A novel p53 target gene, S100A9, induces p53-dependent cellular apoptosis and mediates the p53 apoptosis pathway

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S100A9 (S100 calcium-binding protein A9) is a calcium-binding protein of the S100 family, and its differential expression has been associated with acute and chronic inflammation and several human cancers. Our previous work showed that S100A9 was severely down-regulated in human ESCC (oesophageal squamous cell carcinoma). To further investigate the transcriptional regulation of S100A9, we analysed the S100A9 promoter region and found several putative p53BS (p53-binding sites). Luciferase reporter assays showed that constructs carrying the p53BS exhibited enhanced luciferase activity in response to wild-type p53 activation. Further study demonstrated that S100A9 mRNA and protein expression could be positively regulated in a p53-dependent manner and p53 could bind to p53BS on the S100A9 promoter. Overexpression of S100A9 could induce cellular apoptosis, and this was partly p53-dependent. Knockdown of S100A9 impaired the apoptosis induced by p53. Thus we conclude that a gene down-regulated in ESCC, S100A9, is a novel p53 transcriptional target, induces cellular apoptosis in a partly p53-dependent manner and mediates the p53 apoptosis pathway.

Key words: apoptosis, oesophageal squamous cell carcinoma (ESCC), p53, promoter, S100 calcium-binding protein A9 (S100A9), transcription.

INTRODUCTION

Uncontrolled cellular proliferation and the abolishment of apoptosis are regarded as important incidents in the progress of carcinogenesis [1]. In response to inappropriate growth signals and various kinds of cellular stress, the important tumour suppressor p53 functions by inducing either cell growth arrest or apoptosis [2], mediated mainly through the activation of its downstream target genes. Identification of new p53 downstream genes would therefore shed light on how p53 exerts its tumour-suppressing effects under different cellular stresses.

ESCC (oesophageal squamous cell carcinoma) is one of the most malignant cancers worldwide, especially in China [3]. Mutations in the p53 gene have been reported in over half of human cancers and they seem to occur at an early stage of ESCC [4–6]. In previous studies we have performed a cDNA microarray analysis of the gene expression profile in oesophageal cancer and normal tissues [7–9] and found that S100A9, a member of the S100 gene family, was significantly down-regulated in ESCC tissue samples. Most S100 genes cluster to human chromosome 1q21, a target region of chromosomal rearrangement and instability in common human epithelial malignancies [10]. S100 proteins have attracted substantial interest due to their involvement in inflammation and tumorigenesis [11,12]. For example, altered expression of S100B (S100 calcium binding protein B), S100A2 (S100 calcium binding protein A2) and S100A4 (S100 calcium binding protein A4) was closely related to tumour development and prognosis and all three proteins interact with p53, exerting different effects on p53 activity. S100B and S100A2 are both p53 target genes and mediate p53 downstream signals [13]. We have found that nearly all S100 proteins were differentially expressed in ESCC, indicating the involvement of the S100 family in ESCC development [9,14].

S100A9 displays a wide range of possible intracellular, as well as extracellular, functions. It is expressed during myeloid differentiation, is abundant in granulocytes and monocytes and forms a heterodimeric complex calprotectin with S100A8 (S100 calcium binding protein A8) in a calcium+-dependent manner. Typically, S100A9 is differentially expressed at sites of acute and chronic inflammation and is regarded as a pro-inflammatory cytokine with S100A8 [15–17]. Subsequently, it has been shown that the deregulation of S100A9 is associated with several common human malignancies [13]. Extracellular heterodimers composed of S100A8 and S100A9 can induce cellular apoptosis [18–20]. S100A8 and S100A9 also have functions related to cell migration as they are known to control microtubule reorganization during transendothelial migration of phagocytes [21,22], and S100A9 alone can induce neutrophil adhesion to fibronectin [23]. Primary tumours in the premetastatic lung induce the expression of S100A8 and S100A9, which in turn facilitates adhesion and invasion of tumour cells to the premetastatic sites within the lung parenchyma [24]. However, there is little detailed data available about the altered expression of S100A9 during oesophageal carcinogenesis and its transcriptional regulation.

