Hypoxia-mediated up-regulation of MGr1-Ag/37LRP in gastric cancers occurs via hypoxia-inducible-factor 1-dependent mechanism and contributes to drug resistance

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Our previous study demonstrated hypoxia-inducible factor-1(HIF-1) could prompt multidrug resistance (MDR) phenotype and MGr1-Ag/37LRP, a novel drug-resistance protein was reported by our labortary, associated with multidrug resistance in gastric cancer. Given this association, we hypothesized that MGr1-Ag/37LRP contributed to HIF-1-dependent hypoxia-induced MDR phenotype. Initial experiments revealed that blocking MGr1-Ag/37LRP expression by siRNA in gastric cancer cells effectively reversed multidrug resistance phenotype induced by hypoxia. Subsequent analysis of MGr1-Ag/37LRP mRNA and protein in gastric cancer cells revealed a time-dependent manner increase with hypoxia. While the up-regulation of MGr1-Ag/ 37LRP was abolished by HIF-1 inhibition with siRNA. Studies using luciferase promoter constructs revealed a significant increase in activity in cells subject to hypoxia and such hypoxia inducibility was lost in cells co-transfected siRNA targeting HIF-1. Analysis of the *MGr1-Ag/37LRP* promoter revealed several potential binding sites for HIF-1. Electrophoretic mobility shift assay and chromatin immunoprecipitation demonstrated a functional HIF-1 binding site within *MGr1-Ag/37LRP* gene regulatory sequence located at −16 to −11 relative to the transcriptional initiation point. These observations demonstrate that *MGr1-Ag/ 37LRP* is actively engaged by hypoxia and represent a novel HIF-1 target. Such results suggest hypoxia-elicited MGr1-Ag/37LRP expression as a pathway for resistance of gastric cancer to chemotherapeutics.
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Hypoxia induced drug resistance is a major obstacle in the development of effective cancer therapy.[1–6] Hypoxia-inducible factor 1 (HIF-1) is an essential component in changing the transcriptional response of tumors under hypoxia. HIF-1 is composed of the HIF-1α and HIF-1β subunits. Whereas HIF-1β is constitutively expressed, HIF-1α protein stability and synthesis are regulated by intratumoral hypoxia and genetic alterations. The HIF-1 complex transactivates hypoxia-responsive genes through binding to the hypoxia response elements (HRE) located on the promoter or enhancer regions of hypoxia-inducible genes. Promoter analyses for HRE show that HIF-1α directly transactivates *MDR1* gene contributing to MDR phenotype.[7,8]

Gastric cancer is one of the leading causes of cancer death in China. Like other solid tumors, it has extensive areas of hypoxia. Clinical studies revealed a significant association between the expression of hypoxia and prognosis in gastric cancer.[9–11] Our previous study suggested hypoxia could result in resistance to different chemotherapeutic agents included 5-fluorouracil (5-FU), adriamycin (ADR), cisplatin (CDDP), vincristine (VCR) and etoposide (VP-16) in gastric cancer cells and transfectants of forced HIF-1 expression could increase drug resistance. Resistance acquisition *in vitro* and *in vivo* was suppressed by blocking HIF-1 expression with siRNA.[12] However, the exact HIF-1 dependent mechanism of hypoxia-induced MDR remained elucidated. Although the MDR1 gene was reported as a downstream drug-resistant molecular associated with MDR in colon cancer, our previous study revealed the P-gp expression in gastric cancer is only about 10% in different gastric cancer cell and tissue,[13] which suggested it other mechnism involving in MDR in gastric cancer.

MGr1-Ag/37LRP was previously reported by our lab as an upregulated protein in gastric cancer drug-resistant cell SGC7901/VCR and identified as the 37-kDa laminin receptor precursor (37LRP).[14] Forced expression of MGr1-Ag/37LRP in gastric cancer cells could increase drug resistance toward 5-FU, VCR CDDP, VP16 and ADM.[15] Further study suggested that MGr1-Ag/37LRP involved in drug resistance *via* increasing antiapoptotic protein Bcl-2/Bax ratio or expression of the *MDR1* and *MRP1* gene production in gastric cancer cells.[16] Interestingly, our previous study found an increased expression of HIF-1α in SGC7901/VCR cell under normoxic condition and MGr1-Ag involved in it.[17]

So we hypothesize that *MGr1-Ag/37LRP* may be represented a hypoxia-responsive gene contributing to hypoxia-induced MDR phenotype and HIF-1 induction occurs in this setting. Our analysis firstly revealed that decreased MGr1-Ag/37LRP expression may increase drug sensitivity under hypoxic condition *in vitro*. Extensions of these studies confirmed hypoxia induced *MGr1-Ag/ 37LRP* gene expression and concomitant MGr1-Ag/37LRP protein expression *in vitro* and *in vivo*. Moreover, these data revealed the existence of a previously unappreciated, functional HRE in the *MGr1-Ag/37LRP* gene. Taken together, these data suggest that HIF-1-dependent hypoxia-induced MGr1-Ag/37LRP expression may represent a pathway for resistance of gastric cancer cells to chemotherapeutics.

Material and methods

Cell culture

The following human gastric adenocarcinoma cell lines were employed: MKN45, AGS and MKN28 were obtained from the Shanghai Cell Bank (Shanghai, China), gastric cancer cells MGC803 from American Type Culture Collection (Rockville, Md., USA) and SGC7901 from the Academy of Military Medical Science (Beijing, China). Cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum with sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin-streptomycin mixture at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Oxygen deprivation and animal model of hypoxia

Cells were maintained under hypoxia (1% O_2) condition at 37°C within a modular incubator chamber (Precision Scientific, Winchester, VA)filled with 5% CO_2 and 1% O_2 balanced with N_2.

Mice were exposed to hypoxia (8% atmospheric oxygen) for 0 to 24 hr as described previously.[18] Following exposure, gastric mucosal tissue was removed, dissected along the mesogaster border, washed, and snap frozen for further analysis. All animal procedures were performed according to the guidelines of the Chinese Council on Animal Care and with appropriate institutional certification.

Analysis of drug resistance

The sensitivity of gastric cancer cells under hypoxic conditions to chemotherapeutic drugs was evaluated using the colony-forming assay. Gastric cancer cells in log phase were harvested and plated into 35-mm culture plates (10^3 cells/well). After overnight culture at 37°C for adhesion and 8 hr incubation under hypoxic condition(1% O_2), treated with increased concentrations 0, 0.1, 1, 10,50 μg/ml of vincristine (VCR), 5-fluorouracil (5-Fu), cis-diaminedichloroplatinum (CDDP), etoposide (VP16) and adriamycin (ADM) and cells were incubated for 24 hr under hypoxic condition. The plates were washed twice with serum-free RPMI 1640, grown with complete culture mediuma (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and antibiotics), and incubated for 10 days. The resulting colonies were stained with Coomassie brilliant blue, and the visible colonies were counted. Finally, the concentration of each drug that caused a 50% reduction in the number of colonies (IC_{50}) was calculated.

