

Subcellular distribution of S100A4 and its transcriptional regulation under hypoxic conditions in gastric cancer cell line BGC823

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It is well known that S100A4 is overexpressed in many tumors and involved in tumor invasion and metastasis. But the regulation of it is ill understood. We previously found that hypoxia mimicking cobalt chloride (CoCl₂) enhanced the mRNA and protein expressions of the S100A4 gene in the gastric cancer cell line BGC823. In this study we found that S100A4 also displayed increased expression in BGC823 cells after exposure to real hypoxia (2.5% O₂) as that by CoCl₂ treatment. Moreover, S100A4 protein showed different subcellular distribution under real hypoxia compared with that by CoCl₂ treatment or in normoxic conditions. To investigate the underlying molecular mechanism by which hypoxia regulates the expression of S100A4, we analyzed the regulatory sequences of the genes by bioinformatics and found a putative hypoxia responsive element (HRE) motif in the first intron of S100A4. Furthermore, luciferase reporter assay showed that it is responsive to hypoxia. Electrophoretic mobility shift assay and chromatin immunoprecipitation assays demonstrated that hypoxia-inducible factor 1 (HIF-1) binds to the functional HRE *in vitro* and *in vivo*. The results provide evidence that S100A4 is a hypoxia-inducible gene, whose transcription is stimulated at least partly through the interaction of HIF-1 and HRE located at +329 to +334 of S100A4. (*Cancer Sci* 2010; 101: 1141–1146)

S100A4 protein is a member of the S100 family of calcium-binding proteins involved in the invasiveness and metastasis of tumors.^(1–4) S100A4 expression has been associated with metastasis in animal studies, and a number of studies have examined the utility of S100A4 expression as a prognostic marker in human cancers. Expression of S100A4 is significantly increased in tumors, such as thyroid, ovarian, lung, colorectal, pancreatic, and gastric cancers.^(5–12) It plays a key role in the process of metastasis by stimulating angiogenesis,⁽¹³⁾ promoting the migration of tumor cells,⁽¹⁴⁾ adhesion of cells to matrix, and escape of tumor cells from primary tumors; and inducing expression of protein hydrolase.^(15,16) However, the regulation of S100A4 gene expression is not well understood.

Hypoxia occurs in solid tumors due to uncoordinated tumor growth and angiogenesis. It was reported that hypoxia promoted tumor progression, invasion, metastasis, and resistance to chemotherapy, leading to poor patient prognosis.⁽¹⁷⁾ Whether S100A4 is regulated by hypoxia and mediates the effects of hypoxia in promoting the progression of cancer should be an interesting issue. In a previous study, we treated the gastric cancer cell line BGC823 with CoCl₂ to mimic hypoxia and found that CoCl₂ could up-regulate S100A4 expression,⁽¹⁸⁾ but the effect of real hypoxia on S100A4 expression and underlying molecular mechanism are less well understood.

It is well known that hypoxia-inducible factor 1 (HIF-1) plays a critical role in cell response to hypoxia. HIF-1 is a heterodimeric transcription factor which activates a wide range of genes

encoding important proteins involved in the physiological adaptation to hypoxia.^(19,20) Most HIF-1-regulated genes contain an enhancer sequence termed the hypoxia responsive element (HRE).^(21–25) Hypoxia-induced transcriptional activation of HIF-1 involves, at least in part, a decrease in oxygen-sensitive degradation of the HIF-1 α and an increase in its binding to the HIF-1-binding site (HBS) present in the HRE.⁽²⁶⁾ The present study showed that S100A4 displayed increased expression and different subcellular distribution under real hypoxia (2.5% O₂) conditions compared with that by hypoxia mimetics CoCl₂-treatment or in normoxia. We then explored the mechanism of transcriptional regulation of S100A4 by hypoxia. We found a putative HRE in the first intron of the S100A4 gene and demonstrated that HIF-1 binds to the HRE in the S100A4 gene, by which S100A4 expression was regulated under hypoxic conditions.

