14} & \quad p53 \\
\text{P4} & \quad \text{S12} & \quad \text{S13}
\end{align*}
\]

Relative mRNA level

\[0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5\]

\[\text{HT1080} \quad \text{MCF7}\]

Relative Luciferase Activity

\[0 \quad 0.2 \quad 0.4 \quad 0.6 \quad 0.8 \quad 1 \quad 1.2\]
Chen et al. Figure 6

With wild type p53

A  P=0.012  
B  P=0.001

C  P=0.001  
D  P=0.132

With mutant p53
The involvement of S100A14 in cell invasion by affecting expression and function of matrix metalloproteinase (MMP)-2 via P53-dependent transcriptional regulation

Hongyan Chen, Yi Yuan, Chunpeng Zhang, Aiping Luo, Fang Ding, Jianlin Ma, Shouhui Yang, Yanyan Tian, Tong Tong, Qimin Zhan and Zhihua Liu

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