Annexin-V/propidium iodide staining

The apoptotic indices (AI) of these transfected cells were calculated based on the number of gastric cancer cells in the apoptotic state detected by flow cytometry. In brief, gastric cancer cells in log phase were plated into six-well plates (1×10^6 cells/well) and cultured overnight at 37°C. Prior to hypoxia incubation, the culture medium was replaced with serum deprived RPMI 1640 medium and then with hypoxic incubation for 8h. VCR was added to the each well until a final concentration of 2.5 mg/l was reached. Cell culture was continued under hypoxic condition for 48 hr. Then 5 μl (50 ug/ml) of annexin-V-FITC were added to these cells, and cells were cultured for further 10 min. The cells were washed twice in RPMI 1640. The cells were scratched and resuspended in 490 μl of Tris-HCl buffer. Then 5 μl of propidium iodide (PI) were added to the resuspended cells and cultured at 4°C for further 10 min. The mean fluorescence intensity of annexin-V-FITC/PI was determined by flow cytometry using the excitation and emission wavelengths proposed in the protocol (ClonTech, Palo Alto, CA, USA). Then the AI was calculated at the mean fluorescence intensity.

Western blotting

To examine HIF-1 and MGr1-Ag/37LRP expression, After pre-treated according to experimental demand, whole cells or Mice gastric mucosal tissues were harvested and lysed on ice for 30 min in lysis buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP-40, 5 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). Equal amounts of protein (25 μg) were loaded onto a sodium dodecyl sulfate-polyacrylamide gel (8% polyacrylamide) and subjected to electrophoresis at 200 V for 50 min, transferred to nitrocellulose, and blocked overnight in blocking buffer (250 mM NaCl, 0.02% Tween 20, 5% goat serum, and 3% BSA). For Western blotting, anti-HIF-1α (1:1000; Mouse mAb, Chemicon), MGr1 antibody (1:200; developed in our laboratory)[19,20] or anti-β-actin (1:4000; Sigma) was added for 3 hr; Blots were washed, and species-matched peroxidase-conjugated secondary antibody was added (1:2000). Labeled bands from washed blots were detected by ECL (Amersham).

Real-time reverse transcription (RT)-PCR analysis

Cells were seeded after 0, 2, 4, 8-hr exposure to hypoxia respectively. RNA was extracted by using RNAzol (Biogenesis) according to the manufacturer's instructions. cDNA strand synthesis was performed by using a Moloney murine leukemia virus cDNA synthesis kit (GIBCO BRL). CDNA samples were diluted 1:20, and 10 μl was used as a template for the Taqman real-time PCR technique to quantify mRNA expression by using a qPCR core kit (Eurogentec) and an ABI Prism 7700 sequence detection system (PE Applied Biosystems). Taqman PCR primers were designed for each gene based on the mRNA sequence by using Primer Express software (Perkin-Elmer) supplied by Sigma Genosys. Their sequences were as follows: for actin, ACCATGGATGAT-GATATCGCC and GCCTTGCACATGCCGG; for MGr1-Ag, GCTGGACGATAGCTTGGA and GATGACAGATAGCTGGTG and for GAPDH ACACTCAGACCCCCACCACA and CAT-AGGCCCCTCCCCTCTT.

Plasmid constructs and transfection

The recombinant sense expression vector and the siRNA expression vector for HIF-1α were constructed as described previously.[21,22] In brief, recombinant sense expression vector pcDNA3.1-HIF-1α was constructed by subcloning the complementary deoxyribonucleic acid (cDNA) fragment of HIF-1α that contained the complete coding sequence between *Kpn* I and *Bam*H I of the multiple cloning sites were introduced into SGC7901 or MGC803 cells. Gastric cancer SGC7901 and MGC803 cells were transfected with pSilencerTM neo U6 2.1 vector, containing a HIF-1α-specific targeting sequence (5′-AAA GAGGTGGATATGTCTGGG-3′ and 5′- TTTCTCCACCTATAC AGACCC -3′) and a scramble sequence (5′-TCAGCACGGTG ACT GAGAC-3′) as control. Gastric cancer SGC7901 and MGC803 cells were transfected with pSilencerTM neo U6 2.1 vector, containing an MGr1-Ag/37LRP-specific targeting sequence (5′-AAAGAGGTGGATATGTCTGGG-3′ and 5′-TTTCTCCACCTATACAG ACCC -3′) and a scramble sequence (5′-CTGACACCGTG ACGTA CAG-3′) as control. The sequence was checked against the database to confirm specificity. All cells were transfected by using Lipofectamine 2000(Invitrogen)according to the manufacture's instruction. The cells were harvested 48 hr after transfection and experience hypoxic culture in the 12 to 24 hr.

HIF-1 binding site search

A genomic region of 3000-bp upstream of the MGr1-Ag/37LRP transcriptional initiation site was determined using the NCBI Genomic BLAST program. This DNA sequence was then pasted into DNA Strider 1.0 software, which was employed to locate putative HRE. The search was based on compilations of functional HRE and the HIF-1 binding consensus sequence BDCGTV (B is C/T/G, D is A/G/T, V is G/C/A), in turn established by follows definitions of consensus sequences.[23,24]

Dual luciferase reporter gene assay

To assay the transcriptional activity of MGr1-Ag/37LRP under hypoxic condition, pGL3-2564-Luc containing 5 potential HRE and pGL3-1256-Luc containing three potential HRE, the promoter fragments (nucleotides −1600 to +964 and nucleotides −292 to +964) were cut from pUCm-T-MGr1-Ag/37LRP vector with KpnI and HindIII and subcloned into pGL3 vector (Promega) respectively.[25] SGC7901 or MGC803 cells in a 24-well plate (50,000 cells per well) were co-transfected with or without HIF-1 siRNA, HIF-1 sense vector respectively and the reporter plasmid using the Lipofectamine 2000 (Invitrogen), PRL-TK as a control for transfection efficiency in DMEM without serum. Forty-eight hours post-transfection, cells were exposed to hypoxic or normoxic condition for 24h.The luciferase activity was measured and quantitated in a luminometer using the Dual-Luciferase Reporter Assay System (Promega). Experiments were performed in triplicates. Results are expressed as means of the ratio between the firefly luciferase activity and the renilla luciferase activity.