In the present study, in order to better understand the mechanisms involved in S100A9 differential expression, we analysed the promoter region of S100A9 and, interestingly, we identified response elements for tumour suppressor p53. Further investigation showed that S100A9 expression could be induced by p53 and the physical interaction between the defined promoter response region and p53 did exist. Similar to reported apoptosis induced by extracellular S100A9 protein, intracellular overexpression of S100A9 could also induce cellular apoptosis, and this is partly p53-dependent. Knockdown of S100A9 impaired the apoptosis induced by p53. Therefore identifying S100A9 as a new p53 target gene enriches the relationship of the S100 family and p53 and...
provides evidence for the participation of S100A9 in p53-induced apoptosis and the development of ESCC.

EXPERIMENTAL

Cell lines

Human ESCC cell line KYSE150 [25] was a gift from Dr Y. Shimada (Hyogo College of Medicine, Hyogo, Japan), human colon cancer cell line HCT116 p53+/+ and p53−/− was a gift from Dr Vogelstein (The Johns Hopkins University, Baltimore, MD, U.S.A.) [26]. Human ESCC cell line EC9706 was established in our laboratory as described in [27]. KYSE150, YES2 and EC9706 were maintained in RPMI 1640 medium. HCT116 p53+/+, HCT116 p53−/− and human keratinocyte cell line HaCaT were maintained in DMEM (Dulbecco’s modified Eagle’s medium). Both media were supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin, and cells were kept at 37 °C in a humidified 5% CO2 incubator.

 Luciferase reporter assays

Different lengths of promoter segment were cloned into pGL3-Basic vector (Promega) according to the analysis of p53 BS (p53-binding sites). The pcDNA3.0 plasmid was purchased from Invitrogen. The pcDNA3-p53 plasmid [28] was a gift from Dr Huanran Tan (Peking University, Beijing, China) and Dr Vogelstein. The plasmids of pCB6+, pCB6−/− p53, pCB6−/− p53173L [29] were kindly provided by Professor Karen Vousden (Beatson Institute for Cancer Research, Glasgow, U.K.). Cells (approx. 10^4) were seeded in a 96-well plate and transfected with promoter constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendation. The activities of both firefly and Renilla luciferases were determined 48 h after transfection with the dual luciferase reporter assay system (Promega). The luciferase activities were normalized to the Renilla luciferase activity of the internal control.

 Plasmid construction, transfection, adenovirus infection and camptothecin treatment

The full-length human S100A9 coding region was amplified from normal oesophageal mucosa cDNA and cloned into the HindIII and BamHI sites of the expression vector pEGFP-C1 (Clontech) and the BamHI and HindIII sites of the expression vector pcDNA3.1 (Invitrogen) respectively. The construct pTRE2-GFP-S100A9 was generated by subcloning the GFP (green fluorescent protein)—S100A9 fusion fragment into the NotI and XbaI sites of the responsive plasmid pTRE2 (Clontech). All constructs were confirmed by DNA sequencing. An inducible expression system was used to express proteins. EC9706 cells were first stably transfected with pTet-Off (Clontech) [30], these cells, called TF93, where transfected with pTRE2-EGFP-S100A9 or empty vector were using Lipofectamine 2000. Stable transfectants were selected with 100 μg/ml hygromycin B (Calbiochem) and maintained in medium supplemented with 2 μg/ml doxycycline (Sigma–Aldrich). The S100A9 transfectant pool was verified by Western blot analysis. Ad (adenovirus)—p53 and Ad-GFP were purchased from Shenzhen SiBiona GeneTech Co. and Shanghai GeneChem Co. respectively. Cells were treated with 10 μM hydroxyoxampteothein (Wanle Pharmaceutical) for various times, and harvested for Northern and Western blot analysis. siRNA (small interfering RNA) sequences for S100A9 (Qiagen) were S100709842 (ATGGAGACCTCGGCAACAAAT) and S104317012 (TGCAGCTGGAGACACCAACA). These siRNAs were transfected using Hiperfect (Qiagen) according to the manufacturer’s instructions.