Materials and Methods

Reagents. Cobalt chloride (CoCl₂) was from Sigma-Aldrich (St. Louis, MO, USA). The pGL3-Basic, pRL-TK luciferase reporter vectors and the Dual Luciferase Reporter assay system were obtained from Promega (Madison, WI, USA). The reverse transcription system was from Promega. Rabbit antibodies against HIF-1 α , and rabbit anti- β -actin, secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-S100A4 antibody was from Lab Vision & Neomarkers (Fremont, CA, USA). The DNA 3'-end bio-labeled kit was purchased from Pierce Biotechnology (Thermo Scientific, Rockford, IL, USA).

Cell culture. The gastric adenocarcinoma cell line BGC823 cells were cultured in RPMI-1640 medium supplemented with 10% FCS. The cells were maintained at 37°C and 5% CO₂ in a humidified incubator.

Hypoxia treatment. Cells were incubated in a temperature and humidity-controlled environmental chamber IG750 (Jouan, St-Herblain, France) in an atmosphere containing 2.5% O₂, 5% CO₂, and 92.5% N₂. Hypoxia was also mimicked by treatment with CoCl₂ (200 μ M).

Reverse transcriptase–polymerase chain reaction (RT-PCR). The total RNA of BGC823 cells was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA); 1 μ g of RNA was reverse transcribed using the Promega RT system. The resulting cDNA was amplified by PCR. Primers specific for human S100A4 and β -actin were designed as follows: S100A4 sense 5'-GATGTGATGGTGTCCACCTT-3', antisense 5'-ATTTCTTCCTGGGCTGCTTA-3'; β -actin sense 5'-CTCTTCCAGCCTTCCTTCT-3', antisense 5'-CACCTTCCAGTTCCAGTTT-

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3'. Aliquots of PCR products were checked by electrophoresis on a 1.5% agarose gel with the fragments visualized by ethidium bromide staining.

Immunofluorescence staining. The BGC823 cells were cultured on coverslip. Hypoxia treatment (2.5% O₂) was carried out as described before. The cultured cells were rinsed in PBS and fixed in 10% formaldehyde for 15 min at room temperature. Fixed cells were incubated first with 0.1% Triton X-100 for 10 min followed by incubation in blocking solution (5% BSA) for 30 min, then cells were incubated with a 1:500 dilution of rabbit anti-S100A4 antibody for 2 h, and then incubated with a 1:2000 dilution of FITC-conjugated mouse anti-rabbit IgG (Invitrogen) for 30 min at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Nakarai, Tokyo, Japan). The cells were observed under a fluorescence microscope.

Plasmid constructions. A 937 bp DNA fragment (937 wt) of *S100A4* gene containing the putative HRE motif (from -387 to +550) was amplified by PCR using the human genomic DNA as a template and the primers were: 5'-TCACCACATTCCAGGGCA-3', 5'-GCAAAGTGAGGTGGCAGAG-3'. We employed overlap extension-PCR to generate the mutated 937-bp DNA fragment (937mu) by introducing the same mutations as those described in EMSA to the putative HRE motif using the following primer pairs: 5'-TCACCACATTTCCAGGGCA-3', 5'-GGCACATAATACCAACCTTTACTA-3', 5'-TAGTAAAGGTTGGTATG TATGTGCC-3'; 5'-GCAAAGTGAGGTGGCAGAG-3', and the primers used in the generation of 937wt as described above. The fragments 937wt or 937mu were cloned into the *KpnI/HindIII* sites of pGL3-Basic to generate pGL3-937wt or pGL3-937mu reporter constructs, respectively. All constructs were confirmed by sequencing.

