Krüppel-like factor 8 contributes to hypoxia-induced MDR in gastric cancer cells

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Hypoxia can occur during acute and chronic vascular disease, pulmonary disease, and cancer. Severe or prolonged hypoxia will lead to cell death.(1–3) Tumors become hypoxic because new blood vessels they develop are aberrant and have poor blood flow.(4–6) Although hypoxia is toxic to cancer or normal cells, cancer cells undergo genetic and adaptive changes that allow them to survive and even proliferate in hypoxic conditions.(7,8) These processes contribute to malignant phenotype and aggressive tumor behaviors.(9,10) In solid tumors, hypoxia can promote malignant progression and confer resistance to chemotherapy by altering gene expression.(11–15)

A key mediator of cellular responses to low oxygen is hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits.(16) In normoxia, HIF-1α is hydroxylated by prostyl hydroxylase 2 and ubiquitinated by von Hippel-Lindau protein (VHL), thereby being targeted by proteosomes for degradation.(17–19) In hypoxia, this process is suppressed, resulting in HIF-1α accumulation. Nuclear entry and retention of HIF-1α allow its heterodimerization with the constitutive nuclear HIF-1β subunit. Subsequently, this complex activates numerous genes by binding to hypoxia-responsive elements in their promoters.(20,21) Expression of HIF-1α correlates with poor prognosis and inefficient response to treatment in many cancers.(11) A tumor type that may be particularly susceptible to HIF inhibition is clear cell renal carcinoma, which commonly lacks VHL activity. Loss of VHL function is associated with stabilization of HIF-1α subunits and strong constitutive upregulation of the HIF pathway, similar to hypoxia.(22,23) We previously reported that hypoxia could induce MDR in gastric cancer (GC) cells through HIF-1 dependence.(11)

Krüppel-like factor 8 (KLF8) belongs to the Krüppel-like C2H2 zinc-finger transcription factor family. Several KLF family members, including KLF4, KLF5, and KLF6, have been identified as either onecogenes or tumor suppressors.(24,25) KLF8 is a relatively new member of this family and is a sequence-specific DNA-binding protein that recognizes CACCC-box element and repress active expression.(26) Later studies found KLF8 as a dual transcriptional factor that can either repress or activate transcription of target genes including cyclin D1, KLF4, and E-cadherin.(27,28) Fu et al.(27,29,30) and other researchers have further shown that KLF8 is overexpressed in several types of human cancers, including ovarian and breast cancer, and that it participated in oncogenic transformation and cancer cell invasion. In addition, Fu et al.(29) also found that KLF8 is overexpressed in clear cell renal carcinoma, and
suggested that KLF8 overexpression is associated with HIF-1 stabilization. Our results found KLF8 was highly expressed in GC cells, which is associated with prognosis.\(^{(3)}\) However, whether and how KLF8 might be involved in hypoxia-induced MDR in GC is not fully understood.

In this study, we show that KLF8 contributes to hypoxia-induced MDR in GC cells and the mechanism of KLF8 in this role is elucidated.

Materials and Methods

**Cell culture.** The following human GC cells were used: MKN45 and MKN28 were obtained from Shanghai Cell Bank (Shanghai, China); SGC7901 was from the Academy of Military Medical Science (Beijing, China); and MDR cell variants SGC7901/vincristine (VCR) and SGC7901/adriamycin (ADR) were prepared and characterized in our laboratory. All cell lines were cultured in RPMI-1640 containing 10% FBS. For hypoxic exposure, cells were incubated in a hypoxic incubator with 1% O\(_2\) balanced by CO\(_2\) and nitrogen.

**Hypoxia-inducible factor-1α (HIF-1α) reporter assay.** Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and pSilencer neo U6 2.1 vector, containing a HIF-1α-specific targeting sequence (5′-AAAGAGGTTGATGTCGGAAGCTGGG-3′ and 5′-CTGGTGATATACTGAGACACCC-3′) and an empty sequence. The primers were designed to observe changes in the HIF-1α target gene induced by HIF-1α in hypoxia. HIF-1α siRNA vector and empty vector were transfected into MKN45, MKN28, and SGC7901 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cells were treated with 0.4 mg/mL G418 to select the stably transfected cells.