 RNA extraction and Northern blot

Total RNA was extracted with TRIzol® (GibcoBRL) according to the manufacturer’s instructions. A15 μg amount of total RNA was loaded for each lane and hybridized with biotin-labelled cDNA probe synthesized using a Biotin DecaLabel™ DNA labelling kit (Fermentas) and subjected to the chemiluminescent nucleic acid detection module.

 Preparation of protein extracts and Western blotting

Cell lysates were prepared and Western blotting was performed as described previously [31]. The antibodies were against GFP, p53, PARP1 [poly(ADP-ribose) polymerase 1], caspase-3 (sc-8334, sc-126, sc-7150 and sc-7272 respectively, Santa Cruz Biotechnology) and β-actin (A5441, Sigma–Aldrich) respectively. Rabbit anti-S100A9 polyclonal antibody [32] was a gift from Dr Julio E. Celis (Danish Cancer Society, Copenhagen, Denmark).

 ChIP (Chromatin immunoprecipitation) assays

ChIP assays were performed using Chromatin Immunoprecipitation assay kit (Upstate Biotechnology) following the manufacturer’s instructions. Approx. 10^6 cells were plated per 60 mm dish. After treatments, DNA–protein interactions were fixed with 1% formaldehyde at 37 °C for 10 min. The ChIP assay was carried out using anti-p53 antibody (sc-126; Santa Cruz Biotechnology). Input DNA served as controls for all of the experiments.

 EMSAs (electrophoretic mobility-shift assays)

 Nuclear extracts were obtained from HCT116 p53+/+ and p53−/− cells using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology) according to the supplied protocol. EMSAs were also performed according to the protocol (Pierce Biotechnology). 5′-biotin-labelled double-stranded oligonucleotides (5′-AACAACACGCTCTCTCCACAACCAGTTCACA-3′ and 5′-GGAAGAAGACTGGCGATGCTGCGGCGAGA-3′) were synthesized for S100A9 and p21 probes respectively (TaKaRa). For the specific competition assay, a 100-fold excess of unlabelled oligonucleotides was added to the binding reaction mixture.

 Flow cytometry assay and Annexin V–PI (propidium iodide) assay

Flow cytometry assay was performed by PI staining [31]. FACS analysis was carried out using a FACS Calibur flow cytometer (Becton Dickinson) with CellQuest software. Approx. 10^6 cells were measured per sample. Cells with green fluorescence were sorted to avoid non-transfected cells background. For the Annexin V–PI analysis, Annexin V was incubated for 15 min and PI for 5 min according to the manufacturer’s protocol (Beijing Biosca).

 Growth curve assay and colony formation assay

Transfected TF93 cells, with either S100A9 expression vector or empty vector, were inoculated in 96-well plates at 10^3 cells per well for the growth curve assay and in six-well plates at 500
S100A9 is a p53 target gene

Figure 1  p53 can activate transcription of the S100A9 promoter

(A) Two putative p53BS (represented by a black ellipse and BS1 and BS2 respectively) located in the promoter region of the S100A9 gene are shown. Fragments containing putative p53BS (P1, P2, P3) or a mutated copy of the respective p53BS (M1, M2) were cloned into pGL3-Basic vector respectively. (B) HCT116 p53−/− cells were co-transfected with the S100A9 promoter constructs [P1, P2, P3, M1 or M2, pGL3 (pGL3-basic empty vector)] and pcDNA3-p53. Co-transfection of these constructs and pcDNA3.0 vector served as controls. The upper right inset shows a Western blot of the expression of p53 in the transfected cells with β-actin as a loading control. (C) HCT116 p53−/− (HCT116 −/−) and HCT116 p53+/+ (HCT116 +/+ ) cells were transfected with S100A9 promoter constructs as in (B) and treated as indicated with camptothecin for 24 h (CPT+). Untreated cells served as controls (CPT−). The upper right inset shows the expression of p53 in the transfected cells with or without camptothecin treatment. (D) S100A9 promoter-p53BS transcriptional activity is p53 dose-dependent. Cells were transfected with the construct P2 and infected with Ad-p53 in the concentration of 10^5, 10^6 and 10^7 pfu (plaque-forming units). Ad-GFP served as a control. (E) Wild-type p53, but not mutant p53, increased P2 transcriptional activity. Cells were co-transfected with the construct P2 and pCB6−/−p53, or pCB6−/−p53173L. Co-transfection of P2 with pCB6+ empty vector served as a control.