Chromatin immunoprecipitation assay

HIF-1 binding to MGr1-Ag/37LRP promoter was analyzed by ChIP on gastric cancer cell, using methodologies described previously.[26] SGC7901 cells were fixed with 1% paraformaldehyde, and chromatin derived from isolated nuclei was sheared by using a F550 microtip cell sonicator (Fisher Scientific). After centrifugation, supernatants containing sheared chromatin were incubated with an anti-HIF-1α antibody or control IgG. Protein A sepharose was then added, the incubation was continued overnight, and immune complexes subsequently were eluted. Complexes were next treated with RNase and proteinase K and were extracted with phenol/chloroform and then with chloroform. DNA was precipitated, washed, dried, resuspended in water, and analyzed by PCR. The primers used in this analysis spanned 216 bp around the first possibilities of HIF-1α binding site located -249 bp from translation start site (sense, 5′- GCAGGTACAAGGGCTGGGTA -3′and antisense, 5′- TGCCGGGACTGGGCTTTT -3′) or spanned 216 bp around the second possibilities of HIF-1α binding site located −16bp from translation start site within the MGr1-Ag/37LRP promoter (sense, 5′-CTTCTTCCGCTCGAC TTTC -3′and antisense, 5′- TGACCGGCTTTCATCACTA -3′) and spanned 206 bp around the third possibilities of HIF-1α binding site located +199bp from translation start site within the MGr1-Ag/37LRP promoter(sense, 5′- GTCAGACTGGATCTGTC TCCC -3′and antisense, 5′- AGGATGTTAGCCCGCTTT -3′).

Electrophoresis mobility shift assay

The nuclear extracts were analyzed for HIF-1α-binding to an HRE on the MGr1-Ag/37LRP promoter by gel mobility shift assays as described previously.[27] The double-stranded DNA probes used in the electrophoresis mobility shift assay (EMSA) experiments contained the following sequences: 5′- TATGCA-CAGGGCGTCGCTGTGG -3′ for the first possibility wt HIF-1α binding site and 5′- TATGCACAGGGTTTCGCTGTGG -3′ for the first possibility mutant HIF-1α binding site. 5′- TACATAAG-GACGTCATTTCCTG -3′ for the second possibility wt HIF-1α binding site and 5′- TACATAAGGATTTCATTTCCTG -3′ for the second possibility mutant HIF-1α binding site 5′ -GCGGCC CGCACGTGGCCAGGCT -3′ for the third possibility wt HIF-1α binding site and 5′ - GCGGCCCGCATTTGGCCAGGCT -3′ for the third possibility mutant HIF-1α binding site An unrelated double-stranded oligonucleotide 5′- AGTGCTGCCATGTTGGAC TC-3′was used (ISRE [insulin receptor responsive element]) as a nonspecific competitor. The oligonucleotides were end-labeled with Biotin. SGC7901cells incubated under hypoxic or normoxic conditions were harvested and lysed in extraction buffer (20 mM HEPES [pH 7.9], 1 mM EDTA, 400 mM NaCl [25%], 0.1% NP-40, 1× protease inhibitors cocktail, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mM dithiothreitol). An equal amount of protein (1 μg) from the nuclear extract was used for binding reactions with the biotin-labeled wt or mutant MGr1-Ag/37LRP probe for 20 min at room temperature in binding buffer (8 mM HEPES [pH 7.4], 80 mM KCl, 0.8 mM EDTA, 1 mM dithiothreitol) at a 20μl final volume. For supershift experiments, 1 μg of monoclonal antibody against HIF-1α was added to the reaction mixture before the addition of labeled oligonucleotides. Equivalent amounts of Biotin-labeled probe were used for all samples. For the binding competition experiment, unlabeled oligonucleotides were added into the reaction mixture in a 50-and 200-fold excess. DNA-protein complexes were analyzed in a 4% polyacrylamide gel with 0.5 × Tris-borate-EDTA at 100 V. Then transfer of binding reaction to nylon membrane at 380 mA for 30 min. Cross-link for 15 min on a transilluminator equipped with 312 nm. Detect biotin-labeled DNA by chemilunimscence.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA or unpaired t test with $p < 0.05$ for n independent samples deemed statistically significant.

Results

### Blocking MGr1-Ag/37LRP expression inhibited hypoxia-induced drug resistance

Our previous study represented that hypoxia could prompt MDR in gastric cancer. To investigate the possibility of MGr1-Ag/37LRP in hypoxia-induced MDR in gastric cancer, we firstly investigated the MGr1-Ag expression under normoxia and hypoxia. Protein levels of MGr1-Ag/37LRP was upregulated under hypoxia (Figs 1a and 1b lane 2 compared with lane 1, $p < 0.05$). And then the siRNA expression vector for MGr1-Ag/37LRP was introduced into SGC7901 or MGC803 cells respectively treated with hypoxic condition. The transfection efficiency was analyzed by Western blot. SiRNA of MGr1-Ag was significantly inhibited hypoxia-induced MGr1-Ag overexpression (Figs 1a and 1b lane 3, $p < 0.05$), whereas the expression of MGr1-Ag/37LRP in the scramble control remained unchanged. It suggested that hypoxia could upregulated MGr1-Ag expression, while siRNA of MGr1-Ag could inhibit hypoxia-induced MGr1-Ag expression. To investigate the MGr1-Ag involved in hypoxia-induced MDR in gastric cancer cells. the transfected cells and parental cells were subjected to a colony-forming assay and to an apoptosis analysis *via* Annexin V/PI staining by flow cytometry under hypoxia.

The colony-forming assay data showed significant resistance to 5-fluorouracil, vincristine, cisplatin, etoposide, adriamycin in the two cell lines under hypoxia compared to that of normoxia (Figs 1c and 1d). Hypoxic exposure resulted in a threefold or greater resistance to five drugs than that of normoxia (IC50 value in hypoxia/normoxia #$p < 0.05$). However, blocking MGr1-Ag/37LRP expression by transient transfected vector of siRNA targeting MGr1-Ag/37LRP in 2 cell lines might partly reverse the hypoxia-induced drug-resistance (IC50 value in cells transfected with MGr1-Ag siRNA in hypoxia/parental cells *$p < 0.05$).The mean values of IC50 in the scramble control group SGC7901-c or MGC803-c remained unchanged ($P=$ no significant). These data demonstrated that hypoxia could increased drug resistance in different gastric cancer cell lines, while knockdown of MGr1-Ag/37LRP in gastric cancer cells strikingly elevated sensitivity to various tested antineoplastic agents under hypoxic conditions.