**Dual luciferase reporter assay.** Genomic sequence of MDR1 was determined using the National Center for Biotechnology Information’s Genomic BLAST program: there are two KLF8 binding sites (CACCC box) in the MDR1 promoter. Promoter sequences of MDR1 carrying wild-type or mutated KLF8 binding sequence were cloned into PGL3-Basic vector (Sangon Biotech, Shanghai, China). Specifically, the two binding sites located on the MDR1 promoter sequence were separately or both mutated, and PRL-TK vector served as the control. Six hours post-transfection, cells were exposed to hypoxia or normoxia for 24 h. The luciferase activity was measured and quantified using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation was adopted to analyze whether KLF8 directly binds to the MDR1 promoter sequence in GC cells. SGC7901/KLF8 cells were fixed with 1% paraformaldehyde, chromatin derived from nuclei was sheared using a microtip cell sonicator. After centrifugation, supernatants containing sheared chromatin were incubated with anti-KLF8 or control IgG overnight. Then, DNA precipitation was adopted to analyze whether KLF8 directly binds to the MDR1 promoter sequence in GC cells. SGC7901 cells were transfected into MKN45, MKN28, and SGC7901 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cells were treated with 0.4 mg/mL G418 to select the stably transfected cells.

**Statistical analysis.** Results were presented as mean ± SD. The t-test was carried out to determine the significance of the difference between the covariates. P-values < 0.05 were defined as statistically significant. SigmaStat software (Systat Software Inc, San Jose, CA, USA) was used for statistical analyses.

A detailed description of the materials and methods used in this study can be found in Document S1.

**Results**

**Hypoxia increases KLF8 and HIF-1α expression.** The mRNA and protein levels of KLF8 were detected by RT-PCR and Western blot assays; HIF-1α protein levels were also detected by Western blot assay. Expression of KLF8 increased when MKN45 cells were exposed to hypoxic conditions, but there were no differences in hypoxia induction at 4 h or 24 h (Fig. 1a,b). In MKN28 cells, KLF8 levels increased under hypoxia for 4 h, but decreased after 24 h (Fig. 1c,d). In SGC7901 cells, however, KLF8 levels increased after 4 h of hypoxia induction, and reached a maximum at 24 h (Fig. 1e,f). In these three GC cell lines, we found hypoxia could also increase HIF-1α levels; hypoxia induced the expressions of HIF-1α and KLF8 in a time-dependent manner. These results indicated that hypoxia could upregulate KLF8 and HIF-1α expressions, and KLF8 might be involved in oxygen-regulated gene expression.

**Expression of KLF8 is induced by HIF-1α.** Western blot assay showed that when MKN45, MKN28, and SGC7901 cells were exposed to hypoxia for 4 h, KLF8 and HIF-1α protein levels were upregulated. However, HIF-1α protein levels were decreased by HIF-1α siRNA vector transfected into GC cells, which led to a downregulation of KLF8 expression in hypoxia (Fig. 1g–i). These results showed that HIF-1α could regulate KLF8 expression under hypoxic conditions.

**Expression of KLF8 is increased in drug-resistant human GC cell lines SGC7901/VCR and SGC7901/ADR.** We found mRNA and protein levels of KLF8 in SGC7901/VCR and SGC7901/ADR cells were higher than in SGC7901 cells, and the trend was more obvious in SGC7901/VCR (Fig. 2a,b). These data hinted that KLF8 and MDR in GC cells were closely related.

**Establishment of forced and siRNA expression of KLF8 stable transfection.** To study the functional role of KLF8 in MDR in GC cells under hypoxia, KLF8 overexpression or siRNA vector upregulation or downregulation of KLF8 were confirmed by RT-PCR and Western blot analysis. As shown in Figure 2(c,d), treatment of SGC7901 cells with KLF8 overexpressing vector significantly induced KLF8 expression (lane 2). SGC7901/VCR cells with KLF8 siRNA vectors reduced KLF8 expression (lanes 5 and 6), whereas KLF8 expression was not significantly suppressed by empty vector. Treatment of SGC7901 cells with KLF8 siRNA vectors could inhibit hypoxia-induced KLF8 expression (lanes 9 and 10). In Figure 2(e,f), treatment of SGC7901/ADR cells with KLF8 siRNA vectors could also reduce KLF8 expression.