Transient transfection and luciferase reporter assay. BGC823 cells were seeded at 1.5×10^4 cells per well (24-well plate) and maintained at 37°C in a humidified 5% CO₂ atmosphere. The DNA transfections with 0.8 µg of the pGL3-937wt or pGL3-937mu luciferase constructs with internal control vector phRL-TK (50:1 ratio) were performed using Lipofectin (Invitrogen) 24 h after cell seeding. After incubation for 5 h, the medium was replaced with a fresh one, and these cells were incubated for 24 h under real hypoxic (2.5% O₂) or normoxic (21% O₂) conditions. The cells were then harvested, and the luciferase activities in the cell lysates were determined using dual luciferase assay reagents. The luciferase activities were normalized by the internal control values. The values were represented as the mean ± SD of triplicate samples.

Western blot analysis. BGC823 cells were treated with CoCl₂ (200 µM) or real hypoxia (2.5% O₂), and harvested at different time points for the detection of HIF-1α or S100A4 protein expression, respectively. Then CoCl₂ treatment or real hypoxia treatment as well as untreated BGC823 cells were lysed with lysis buffer. Protein concentrations were measured using a modified Lowry procedure with bovine serum albumin (BSA) as the standard. Equal amounts of proteins in whole cell lysates were separated on 12% SDS-polyacrylamide gels, transferred onto PVDF membranes (Millipore, Bedford, MA, USA), and blocked with TBST supplemented with 5% nonfat milk. Membranes were incubated with the rabbit anti-HIF-1α, S100A4, or anti-actin antibodies at 1:1000 dilution for 16 h. After extensive washing with TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000)

in blocking solution. The blots were visualized using the Amersham ECL system.

Electrophoretic mobility shift assay (EMSA). The nuclear extracts were prepared from BGC823 cells maintained under normoxic or hypoxic conditions for 24 h using the NE-PER Nuclear Extraction Reagent Kit (Pierce Biotechnology). Wild-type and mutated oligonucleotides corresponding to the S100A4-HRE region were designed according to the *S100A4* gene sequence (GenBank accession no. 6275). The sequences of the sense strands used for EMSA are shown in Table 1. The probes were labeled using the Biotin 3' End DNA Labeling Kit according to the manufacturer's instructions. The binding reactions were carried out with 15 µg of nuclear extracts and biolabeled S100A4-HREwt, S100A4-HREmu, and consensus probes (Epo-HRE). The mixtures were incubated for 1 h at room temperature. The supershift analysis was performed using 1 µg of rabbit antibody against HIF-1α. For the oligonucleotide competition experiments, unlabeled S100A4-HREwt was used. The complexes were resolved in a 6% nondenaturing polyacrylamide gel in 1× Tris-Borate-EDTA electrophoresis buffer.

Chromatin immunoprecipitation (ChIP). BGC823 cells were treated with CoCl₂ (200 µM) for 24 h to induce the expression of HIF-1, with the untreated cells as control. DNA and proteins were cross-linked by the addition of 1% formaldehyde for 20 min before harvesting. Cells were washed twice with ice cold PBS, scraped off the plates, resuspended in 300 µL of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH8.0], and a protease inhibitor mixture), then sonicated to generate 100–1000 bp fragments. After centrifugation, the cleared supernatants were diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). The cell lysates were precleared by incubation at 4°C with 15 µL protein G beads pre-adsorbed with sonicated single-stranded DNA and BSA. The cleared lysates were incubated overnight with anti-HIF-1α antibody or without any antibodies. Immune complexes were precipitated with 30 µL protein G beads pre-adsorbed with sonicated single-stranded DNA and BSA. After centrifugation, the beads were washed, and the antigen was eluted with 1% SDS, 100 mM sodium carbonate. DNA-protein cross-links were reversed by heating at 65°C for 4–5 h, and DNA was phenol-extracted and analyzed by PCR with Taq DNA Polymerase (Promega) using the following primers: forward 5'-ATGTGGAAGACAGCATAGACC-3' and reverse 5'-AGAGGCAAAGAGGA AAACCACCA-3'.