To assess the role of MGr1-Ag/37LRP in hypoxic protection of SGC7901 cell from apoptosis induced by VCR, Annexin V/PI staining assay revealed that SGC7901 and MGC803 cells transient transfected with siRNA targeting MGr1-Ag/37LRP could enhance drug-induced apoptosis under hypoxic condition. As it shown in Figure 1e and 1f, the induction of apoptosis by VCR was19.1 and 24% in SGC7901 and MGC803 cells respectively incubated under hypoxic conditions. However, transiently transfected MGr1-Ag/37LRP siRNA vector into SGC7901 and MGC803 cells reached apoptosis to 39.5 and 40.5% respectively under hypoxic condition. These data demonstrated that MGr1-Ag/37LRP play a role in preventing gastric cancer cell lines from VCR-induced apoptosis and its up-regulation by hypoxia may be an important contributor to drug resistance in gastric cancer cell lines.

Hypoxia increased expression of HIF-1 and MGr1-Ag/37LRP protein *in vitro and* in vivo

Western Blot analysis of lysates derived from the gastric cancer cells SGC7901 cultured for 1/2 to 16 hr under hypoxia, HIF-1 α represented an increased expression up to 1/2 hr under hypoxia. There were no major changes in the level of MGr1-Ag/37LRP protein expression in 1 hr. However, hypoxia induced an increased expression of MGr1-Ag/37LRP exposed to hypoxia for 2 to 16 hr (Fig. 2a). When the gastric cancer cells MGC803, a gastric cancer cell low expressed MGr1-Ag/37LRP, subjected to hypoxia (range from 1 to 16 hr) also revealed a time-dependent increase in expression of MGr1-Ag/37LRP protein (Fig. 2b). To determine the generality of this induction by hypoxia, we exploited further gastric cancer cell lines (Fig. 2c). The results indicated that AGS, MKN45 and MKN28 cells exposed to hypoxia for 8 hr induced an increase expression of MGr1-Ag/37LRP protein. Similarly, in gas-
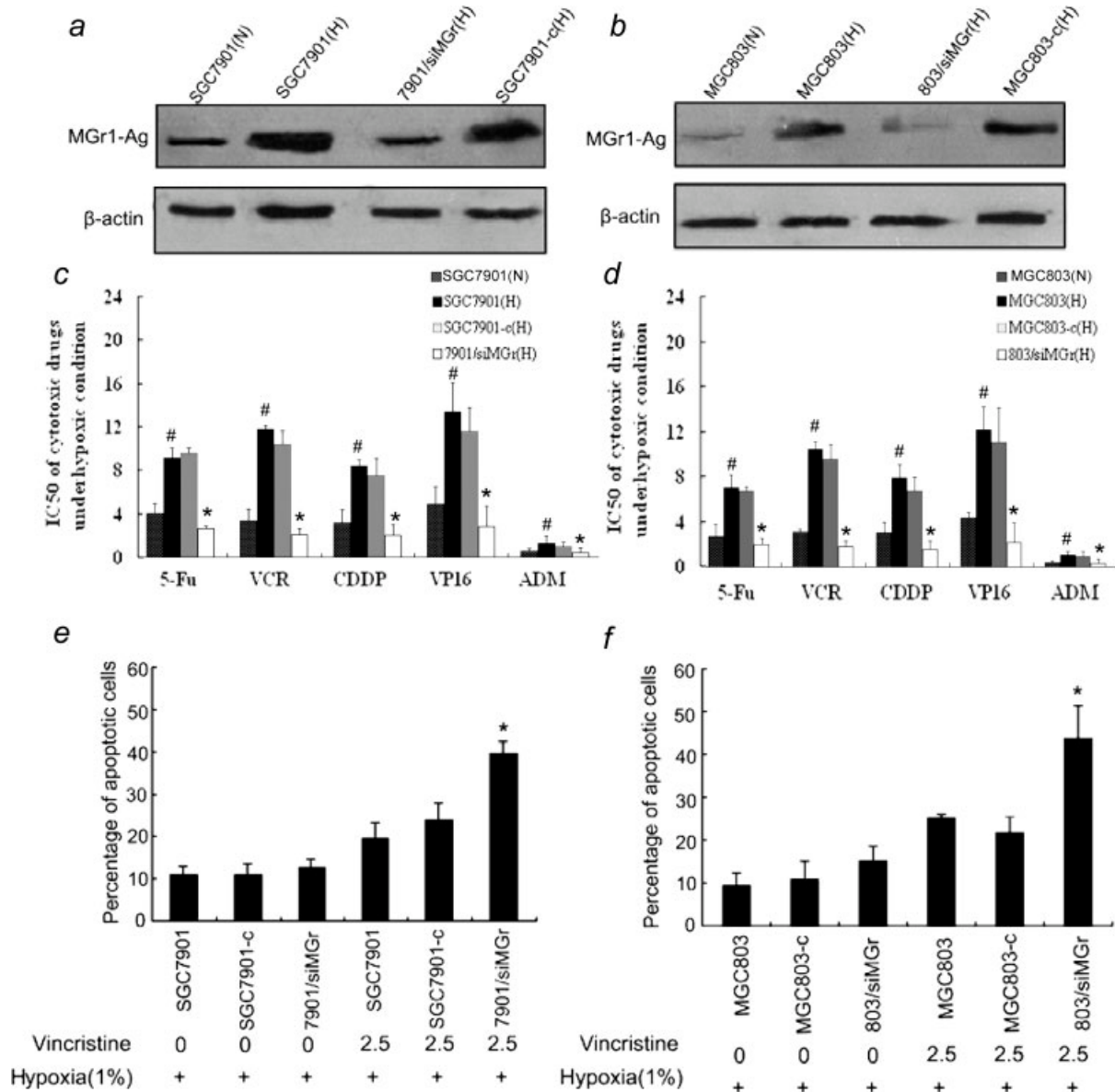
FIGURE 1 – Impact of down-regulated MGr1-Ag/37LRP on response to different drugs under hypoxia. The concentration of each drug that caused a 50% reduction in the number of colonies (IC50) was calculated. 5-FU, 5-Fluorouracil; VCR, vincristine; CDDP, cisplatin; VP16, etoposide; ADM, adriamycin.(*a* and *b*) Vector-based siRNA reduces expression of MGr1-Ag in SGC7901 or MGC803 cells under hypoxic condition. Analysis of MGr1-Ag protein expression levels in cells subjected to hypoxia (1% O2). (*c* and *d*) Mean values of IC50 ($\pm$SD) of cytotoxic drugs in gastric cancer cell lines SGC7901and MGC803, transfectants of SGC7901-c, MGC803-c and 7901/siMGr, MGC803/siMGr cells under hypoxic and normoxic condition.# *vs.* IC50 of SGC7901 and MGC803 cells in normoxia $p < 0.05$; * *vs.* IC50 of SGC7901-c and MGC803-c cells in hypoxia, $p < 0.05$. (*e* and *f*) The percentage of apoptotic SGC7901and MGC803, SGC7901-c and MGC803-c or 7901/siMGr and 803/siMGrcells in hypoxia pretreated with or without Vincristine (2.5 μM). *$p < 0.05$: 7901/si-HIF *vs.* SGC7901-c; 803/siMGr *vs.* MGC803-c. Data are means from three separate experiments. N means Normoxia and H means Hypoxia.

tric mucosal tissue taken from mice exposed to whole animal hypoxia (0 to 24 hr), both HIF-1α and MGr1-Ag/37LRP levels increased (Fig. 2*d*). This data revealed that hypoxia could upregulated MGr1-Ag protein expression and HIF-1 may be involved in it.