**Kruppel-like factor 8 (KLF8) increases hypoxia-induced MDR in GC cells.** Forced expression of KLF8 increased its IC\(_{50}\) value towards that of cisplatin, 5-fluorouracil (5-FU), VCR, and ADR in SGC7901-KLF8 cells (Fig. 3a). In contrast, treatment of SGC7901, SGC7901/VCR, and SGC7901/ADR cells with KLF8 siRNA vectors could abolish it in normoxia and hypoxia (Fig. 3b–f). The mean values of IC\(_{50}\) in SGC7901-NC, SGC7901/VCR-NC, or SGC7901/ADR-NC cells remained unchanged.

What is more, we examined the apoptotic index in GC cells using annexin V/propidium iodide staining assay. As Figure 4(a) shows, SGC7901 cells treated with VCR growing under normoxic conditions reached an apoptosis level of 46%. Interestingly, in SGC7901-KLF8 cells and SGC7901 cells exposed to hypoxia, the induction of apoptosis was significantly reduced to 12% and 20%, respectively. SGC7901 cells treated with KLF8 siRNA vectors abolished apoptosis in hypoxia (Fig. 4b).

Finally, the ADR releasing rate in GC cells was detected by an ADR accumulation and retention assay. SGC7901 and SGC7901-NC cells subjected to hypoxia and SGC7901-KLF8...
cells all showed a significantly increased ADR releasing index. However, SGC7901-siKLF8 cells showed opposite results in hypoxia (Fig. 4c,d).

These data suggested that KLF8 might participate in the MDR phenotype in GC cells, but knockdown of KLF8 could reverse hypoxia-induced MDR.

Kruppel-like factor 8 (KLF8) induces anti-apoptotic BCL-2 and inhibits pro-apoptotic Bax and caspase-3. To further elucidate the mechanisms of hypoxia-induced MDR in GC cells and the effect of KLF8 in this event, apoptosis-related proteins (Bcl-2, Bax, and caspase-3) were detected in GC cells by Western blot assay. As shown in Figure 5(a), SGC7901 cells exposed to hypoxia or SGC7901-KLF8 cells increased Bcl-2 and decreased Bax and caspase-3 expressions. In contrast, SGC7901 cells treated with KLF8 siRNA vectors could reverse it in hypoxia. These data showed that KLF8 may be involved in hypoxia-induced MDR in GC cells by inhibiting apoptosis.

Kruppel-like factor 8 induced MDR1 and P-gp expression. MDR1 played major roles in MDR acquisition in various types of cancer cells, and cisplatin, 5-FU, VCR, and ADR were its substrates. P-gp is the gene encoding product of MDR1. Therefore, we examined the possibility that the protein was upregulated by KLF8. Both RT-PCR and Western blot analysis revealed that SGC7901 cells cultured in hypoxia and SGC7901-KLF8 cells upregulated mRNA and protein expression of MDR1, whereas SGC7901-siHIF-1α(1) and SGC7901-siHIF-1α(2) cells repressed hypoxia-induced MDR1 (Fig. 5b,c). These results suggested that KLF8 increased the P-gp level, contributing to hypoxia-induced MDR in GC cells.

Kruppel-like factor 8 activates transcription of MDR1. We have proved that KLF8 could increase the mRNA and protein expression of MDR1; we intended to investigate whether KLF8 could directly regulate MDR1 transcription. Results of luciferase reporter assay showed increased luciferase activity in cells transfected with MDR1 promoter (nucleotides –1000 to –200). The two possible KLF8 binding sites (CACC box) are located at –163 bp to –159 bp, and –174 bp to –170 bp from MDR1 TSS. The relative luciferase activity of MDR1 in