PCR and sequencing. DNA was extracted from 25 cases of gastric carcinoma tissues. The fragment of the *S100A4* gene containing the core HRE motif was analyzed by the combined PCR and sequencing techniques. The PCR primers were: forward 5'-TTCTTGGTTTGGTGGAGTTGTG-3' and reverse 5'-GCAAAGTGAGGTGGCA GAG-3'.

Results

S100A4 expression was up-regulated and showed distinct subcellular distribution under real hypoxic conditions. RT-PCR showed that the level of S100A4 mRNA at 24 h after exposure to hypoxia was the highest among the time points observed (Fig. 1a). Therefore, we analyzed S100A4 protein expression at 24 h after exposure to hypoxia by western blotting and immunofluorescence staining, which showed up-regulation of S100A4

Table 1. Oligonucleotides used for EMSA

S100A4-HREwt	5'-CACACGCTGTTGCTA TAGT ACG TGTTGGTATGTA-3'
Erythropoietin (Epo-HRE)	5'-CTATAGTACGTTGGTA-3'
S100A4-HREmu	5'-CACACGCTGTTGCTATAGTAA AGG TGTTGGTATGTA-3'

Core sequences of the S100A4-HRE are shown in bold and mutant bases are underlined. HRE, hypoxia responsive element.

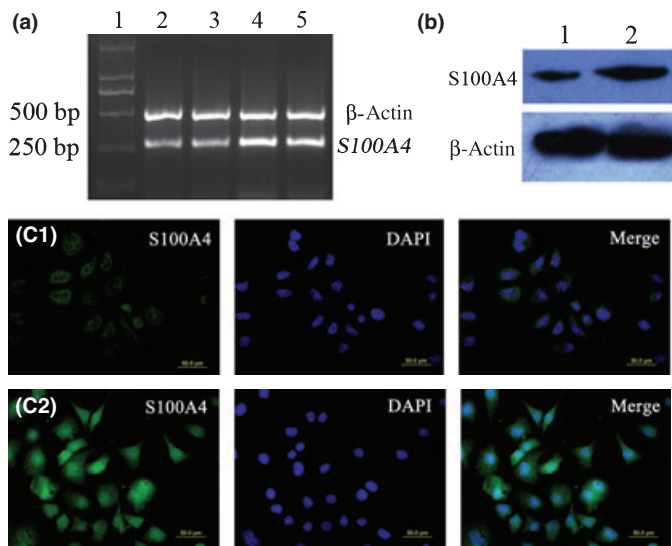


Fig. 1. Effects of real hypoxia (2.5% O₂) on S100A4 expression and subcellular distribution of S100A4 protein. BGC823 gastric cancer cells were cultured under hypoxic conditions (2.5% O₂), and S100A4 mRNA and protein expression were analyzed by RT-PCR (a), western blot analysis (b), and immunofluorescence staining (c) as described in the Materials and Methods. (a) Lane 1: M, DNA marker – DL2000; Lane 2: sample from untreated cells; Lanes 3 to 5: samples representing 6, 24, and 48 h after exposure to hypoxia, respectively. (b) Lane 1: sample from untreated cells; Lane 2: sample representing 24 h after exposure to hypoxia. (c) C1, C2: samples representing untreated cells, 24 h after exposure to hypoxia, respectively. The primary antibody used in this experiment was anti-S100A4 antibody (green). DAPI was used to visualize nuclear staining (blue). Merged images of green and blue signals are shown in the right panels.

protein (Fig. 1b,c). Moreover, S100A4 protein displayed distinct subcellular distribution after real hypoxia exposure. High nuclear S100A4 protein expression was observed in BGC823 cells under real hypoxic conditions (Fig. 1c).

Identification of the putative HRE motif in the first intron of the human S100A4 gene. Bioinformatic analysis of the promoter region and the first intron sequences of the S100A4 gene showed that there was HIF-1 binding site, named HRE motif, located at +329 to +334 (Fig. 2) in the first intron, suggesting that it may have an oxygen-dependent regulation. Potential HRE motif was identified using the programs at URL: <http://www.gene-regulation.com>.