RESULTS

p53 can activate transcription of the S100A9 promoter

After analysing the S100A9 promoter region, we found several putative p53BS. Specific p53BS typically contain two copies of a 10-bp motif that conforms to the sequence, 5′-RRRC(A/T)(T/A)GYYY-3′ separated by 0–13 nucleotides [2]. There are several potential p53BS in the S100A9 promoter, matching 17, 16 and 15 nucleotides in the p53 consensus binding sequence respectively. To examine which S100A9 p53BS might be functionally responsible for mediating p53-dependent transactivation, three fragments (P1, P2 and P3) carrying different S100A9 promoter p53BS were cloned into the pGL3-Basic vector (Figure 1A). As shown in Figure 1(B), when the individual reporter construct was transiently co-transfected with empty pcDNA3.0 or pcDNA3-p53 vector into HCT116 p53−/− cells, pcDNA3-p53 significantly increased the luciferase activity of the reporter plasmid carrying S100A9 promoter-p53BS. P2, which contains two sequences matching 17 nucleotides in the p53 consensus sequence (17/20 p53BS) showed the highest luciferase activity, nearly 6-fold that of the control, whereas P1, with the most p53BS, had lower activity. This may be due to the...
existence of some unknown negative transcriptional factors. P3, which has no p53BS, showed the lowest luciferase activity. In order to verify which of the 17/20 p53BS is mostly responsible for the activation, we cloned two knockout constructs, M1 and M2 respectively (Figure 1A). The results showed that there is little difference between these two S100A9 p53BS as each exerts nearly half of the total P2 luciferase activity (Figure 1B). Moreover, to examine the effects of endogenous p53 activation on transactivation of these reporter vectors, we treated wild-type, p53-containing cells, HCT116 p53+/−, and p53-knockout cells, HCT116 p53−/−, with the DNA-damaging agent camptothecin. The increased luciferase activity was observed in HCT116 p53+/− cells, but not in HCT116 p53−/− cells (Figure 1C). Furthermore, we infected HaCaT cells with Ad-p53 and Ad-GFP at increased adenovirus concentrations. The P2 construct exhibited dose-dependent transcriptional activity in response to increased Ad-p53 concentration (Figure 1D). In addition, to further demonstrate the effect of p53 on the S100A9 transactivation, we co-transfected HCT116 p53+/− cells with P2 and wild-type p53 or mutant p53, respectively. It showed that transfection of pCB6−/−p53, but not pCB6+ or pCB6−/−p53173L (a DNA-binding p53 mutant), significantly increased the luciferase activity (Figure 1E). These observations revealed that S100A9 promoter activity is dependent on functional p53.

S100A9 expression is p53-dependent

To further confirm the effect of p53 on S100A9 expression in vivo, we examined S100A9 mRNA and protein expression levels after p53 stimulation. Consistent with the luciferase activity data, HaCaT cells infected with Ad-p53 at a MOI (multiplicity of infection) of 40 exhibited a significant increase in S100A9 mRNA levels relative to cells infected with Ad-GFP (Figure 2A). This increase was evident as soon as p53 became stably expressed, usually at 36–48 h following Ad-p53 infection (Figure 2B). Similar to the change in mRNA level, S100A9 protein levels increased coincidentally with p53 protein level (Figure 2B). To verify that S100A9 could also be up-regulated in response to endogenous p53 activity, we treated HCT116 p53+/− and HCT116 p53−/− cells with 10 μM camptothecin. HCT116 p53+/− cells treated with camptothecin responded with a rapid increase in p53 protein, which was first detectable at 3 h and increased to its maximum level at 12 h (Figure 2D). In p53-wild-type HCT116 p53+/− cells, S100A9 mRNA increase was induced by camptothecin as early as 9 h. This induction, however, was not observed in p53-deficient HCT116 p53−/− cells treated under the same conditions (Figure 2C). The change of protein levels was consistent with the change in mRNA levels (Figure 2D). Further immunofluorescence analysis showed that EC9706 cells transiently transfected with pcDNA3-p53 expressed more S100A9 protein consistently (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/422/bj4220363add.htm).