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**Fig. 1.** Krüppel-like factor 8 (KLF8) and hypoxia-inducible factor-1α (HIF-1α) levels were detected in gastric cancer (GC) cells under hypoxic conditions. (a,b) KLF8 expression increased in MKN45 cells exposed to hypoxia for 4 h and 24 h. *P < 0.05. (c,d) In MKN28 cells, KLF8 expression increased after 4 h of hypoxia induction, but decreased after 24 h. *P < 0.05. (e,f) KLF8 expression increased at 4 h after hypoxia induction in SGC7901 cells, reaching a maximum at 24 h. **P < 0.01; *P < 0.05. In these three GC cell lines, hypoxia also increased HIF-1α levels. (g–i) Western blot assay detected HIF-1α and KLF8 expression in MKN45, MKN45-NC, and MKN45-siHIF-1α (g), MKN28, MKN28-NC, and MKN28-siHIF-1α (h), and SGC7901, SGC7901-NC, and SGC7901-siHIF-1α(1) (i) cells exposed to hypoxic conditions at 4 h. In RT-PCR and Western blot assays, GAPDH and β-actin were used as controls, respectively, NC, negative control.
SGC7901-KLF8 cells or SGC7901 and SGC7901-NC cells cultured in hypoxia was higher, and the relative luciferase activity of the other three mutative sequences in these cells was lower. Similarly, the relative luciferase activity of MDR1 in SGC7901-siKLF8(1) and SGC7901-siKLF8(2) cells was lower in hypoxia (Fig. 5d).

To assess the contribution of the two proximal putative KLF8 binding sites of the MDR1 promoter, ChIP assay was used to analyze SGC7901-KLF8 cells. As shown in Figure 5(e,f), ChIP analysis of nuclei derived from SGC7901-KLF8 cells revealed dominant bands containing the two possible binding sites. These results suggested that there were two proximal KLF8 binding sites in the MDR1 promoter.

Taken together, these findings indicated that the MDR1 promoter was directly activated by KLF8 and the two KLF8 binding sites were essential for the transcriptional activation of MDR1 promoter.

Discussion

Gastric cancer is the fourth most common cancer worldwide and the second leading cause of cancer-related death, with the highest incidence reported in Japan, Korea, and China. The only potentially curative treatment for local GC is surgery. However, most cases of GC are diagnosed at an advanced stage where the only possible management of the disease is chemotherapy. The rapid development of new chemotherapeutic agents has done little to improve the prognosis of advanced and recurrent GC. It is evident that new treatment options are urgently needed.

Hypoxia is a common feature of solid tumors and has the potential to promote malignant progression by altering gene expression. These alterations promote the expression of tumorigenic proteins, including cell cycle regulatory proteins, angiogenesis regulatory proteins, metastasis-promoting proteins, metabolic enzymes, and transcription factors. Tumor hypoxia can also act as a trigger of enhanced growth, metastasis, radiation resistance, and chemotherapy resistance.

Hypoxia-induced MDR is a major obstacle in the development of effective cancer therapy. It was reported that some chemotherapeutic agents, such as 5-FU, ADR, bleomycin, etoposide, and VCR are examples of drugs that are dependent on cellular oxygenation for their maximal efficacy.

Hypoxia-induced MDR has been studied with respect to stimulus for some genes associated with drug resistance. However, the key transcriptional factors that modulate drug resistance-associated proteins remain to be identified. In this study, we found KLF8 is a major transcriptional factor activated by hypoxia and could also be inhibited by HIF-1α siRNA in hypoxia. We previously reported KLF8 was overexpressed in GC cells. Further study confirmed it is involved in metastasis and invasion in GC cells. However, whether KLF8 is involved in hypoxia-induced MDR was not clear. Our results showed that overexpression of KLF8 promoted the MDR phenotype in GC cells, and knockdown of KLF8 reversed it in hypoxia.

The mechanisms of MDR in tumor cells mainly include the following aspects: (i) drug-resistant proteins including P-gp, lung resistance protein and breast cancer resistance protein inhibit drugs from entering the cell nucleus, promote drug