Transcriptional regulation of the region harboring the putative HRE in the S100A4 gene by hypoxia. The promoter activity of pGL3-973wt increased by 2.6-fold upon exposure to hypoxia compared with normoxia, and activity was markedly abrogated by the mutation of the HRE (pGL3-973mu) (Fig. 3). The data indicated that the HRE motif was directly involved in the activation of S100A4 transcription under hypoxia.

HIF-1 protein expression in BGC823 cells at different time points after exposure to CoCl₂. Western blot showed up-regulation of HIF-1 protein after CoCl₂ exposure compared with non-treated cells. The up-regulation of HIF-1 protein in BGC823 cells occurred 6 h after cells were exposed to CoCl₂, reached a peak at 24 h, and the overexpression lasted for 48 h (Fig. 4). Therefore, we treated BGC823 cells with 200 μM CoCl₂ for 24 h for EMSA and ChIP.

Analysis of binding of nuclear protein HIF-1 to the predicted HRE by EMSA and ChIP. To determine whether the HRE identified within the first intron of the S100A4 gene binds the HIF-1 protein, EMSA was performed with the nuclear extracts obtained from CoCl₂-treated BGC823 cells. As shown in

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1 attcttcccc tctctacaac cctctctctc cagcgcttct tctttcttgg ttgttgagt+
61 tgtgttggcc tgactggcat gcaaggsgtg tcaagaggcca gggctggsga
aggagaaggg+
121 gagcctgggt ggggcccagat gtgctaaaga gatccagatg tgagattctg
atgtggaact+
181 ctgggtggat tgtgtgcctg ggtgtgcatg gcacacacac acatgcacgt
aagacggagg+
241 aaaaaacaaa caaaaaagtg agcaagtac tgaatttgag ctctccaggt
gcttctgaga+
301 tgtgggcttg cacacgctgt tgctatagta cggtttggta tgtatgtgcc
tgtgggtatc+
361 tgcaactggc catgttttgc gggattgcga ctggggagca ggaagcaaa
gaaaggcaga+
421 aggcaactgt gggcctttgt ctgggtgtgt gccccatgag cctctgccct
gcacgcagca+
481 gccacgctcg agaaggtgca tggcctctgc agcttctctt ccaacccttg
cctctgccac+
541 ctcaactttg cctctcccat gctgagagct aagcggctgt gctggtttt
tccactcgag+

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Fig. 2. Prediction of the hypoxia responsive element (HRE) motif in the first intron of the S100A4 gene. The sequence containing the entire first exon and part of the first intron in the S100A4 gene is shown, in which the putative HRE motif is indicated.

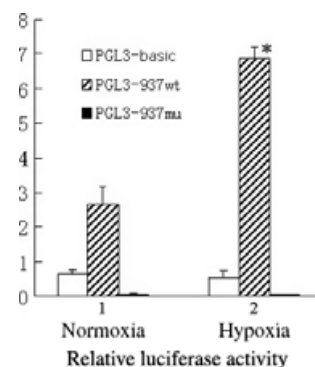


Fig. 3. Luciferase reporter assay demonstrating that the putative hypoxia responsive element (HRE) is responsible for the transcriptional activation of S100A4 under hypoxia. Each bar represents the relative luciferase activity under hypoxic and normoxic conditions. The relative luciferase activity is represented by the ratio of luciferase activity of firefly (from the various pGL3 vectors) to that of Renilla (from phRL-TK construct) as an internal control. The data represent the mean ± SD from three experiments (*P < 0.05).