To find out whether the regulation was in a cell-type- or tissue-specific manner, we examined the regulation of p53 on S100A9 in six different kinds of cell lines. p53 protein levels increased in all cells at 24 h or 48 h following Ad-p53 infection, and S100A9 mRNA was induced in all the six cell lines at 24 h or 48 h after infection, as shown by real-time reverse transcription-PCR results (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/422/bj4220363add.htm). The results indicated that the regulation of p53 on the S100A9 gene was not cell type- or tissue-specific. Together, these results demonstrated that increased levels of S100A9 mRNA and protein correlate directly with p53 activation and suggest that the S100A9 gene may be a transcriptional target of p53.

Recruitment of p53 protein at the S100A9 promoter

To determine whether p53 actually binds to the S100A9 promoter-p53BS in cells on p53 activation, we performed ChIP assays to detect formation of S100A9 promoter-p53BS binding complex
we tested whether p53 was able to bind the control. Nuclear protein was extracted from HCT116 p53 site si nav e r i fi e dt a r g e tg e n e Oligonucleotides corresponding to the p53 consensus binding promoter-p53BS were synthesized, and EMSA was performed. p53BS in vitro element.

that p53 protein bound to band increased upon p53 activation. The result clearly showed that was performed to test whether p53 was able to bind the S100A9 promoter-p53BS (A9 probe) B

p53-specific antibody in HCT116 p53 S100A9 shown in Figure 3(A), we detected DNA fragments containing anti-p53 antibody (p53 antibody) or unlabelled probes as indicated. reactions were carried out with nuclear extracts and various oligonucleotides in the presence of Oligonucleotides corresponding to p21-p53BS (p21 probe) served as positive controls. Binding

The EMSA results demonstrated that HCT116 p53 in vitro . Protein was extracted from HCT116 p53 camptothecin. Nuclear protein from HCT116 p53

A, the cells were maintained in medium supplemented with 2 μg/ml doxycycline to obtain the stable transfectant pool. The expression of transfected proteins was verified by Western blot analysis using anti-PARP1 and anti-caspase-3 antibodies. In

with p53 in HCT116 p53−/− cells infected with Ad-p53 and in HCT116 p53+/+ cells treated with camptothecin respectively. As shown in Figure 3(A), we detected DNA fragments containing S100A9 promoter-p53BS from genomic DNA precipitated with p53-specific antibody in HCT116 p53−/− cells infected with Ad-p53 but not from those infected with Ad-GFP. When HCT116 p53+/+ cells were treated with 10 μM camptothecin for 12 h, ChIP assays were performed using an anti-p53 antibody. (B) EMSA was performed to test whether p53 was able to bind the S100A9 promoter-p53BS (A9 probe) in vitro. Protein was extracted from HCT116 p53+/+ cells 12 h after treatment with 10 μM camptothecin. Nuclear protein from HCT116 p53−/− cells was extracted as a negative control. Oligonucleotides corresponding to p21-p53BS (p21 probe) served as positive controls. Binding reactions were carried out with nuclear extracts and various oligonucleotides in the presence of anti-p53 antibody (p53 antibody) or unlabelled probes as indicated.