Hypoxia increased the levels of MGr1-Ag/37LRP mRNA

RT-PCR was used to investigate the mechanism by which the MGr1-Ag/37LRP protein was up-regulated by hypoxia. Figs 2*e* and *f* showed that in SGC7901 and MGC803, hypoxia induced an increased levels of MGr1-Ag/37LRP mRNA in a time-dependent manner compare to that for normoxia (Mean $\pm$ SE, $n = 3$). These

data provoked an investigation into the possibility of hypoxia-mediated transcriptional activation.

HIF-1-mediated hypoxia-induced MGr1-Ag/37LRP expression and transcriptional activation

In order to explore the relationship between HIF-1 function and the hypoxia-mediated up-regulation of MGr1-Ag/37LRP, SGC7901 and MGC803 were transiently transfected with either a vector containing a scrambled siRNA or a vector containing a HIF-1α targeting sequence 48 hr before incubation under normoxic or hypoxic conditions for 8 hr Figs. 3*a* and 3*b* showed a substantial reduction expression of HIF-1α protein in hypoxic cul-
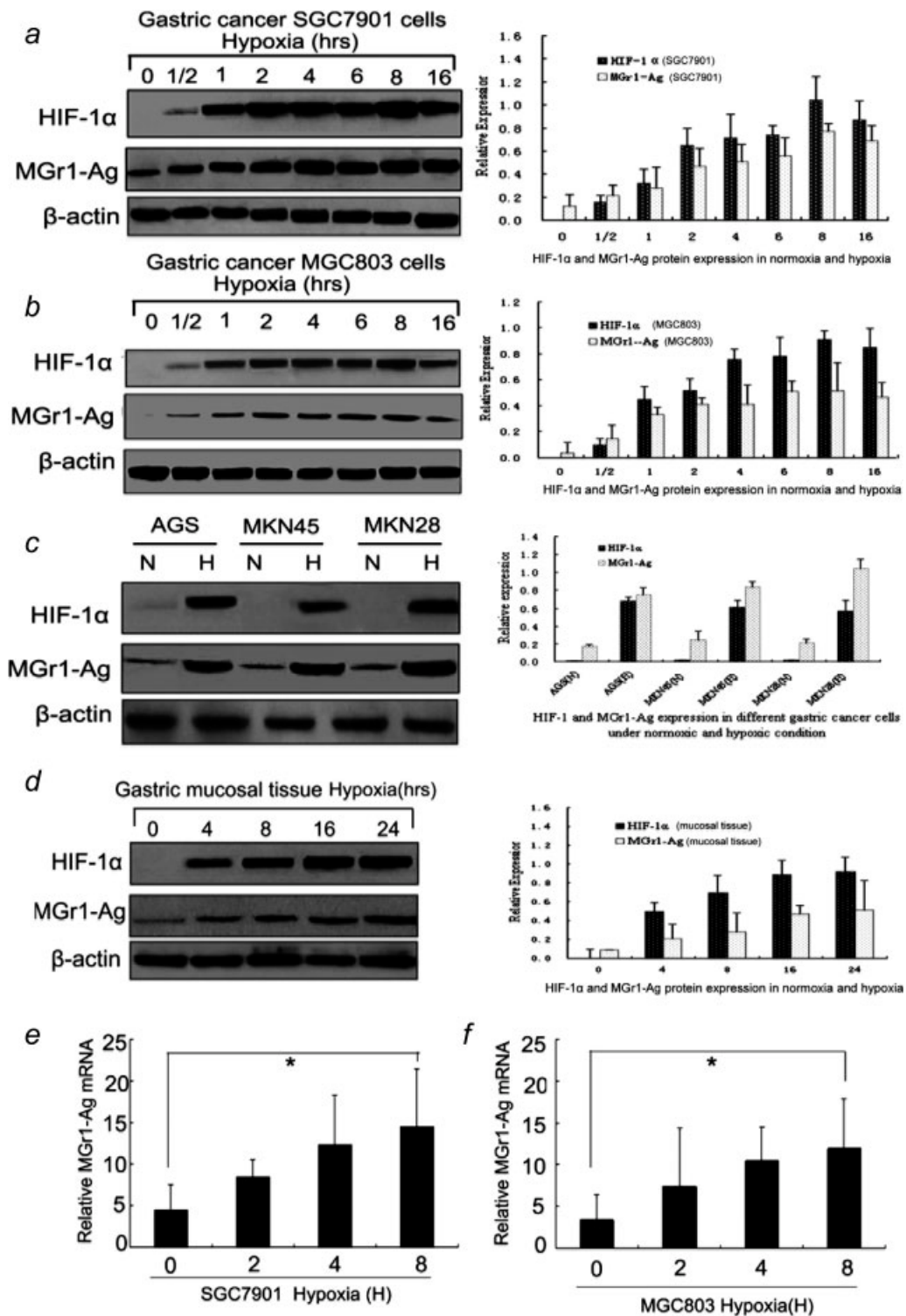
FIGURE 2 – Effect of hypoxia on MGr1-Ag/37LRP protein expression and mRNA levels. *a* and *b*. Gastric cancer SGC7901 or MGC803 cells were incubated in hypoxia at indicated times; *c*. Gastric cancer AGS, MKN45 or MKN28 cells were incubated in hypoxia or normoxia for 8 hr. *d*. Mice were exposed to whole animal hypoxia (8%) for 0 to 24 hr. Gastric mucosal tissue was removed, homogenized, and tissue lysates were prepared. Lysates were immunoblotted for HIF-1α, MGr1-Ag/37LRP or β-actin proteins. Twenty-five microgram of protein per sample was loaded, and equal loading was confirmed by using actin as a control. The graphs on the right side show the grey value of the HIF-1 and MGr1-Ag proteins for Fig. *a*, *b*, *c*, and *d*, respectively. (*e* and *f*) To study on effect