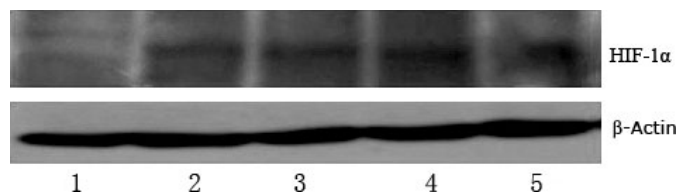


Fig. 4. Hypoxia-inducible factor 1 (HIF-1) protein expression at different time points after exposure to CoCl₂ in BGC823 cells as measured by western blot analysis. Lane 1: sample from untreated cells; Lanes 2 to 5: samples representing 6, 12, 24, and 48 h after exposure to CoCl₂.

Figure 5, a specific complex with retarded migration appeared exclusively when CoCl₂-treated nuclear extracts were incubated together with the labeled probe containing the intact HRE motif. No complex was visualized when mutated oligonucleotides were used as the probe. In addition, the specific binding of CoCl₂-treated nuclear extracts to the wild-type probe was eliminated

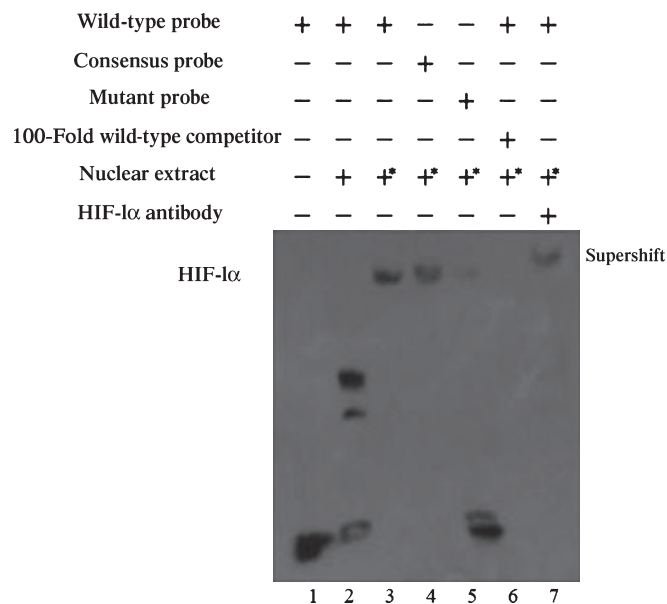


Fig. 5. Analysis of hypoxia-inducible factor 1 (HIF-1) binding to the hypoxia responsive element (HRE) motifs in the *S100A4* gene *in vitro* using EMSA. BGC823 cells were exposed to normoxic or hypoxic mimicking conditions for 24 h prior to preparation of nuclear extracts. Various probes were incubated with nuclear extracts from CoCl_2 -treated or nontreated BGC823 cells. Lane 1: labeled *S100A4*-HREwt probe without nuclear extract; Lane 2: labeled *S100A4*-HREwt probe incubated with the nuclear extracts from nontreated cells formed no specific complex; Lane 3: labeled *S100A4*-HREwt incubated with the nuclear extracts from CoCl_2 -treated cells forming a complex; Lane 4: labeled Epo-HREwt as consensus oligonucleotide probe incubated with the nuclear extracts from CoCl_2 -treated cells forming a complex; Lane 5: labeled *S100A4*-HREmu probe did not form a complex; Lane 6: competition experiments performed with the *S100A4*-HREwt probe using a 100-fold molar excess of unlabeled *S100A4*-HREwt oligonucleotides; Lane 7: supershift analysis of HIF-1 bound to the *S100A4*-HREwt probe performed using anti-HIF-1 α antibody; *nuclear extracts from the CoCl_2 -treated cells.

by competition with an excess of homologous unlabeled probe. These results demonstrate that HIF-1 binds to the consensus HRE. To identify proteins involved in the formation of these complexes, we performed supershift assays which showed that a HIF-1 α antibody decreased the formation of the hypoxia-inducible complex and caused the appearance of a supershifted complex. These results indicate that HIF-1 α was present in these hypoxia-induced complexes.