Apoptosis induced by S100A9 is partly p53-dependent

since overexpression of S100A9 can induce cellular apoptosis, we further investigated whether this effect was dependent on the activation of endogenous p53. We transiently transfected pEGFP-C1-S100A9 into ESCC cell lines KYSE150 and EC9706, and found that S100A9 could remarkably induce apoptosis by flow cytometry analysis (Figure 4A) with the percentage of sub-G1 cells at 21.7% and 20.7% respectively. In these experiments, cells with green fluorescence were sorted to avoid non-transfected negative background. Considering its apoptosis-inducing effect we did further experiments using an inducible expression system. EC9706 cells were first stably transfected with pTet-Off (designated TF93), then stably transfected with pTRE2-GFP and pTRE2-GFP-S100A9 expressing GFP and GFP–S100A9 fusion protein respectively. After selection with 100 μg/ml hygromycin B, the cells were maintained in medium supplemented with 2 μg/ml doxycycline to obtain the stable transfectant pool. The expression of transfected proteins was verified by Western blot analysis (Figure 4B). To determine whether S100A9 overexpression affected the growth of cells, a cell growth curve assay, for different clones, was constructed. We observed that the S100A9 transfected pool grew more slowly than the parental and vector control cells (Figure 4C). Furthermore, a colony formation assay showed consistent results (Figure 4D). All these data suggested that S100A9 could reduce cell growth and might act as a negative regulator in the development of human ESCC.
Figure 4  Overexpression of S100A9 is sufficient to induce cellular apoptosis and inhibit cell growth

(A) ESCC cell lines KYSE150 and EC9706 were transiently transfected with S100A9 to detect apoptosis by flow cytometry analysis and the percentage of sub-G1 cells is indicated. (B) pTel-off transfected EC9706 cells (TF93) were transfected with pTRE2-GFP-S100A9 to create cells with inducible GFP–S100A9 fusion protein. S100A9 was induced after removal of doxycycline, and cleaved PARP (PARP; 89 kDa) bands were detected. β-Actin was loaded as a control. kD, bands of indicated kDa molecular mass. (C and D) Induction S100A9 expression in the system described in (B) caused a significant retardation of cell growth rate as shown by (C) growth curve analysis and (D) colony formation assays. TF93 and empty vector pool (Vector) served as controls. Results are means ± S.D. *P < 0.001; n.s.d., no significant difference. OD (490 nm), D490.

pro-caspase-3 (31 kDa) was observed in both HCT116 p53+/+ and HCT116 p53−/− transfected cells, but there was no significant decrease between the cells types. The above results demonstrated that S100A9 induces cellular apoptosis in a p53-dependent manner.

S100A9 mediates p53 apoptosis signals

Since S100A9 was confirmed as a p53 target gene and it could induce apoptosis, we further investigated the role of S100A9 in mediating p53-induced apoptosis. We evaluated the effect of
endogenous S100A9 inhibition on p53 induced apoptosis upon knockdown of S100A9 expression. To test the effect of S100A9 depletion on a purely p53-dependent apoptotic response, human ESCC cell line YES2, which contains endogenous wild-type p53 but is not sensitive to the DNA-damaging agent [33], was transiently transfected with S100A9 siRNA, and the ability of exogenous p53 expression (by Ad-p53 infection) to induce apoptosis was assessed. S100A9 siRNA was effective in interfering with S100A9 expression as compared with non-silencing siRNA. Ad-p53 could increase p53 in YES2 cells (Figure 6A). In three independent apoptotic assays, S100A9 siRNA transfected YES2 cells showed a significant reduction in the percentage of Annexin V–PI positive cells compared with non-silencing siRNA controls 48 h after Ad-p53 infection (Figure 6B). The result demonstrated that S100A9 participated in p53-mediated apoptosis and suggested that S100A9 be a downstream target of the p53 gene.

**DISCUSSION**

S100A9, a member of the S100 calcium-binding protein family, has been shown to be down-regulated in different kinds of squamous cell carcinomas [34–36] and up-regulated in some adenocarcinomas [37–41]. We have previously found that S100A9 was significantly down-regulated in human ESCC. Thus we further investigated the regulation of S100A9 in ESCC cells.

There are several reasons for the consistent reduction of gene expression levels. In the present study, we focused on the transcriptional regulation of S100A9, and interestingly, we found that the S100A9 promoter contained several putative p53 BS. Among them, two 17/20 p53BS showed higher transcriptional activity in response to wild-type p53 activation using a luciferase reporter assay. Deletion mutations of these two p53BS revealed that they contributed even more to p53-mediated S100A9 activation, and the absence of both p53BS resulted in complete loss of activity. We also found that induction of S100A9 mRNA and protein expression corresponded with the increase in p53 expression upon Ad-p53 infection or treatment with the DNA-damaging reagent camptothecin. This was not observed in the control experiments of Ad-GFP infection or camptothecin treatment of p53-knockout cells. ChIP assays and EMSA further revealed that p53 could bind to the S100A9 promoter both in vivo and in vitro. Taken together, the data clearly demonstrate that the S100A9 gene is a direct transcriptional target for p53 and p53 could be at least one of the reasons for the reduction of S100A9 expression in ESCC.