of hypoxia on MGr1-Ag/37LRP mRNA levels, Gastric cancer SGC7901, MGC803 cells were exposed to the indicated periods of ambient hypoxia (1% O2). RT-PCR was used for analysis of MGr1-Ag/37LRP mRNA levels in cells. The results shown are representative of 3 independent experiments. N means Normoxia and H means Hypoxia.
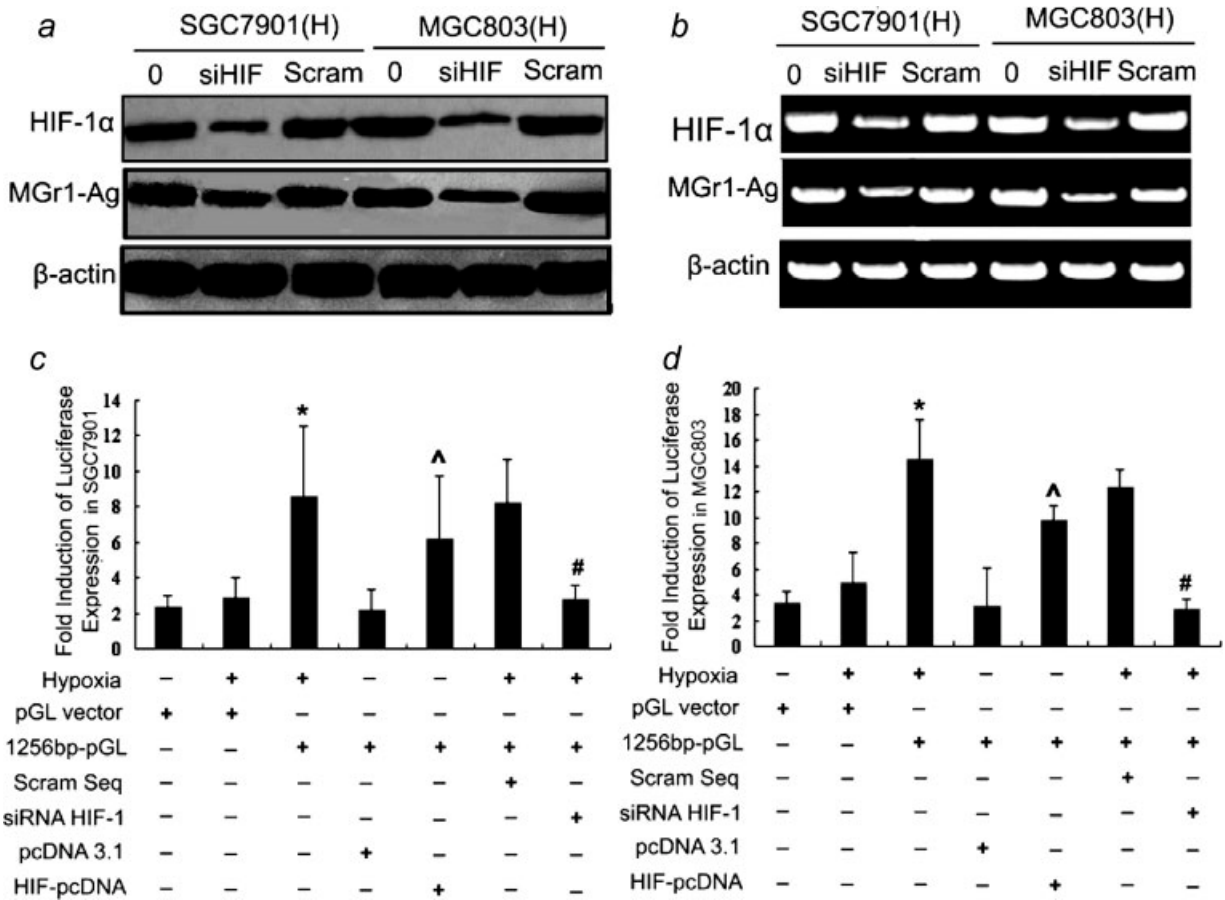
FIGURE 3 – HIF-1-dependent up-regulation of MGr1-Ag/37LRP by hypoxia. SGC7901 and MGC803 were transiently transfected with either a vector containing a HIF-1α scrambled or a vector containing a HIF-1α targeting sequence 48 hr before incubation under normoxic or hypoxic conditions for 8 hr. *a*. Lysates were then prepared and immunoblotted for HIF-1α and MGr1-Ag/37LRP. *b*. Semiquantitative RT-PCR was used for analysis of HIF-1α and MGr1-Ag/37LRP mRNA levels in SGC7901 and MGC803 cells, respectively. *c* and *d*. SGC7901 and MGC803 cells were respectively transfected with plasmids expressing sequence corresponding to pGL3-1256-Luc in the presence or absence of HIF-1 siRNA or HIF-1 cDNA expressed plasmid. Forty-eight hours later cells were exposed to hypoxia or normoxia for 24 hr and assessed for luciferase activity. Results shown represent the Mean ± SE of 3 independent experiments performed in triplicate (*$p < 0.05$ hypoxia compared with normoxic control; ^$p < 0.05$ cotransfected HIF-1 plasmid compared with pcDNA plasmid; #$p < 0.05$ cotransfected HIF siRNA compared with hypoxic indicibility). N means Normoxia and H means Hypoxia.
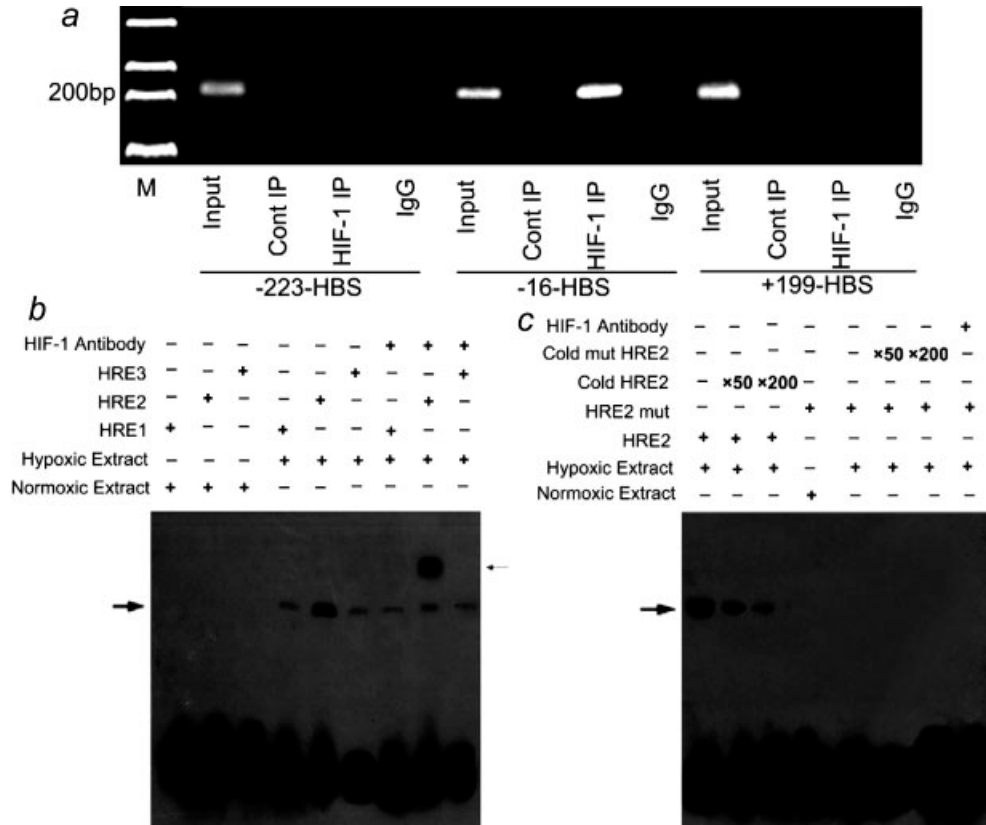
tured SGC7901 and MGC803 cells transfected HIF-1α siRNA, which correlated with the inhibition of MGr1-Ag/37LRP protein expression and mRNA level. The data demonstrate that the up-regulation of MGr1-Ag/37LRP is a HIF-1depenent event in several types.