ChIP experiments were performed to check the binds of HIF-1 proteins and HRE motifs *in vivo*, using HIF-1 α antibody, with the IgG antibody as a negative control. Positive signals were found when the HIF-1 α antibody but not the IgG antibody was used in BGC823 cells subjected to hypoxic conditions, demonstrating that HIF-1 interacts with the *S100A4* gene *in vivo* (Fig. 6). Significantly weaker signals were found in cells exposed to normoxic compared to hypoxic conditions, due to the up-regulation of HIF-1 under hypoxic conditions.

Sequence analysis of fragments harboring HRE of *S100A4* in gastric carcinoma tissues. The combined PCR and sequencing techniques were used to analyze the fragment of the *S100A4* gene (+44 bp to +507 bp) containing the core HRE motif in 25 gastric carcinomas, but no variation was detected among all the samples.

Discussion

Our previous study demonstrated that the *S100A4* gene was induced during chemical induction of hypoxia in BGC823

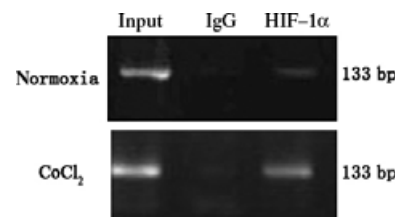


Fig. 6. Analysis of the hypoxia-inducible factor 1 (HIF-1) binding to the hypoxia responsive element (HRE) motif of the *S100A4* gene *in vivo* by ChIP assay. BGC823 cells were grown under normoxic or hypoxic conditions for 24 h. Lysates of BGC823 cells were immunoprecipitated with HIF-1 α antibody or normal rabbit IgG. Subsequently, a DNA segment of the first intron of the *S100A4* gene containing the HRE was amplified by PCR. Lysates without immunoprecipitation were used as the control for DNA input in each sample. Binding of HIF-1 α was increased by CoCl_2 treatment compared with untreated samples.

cells.⁽¹⁸⁾ In the present study, we found that real hypoxia (2.5% O_2) could also up-regulate *S100A4* expression at both mRNA and protein levels in BGC823 cells. Moreover, the subcellular distribution of *S100A4* by real hypoxia was different from that by hypoxia mimicking CoCl_2 treatment in our previous study. Increased *S100A4* protein occurred mainly in the cytoplasm of BGC823 cells after CoCl_2 treatment, whereas it occurred mainly in nuclear after real hypoxia exposure. The different subcellular distribution of *S100A4* protein maybe due to different properties of hypoxia mimics and real hypoxia, although they share some commonality. It seems that nuclear overexpression of *S100A4* under a real hypoxic condition maybe an interesting issue. Maelandsmo *et al.*⁽⁶⁾ reported that nuclear *S100A4* expression in tumor cells was associated with more aggressive ovarian carcinomas. Furthermore, it has been reported that *S100A4* regulated the expression of many genes.⁽²⁷⁾ So we can speculate that hypoxia induces *S100A4* expression and translocation to the nucleus, where it regulates many gene expressions and promotes the progression of cancer.

Our previous and present studies showed that real hypoxia and CoCl_2 are consistent in promoting the expression of *S100A4*. So we wanted to explore the mechanism by which hypoxia regulates *S100A4* expression. One possible mechanism by which hypoxia regulates *S100A4* expression is through the transcriptional factor HIF-1. Previous studies have shown several regulatory elements such as kB-related, specificity protein 1 (Sp1), core-binding factor (Ap-1), core-binding factor (CBF), and κ recognition component (KRC) binding sites etc., in the first intron of *S100A4*,⁽²⁸⁻³⁰⁾ which suggest that the first intron is an important region for gene regulation. In the present study, bioinformatics analysis was used to analyze the promoter region and sequences downstream of the transcription initiation site, including the first intron of the *S100A4* gene in the search for HREs. The core sequence of the potential HRE motif was found in the first intron of the *S100A4* gene, located at +329 to +334 (TACGTG) which conforms to the consensus sequence of the HIF-1-binding site 5'-RCGTG-3' found in other genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), endothelin-1, endothelial nitric oxide synthase (eNOS), etc.⁽²²⁻²⁵⁾ It might be essential to the regulation of *S100A4* under hypoxic conditions in BGC823 cells.