As an important transcriptional factor, stress activation of p53 induces the expression of a large number of p53 target genes, some of which play important roles in mediating the various responses to p53 [42]. According to our experiments, overexpression of S100A9 in ESCC cell lines did not lead to cell-cycle arrest (results not shown) but significantly induced cellular apoptosis. Moreover, we demonstrated that the apoptosis induced by S100A9 is partly p53-dependent as there was a sharp contrast in the apoptotic degree between wild-type p53 and p53-knockout cell lines. siRNA-directed inhibition of S100A9 diminished p53-mediated apoptosis, placing S100A9 among other pro-apoptotic p53 targets. More detailed studies are needed to elucidate how S100A9 is involved in the p53-mediated apoptosis network.

It was proposed by Lin et al. [43] that mouse S100A9 might be regulated by p53, as mouse S100A9 had consensus p53-binding sequences in its promoter, but this study did not confirm this by experiment and the present study is the first to positively identify S100A9 as a novel p53 target gene. In fact, several members of the S100 family have been shown to interact with p53 and enhance p53-dependent apoptosis, which might functionally interact with p53 in the regulation of p53-dependent cell growth arrest and apoptosis [44]. S100B has been found to accelerate the loss of wild-type p53 function in tumours [46]. According to our experiments, S100A9 showed growth-inhibitory and apoptosis-inducing activities in various cell types with its heterodimer partner S100A8 [19]; together they induce apoptotic activity through the classical mitochondrial (intrinsic) pathway [18,20]. All of these studies were carried out by way of extracellular S100A8/A9 addition. In this study, similar apoptotic effects were observed when we overexpressed intracellular S100A9 alone as cleaved PARP1 was apparently detected, indicating that intracellular S100A9 alone could also induce apoptosis.
The function of nuclear S100A9 remains unknown and requires more detailed study. The introduction of S100A9 into ESCC cells resulted in the suppression of growth in the tumour cells, suggesting a relationship between S100A9 expression and malignancy of human oesophageal carcinoma. These findings imply an involvement of S100A9 in tumour progression and suggest the possibility that S100A9 may serve as a potential molecular target for therapy of carcinomas with underexpressed S100A9.

In conclusion, the present study demonstrates that S100A9 is a novel p53 target gene that can induce cellular apoptosis, in a partly p53-dependent manner, and mediate p53-induced apoptosis. This work therefore adds to our understanding of the relationship between the S100 family and tumour suppressor p53 and the role of S100A9 in tumour development.

**AUTHOR CONTRIBUTION**

Chunsun Li and Zhihua Liu were responsible for the study concept and design. Chunsun Li, Hongyan Chen and Fang Ding performed the experiments and were responsible for the analysis and interpretation of results. Chunsun Li wrote the manuscript and Zhihua Liu was responsible for critical revision of the manuscript. Statistical expertise was provided by Aiping Luo. Yu Zhang and Mingrong Wang provided technical and material support. Zhihua Liu supervised the study.
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SUPPLEMENTARY ONLINE DATA

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Figure S1  S100A9 expression is p53-dependent

EC9706 cells were transfected with pcDNA3.0 (Vector) or pcDNA3-p53 (p53 transfected). Immunofluorescence analysis (with antibodies against S100A9 or p53) showed that EC9706 cells transiently transfected with pcDNA3-p53 expressed more S100A9 protein.

Figure S2  p53-mediated regulation of S100A9 was not cell-type- or tissue-specific

(A) Western blot showing that p53 protein levels increased in all the six different kinds of cell lines at 24 h or 48 h following Ad-p53 infection. (B) Real-time reverse transcription–PCR results showed that S100A9 mRNA was induced in all the six cell lines at 24 h or 48 h after infection.

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