In an attempt to gain specific insight into the mechanisms of MGr1-Ag/37LRP induction, the luciferase reporter construct expressing 2564bp(pGL3-2564-Luc; the promoter fragments nucleotides −1600 to +964 containing 5 possibilities HREs) and the luciferase reporter construct expressing 1256bp (pGL3-1256-Luc; nucleotides −292 to +964 containing 3 possibilities HREs) were used to address hypoxia inducibility, and specifically, the role of HIF-1. As shown in Figure 3*c*, SGC7901cells transfected with pGL3-1256-Luc promoter showed a 3.53 ± 0.57-fold increase in luciferase activity over normoxia controls when compared with cells subjected to 24 hr hypoxia ($p < 0.05$). Likewise MGC803 cells transfected with pGL3-1256-Luc showed a 4.25 ± 0.76-fold increase in luciferase activity over normoxia controls when compared with cells subjected to 24 hr hypoxia ($p < 0.05$ Fig. 3*d*).Cells transfected with pGL3-2564-Luc showed an equal increase luciferase activities comparing to that of pGL3-1256-Luc construct (p = not significant, data not shown).

We next performed co-transfection assays by using the reporter pGL3-1256-Luc with HIF-1. HIF-1 overexpression transactivated the MGr1-Ag/37LRP promoter by more than 3-fold over the MGr1-Ag/37LRP promoter activity in normoxia ($p < 0.05$). However, transient transfected vector containing a HIF-1α targeting sequence, which block HIF-1 mRNA expression, resulted in a nearly complete loss of hypoxia inducibility ($p < 0.05$ compare with the hypoxic inducibility, Fig. 3*c* and 3*d*). These findings suggested a role of HIF-1α in hypoxia-induced expression of MGr1-Ag/37LRP.

Confirmation of HIF-1 binding sites in MGr1-Ag/37LRP promoter

Results of luciferase reporter assay showed that an increased luciferase activity transfected with *MGr1-Ag/37LRP* promoter (nucleotides −292 to +964). So, the 3 possibilities of HIF-1α binding site is located −292 to +964 from translation start site of the human *MGr1-Ag/37LRP* gene. The first possible site contains the HIF-1 core sequence 5′-GCGTG-3′ between nucleotides −249 to −244 and the HIF-1 ancillary sequence 5′-CAGAC-3′. The second possible site contains the HIF-1 core sequence 5′-ACGTG-3′ between nucleotides −16 to −11 bp and the HIF-1 ancillary sequence 5′-

a

M | Input | Cont IP | HIF-1 IP | IgG | Input | Cont IP | HIF-1 IP | IgG | Input | Cont IP | HIF-1 IP | IgG
200bp

-223-HBS   -16-HBS   +199-HBS

b

HIF-1 Antibody	−	−	−	−	−	−	+	+	+
HRE3	−	−	+	−	−	+	−	−	+
HRE2	−	+	−	−	+	−	−	+	−
HRE1	+	−	−	+	−	−	+	−	−
Hypoxic Extract	−	−	−	+	+	+	+	+	+
Normoxic Extract	+	+	+	−	−	−	−	−	−

c

HIF-1 Antibody	−	−	−	−	−	−	−	+
Cold mut HRE2	−	−	−	−	−	×50	×200	−
Cold HRE2	−	×50	×200	−	−	−	−	−
HRE2 mut	−	−	−	−	+	+	+	+
HRE2	+	+	+	−	−	−	−	−
Hypoxic Extract	+	+	+	−	+	+	+	+
Normoxic Extract	−	−	−	+	−	−	−	−

FIGURE 4 – Identification of HRE in the *MGr1-Ag/37LRP* promoter. *a*. Chromatin immuno-precipitation was used to examine HIF-1α binding to the MGr1-Ag/37LRP promoter in normoxic and hypoxic SGC7901 cells. Reaction controls included immunoprecipitations performed by using a nonspecific IgG monoclonal antibody (Cntl IP) and PCR performed by using whole cell genomic DNA (Input). *b* and *c* Biotin-labeled HRE1, HRE2 and HRE3 Oligonucleotide was used as a probe. For the competition assays, a 50-fold or 200-fold molar excess of the HRE was used. For the supershift assay, anti-HIF-1α antibody (1 μg) was added to the binding reaction. The thick arrow indicates HIF-1/DNA complexes, and the thin arrow indicates the supershift. All results are representative of at least 3 independent repeat experiments.

CAGAC-3′.The third possible site contains the HIF-1 core sequence 5′-CACGTG-3′ between nucleotides +199 to +204 bp.

To assess the contribution of the 3 proximal putative HREs of the *MGr1-Ag/37LRP* promoter, the chromatin immunoprecipitation (ChIP) was performed to analyze in gastric cancer cell subjected to condition of hypoxia. As shown in Fig. 4*a*, ChIP analysis of nuclei derived from SGC7901 cells revealed a dominant band of 216 bp containing the second possible binding (−16 to −11) site in hypoxic condition. No bands were evident in other 2 possible binding sited and control IgG immunoprecipitates. These results suggested that the proximal HRE at -16 is the main HIF-1α binding site in the *MGr1-Ag/37LRP* promoter.