To identify whether the putative HRE motif found in the first intron of *S100A4* gene was indeed responsible for the transcriptional activation under hypoxia, we performed luciferase reporter assays. The results showed that transfection of pGL3-937wt containing the regulatory fragment with the wild-type HRE motif enhanced the reporter gene's activity under hypoxia compared with that in normoxia. Meanwhile, the pGL3-937mu construct with the mutated HRE exhibited

far lower luciferase activity than the pGL3-937wt construct in hypoxia, and no significant difference in the regulatory activity of pGL3-937mu construct was found between normoxic and hypoxic conditions, indicating that the putative HRE motif in *S100A4* is essential for the regulatory activity under hypoxia. It is interesting that the regulatory activity of the pGL3-937mu construct decreased dramatically compared to the pGL3-937wt and basic pGL3 construct even under normoxia. But no known human regulatory elements were found in the mutated HRE motif and its neighboring sequences by bioinformatics analysis. Therefore, we speculated that wild-type HRE may antagonize the effect of a potential repressor element in *S100A4*. When it was mutated, not only was the induction of HRE disrupted and abolished under hypoxia, but also the activity of related repressor element(s) increased and caused the reduced luciferase activity from the pGL3-937mu construct even in normoxic conditions. However, the exact molecular mechanism remains to be clarified.

It is known that CoCl₂ can also induce HIF-1 α expression in many different kinds of cells.⁽³¹⁾ We used 200 μ M CoCl₂ to mimic the hypoxic conditions and detected the HIF-1 α protein expression in BGC823 cells at different time points after exposure to CoCl₂. We determined that the peak expression of the HIF-1 α protein occurred at 24 h after exposure to CoCl₂. We therefore treated cells with CoCl₂ for 24 h in subsequent studies.

To identify the binding of nuclear protein HIF-1 to the HRE motif, EMSA and ChIP were performed. EMSA showed that a specific DNA-protein complex, absent with control extracts from normoxic cells, was formed with nuclear extracts from CoCl₂-treated gastric cancer cells. Mutant oligonucleotides failed to form the complex with nuclear extracts from CoCl₂-treated gastric cancer cells as well. Additionally, the complex was prevented by an excess of cold *S100A4*-HREwt probes.

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Anti-HIF-1 α was able to “supershift” the hypoxia-induced binding activity, showing that HIF-1 α could bind the HRE motif *in vitro*. The ChIP assay results also showed that HIF-1 could bind to the HRE of the *S100A4* gene *in vivo* and that CoCl₂ treatment could enhance their interactions. Collectively, these data indicate that HIF-1 can bind the HRE motif of *S100A4* both *in vivo* and *in vitro*.

Furthermore, we hypothesized that there might be sequence variations in the HRE of the *S100A4* gene which may affect the *S100A4* hypoxic response in gastric carcinoma tissues. To test this possibility, we screened 25 primary gastric carcinoma tissues by amplifying and sequencing a fragment of approximately 500 bp containing the HRE of the *S100A4* gene. However, no sequence variations were found in the fragments in any of the gastric carcinoma tissues investigated. The results did not seem to support our hypothesis. However, the sample number in our study was small; further study by increasing sample numbers is needed to clarify this.

In summary, our data showed that real hypoxia (2.5% O₂) induced *S100A4* expression at both the mRNA and protein level. Moreover, real hypoxia led to nuclear overexpression of *S100A4*, which is different from subcellular distribution of *S100A4* by CoCl₂ treatment or in normoxia. Our data also showed that HIF-1 could bind the HRE at position +329 to +334 of the *S100A4* gene *in vivo* and *in vitro*. The results provide important insights into the molecular mechanism by which human *S100A4* gene expression is induced under hypoxic conditions.

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