This conclusion was confirmed by electrophoretic mobility shift assay (EMSA). Three oligonnucleotides corresponding to nt −232 to −210(HRE1), −24 to −2(HRE2), +191 to +213 (HRE3) of the *MGr1-Ag/37LRP* promoter were incubated with nuclear extract derived from SGC7901 exposed to hypoxia for 8 hr. The HRE2 bound strongly to the HIF-1 complex (Fig. 4*b*; lane 5) and also was supershifted by using anti-HIF-1 antibody (Fig. 4*b* lane 8) whereas the HRE1 and HRE3 construct no specific binding to the recombinant HIF-1 complex(Fig. 4*b*; lane 4 and 6). The binding between HIF-1α and the MGr1-Ag/37LRP HRE2 was prevented by 50-fold and 200-fold excess unlabeled oligonucleotides (Fig. 4*c*; lane 2 and 3), indicating the specificity of the interaction. In the absence of HIF-1α in normoxic cell extracts, there is no complex formation on the MGr1-Ag/37LRP HRE (Fig. 4*b*; lane 1–3).

To further substantiate the binding of the HIF-1 MGr1-Ag/37LRP HRE2 complex, the MGr1-Ag/37LRP HRE consensus site was mutated(3 point mutation) and no longer supported complex

formation with HIF-1α (Fig. 4*c*; lane 4–8). Taken together, these findings indicated that the *MGr1-Ag/37LRP* promoter is directly activated by HIF-1 and the HRE at -16bp is essential for the transcriptional activation of the MGr1-Ag/37LRP promoter.

Discussion

The presence of hypoxic cells in solid tumors has long been recognized and there is a large body of evidence indicating that hypoxic cells are relatively resistant to conventional chemotherapy.[28–31] Consistent with these reports, our previous study showed that 5-Fu, VCR, CDDP, VP16 and ADM, 5 common anticancer drugs exhibited less effective in hypoxia or in gastric cancer cells SGC7901 exogenous expressed of HIF-1α. Knockdown of HIF-1α expression strikingly elevated sensitivity to various drugs under hypoxic conditions, which suggested that HIF-1α might be a factor involved in hypoxia-induced MDR phenotype in gastric cancer. It was widely known that HIF-1 is a key transcriptional factor in hypoxia which might tranactivated *MDR1* gene involving in MDR. Our previously study revealed that HIF-1 could unregulated MDR and MRP in *vitro* and *in vivo* and subsequently contributed to MDR in gastric cancer. However, blocking the both protein could not absolutely reverse hypoxia-induced MDR. Consistented with study, the previous study by our lab suggested that there is only 10 and 12% expressed rate of p-gp and MRP respectively in gastric cancer. It suggested other downstream molecular regulated by HIF-1 contributed to hypoxia-induced MDR..

A series of studies related to MDR in gastric cancer were conducted by our lab, MGr1-Ag/37LRP is up-regulated protein in

drug-resistant cell SGC7901/VCR which was derived from the human gastric adenocarcinoma cell line SGC7901 by stepwise selection *in vitro*, using vincristine as inducing reagent.[32] Immunohistochemistry staining showed that MGr1-Ag is overexression in gastric cancer section and the positive rate of MGr1-Ag is 18% in gastric cancer.[13] Further study revealed that MGr1-Ag/37LRP overexpression increased resistance to gastric cancer cells, while inhibition of MGr1-Ag expression could reverse the MDR phenotype in gastric cancer drug-resistant cell lines. Mechanistic study represented that it may promote MDR of gastric cancer cells *via* a decrease in intracellular drug accumulation and inhibition of drug-induced apoptosis.

MGr1-Ag, also termed 37LRP or p40, appears to be involved in 2 seemingly unrelated activities-cell adhesion and ribosomal biogenesis. Apart from their role of ribosome-associated protein,[33] numerous studies have linked the overexpression of the MGr1-Ag/ 37LRP to tumor growth and proliferation as precursors for laminin receptors.[34] Recently, researchers follow interest with the effect of uniform microenvironment in solid tumor on the drug resistance, which is largely different in monolayer tumor cell *in vitro* and in real global tumor *in vivo*.[35] Cells in solid tumors are exposed to various microenvironments and modulation of the composition and structure of the extracellular matrix (ECM) can slow down the movement of molecules within the tumor. These characteristics of the tumor microenvironment limit the delivery of anticancer drugs to cells. Cancer cell adhesion to ECM, which also influences the execution of the apoptotic program through the actions of adhesion receptors, confers a novel acquired chemotherapeutic drug-resistant phenotype referred to as cell adhesion-mediated drug resistance (CA-MDR).[36,37]

Our previous study firstly reported that MGr1-Ag/37LRP promoted CA-MDR of gastric cancer cells *via* enhancing cell-adhesion and then inhibited drug-induced apoptosis[38] To further explore whether hypoxia-induced MDR in gastric cancer cells was mediated by up-regulation of MGr1-Ag/37LRP expression, initial studies confirmed that hypoxia could increased MGr1-Ag/37LRP expression, while blockage MGr1-Ag with siRNA might inhibit hypoxia-upregulated it. Functional analysis revealed that blocking MGr1-Ag/37LRP expression by siRNA in gastric cancer cells could abolish hypoxia-induced drug resistance toward 5-FU, VCR CDDP, VP16, ADM and hypoxia-protected drug-induced apoptosis. It suggested that hypoxia prompt MDR in gastric cancer and MGr1-Ag might contribute to it.

The subsequent result revealed that hypoxia induced MGr-1Ag/ 37LRP upregulated *via* HIF-1-dependnt.

Given the temporal and robust hypoxia response observed in the induction of MGr1-Ag/37LRP, a candidate regulator was HIF-1 for the reason of its contribution to MDR in gastric cancer cells. A search of the cloned gene promoter revealed 5 HIF-1 possible binding sites at *MGr1-Ag/37LRP* gene promoter. The dual luciferase reporter gene assay revealed that hypoxia could induce MGr1-Ag promoter activity and also the induction could blockage by HIF-1 siRNA, which suggested it a HIF-1-dependent mannder. Results from the combination of ChIP and EMSA narrowed the sequence −16 to −11 as a classic HRE, and mutation of this HIF-1 site resulted in a completed blockade of HIF-1 binding activity. So the results make me presumed that HIF-1 transactiviated MGr1-Ag/37LRP expression and subsequently its function of gastiric MDR phenotype. However, the exact mechanism of MGr1-Ag involving in hypoxia-induced MDR of gastric cancer remains not fully understood.

In summary, these data presented here *MGr1-Ag/37LRP* as a novel HIF-1 target gene provide an explanation for MDR of hypoxic gastric cancer cells. The HIF-1-dependent induction of MGr1-Ag/37LRP in hypoxic tumor cells offers a new insight to antitumor strategies target to treatment of solid tumor.

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