Fig. 2. Kruppel-like factor 8 (KLF8) expression was detected in SGC7901, MDR cell variants SGC7901 vincristine (VCR) and SGC7901/adriamycin (ADR), and transfected gastric cancer cells using RT-PCR and Western blot assays. (a,b) KLF8 expression was detected in SGC7901, SGC7901/VCR, and SGC7901/ADR cells. *P < 0.05; ***P < 0.01. (c-f) KLF8 expression was detected in transfected cell cultures under normoxia and hypoxia. Cells were subjected to hypoxia for 24 h, as indicated, *P < 0.01 versus SGC7901 and SGC7901-NC; **P < 0.05 versus SGC7901/VCR and SGC7901/VCR-NC or SGC7901/ADR; ***P < 0.05 versus SGC7901 and SGC7901-NC in hypoxia. GAPDH and β-actin were used as controls. NC, negative control.
release, and finally result in resistance toward chemotherapy drugs\(^3\)–\(^4\); (ii) imbalance in the Bcl-2\(^\text{I}/\text{Bax}\) rate and p53 mutation restrain apoptosis\(^4\); (iii) glutathione makes chemotherapy drugs rapidly inactivated in cancer cells participating in the form of MDR\(^4\); and (iv) tumor tissue growth environments, such as pH value, temperature, and hypoxia, have changed. Experts have found that MDR in cancer cells is not only the result of the changes in cancer cells’ internal structure, but also the result of cancer cells interacting with the external environment. Through the experiments we found KLF8 is not only an MDR-related molecule, but also a hypoxia-regulated molecule. Moreover, the mechanism of KLF8’s involvement in hypoxia-induced MDR in GC cells has been researched.

There are two important apoptotic pathways: the intrinsic pathway, or mitochondria/cytochrome c-mediated apoptosis pathway; and the extrinsic pathway, or death receptor-mediated apoptosis pathway. Apoptosis-related genes (Bcl-2, Bax, and caspase-3) play an important role in apoptosis pathways.\(^4\) Krüppel-like factor 8 could increase Bcl-2 expression and decrease Bax and caspase-3 expressions in GC cells. Knockdown of KLF8 could reverse the protein levels in hypoxia. Bcl-2 and Bax could form a dimer and play a molecular switch role in the apoptosis pathway. In the intrinsic apoptotic pathway, apoptosis is accompanied by the release of cytochrome c (Cyt c) from mitochondria to the microenvironment around mitochondria; Cyt c could combine with apoptosis protease activating factor-1 to activate caspases, which induce apoptosis. Bcl-2 could close the permeability transition pore located in mitochondria and inhibit Cyt c release, result in inhibiting apoptosis. So, we could infer that KLF8 could inhibit apoptosis in GC cells through the intrinsic apoptotic pathway. We also found that hypoxia could inhibit VCR-induced apoptotic index and promote the ADR releasing rate through increased P-gp expression. However, the exact mechanism of hypoxia-induced MDR in GC cells remains poorly understood.\(^1\)

Given the temporal and robust hypoxia response observed in the induction of MDR1, a candidate regulator was KLF8 for the reason of its contribution to MDR in GC cells. Previous research has confirmed that KLF8 directly regulates the

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**Fig. 3.** Sensitivity of gastric cancer cells to chemotherapy drugs was evaluated by MTT assay. The concentration of each drug that caused IC\(_{50}\) was calculated. (a-c) IC\(_{50}\) values of chemotherapy drugs in SGC7901, SGC7901/vincristine (VCR), SGC7901/adriamycin (ADR), and transfected cells in normoxic conditions. \(* P < 0.05\) versus SGC7901 and SGC7901-NC, \(\# P < 0.05\) versus SGC7901/VCR and SGC7901/VCR-NC, \(\& P < 0.05\) versus SGC7901/ADR and SGC7901/ADR-NC. 5-FU, 5-fluorouracil; CDDP, cisplatin; NC, negative control.
transcription of Bcl-2 and Bax by combining to the CACCC box located on their promoter regions. A search of the cloned gene promoter revealed two KLF8 possible binding sites at the MDR1 gene promoter. Three approaches were used to define a role for KLF8 in the induction of MDR1: (i) use of siRNA resulted in a nearly complete blockade of MDR1 induction acquired by hypoxia; (ii) luciferase reporter construct transfections were used to limit the hypoxia-responsive region of the MDR1 promoter; and (iii) ChIP assay was used to identify KLF8 binding sites in the MDR1 promoter. Results from these studies narrowed the sequences −163 bp to −159 bp and −174 bp to −170 bp as classic binding sites, and anti-KLF8 resulted in a completed blockade of the binding activity.

In summary, we are the first to report that KLF8 played an important role in hypoxia-mediated MDR in GC cells by inhibiting apoptosis and decreasing cytotoxic drug activity in vitro. In addition, MDR1 as a novel KLF8 target gene provides an explanation for hypoxia-induced MDR in GC cells. All results may be of interest for developing new sensitizers based on KLF8 in GC treatment.
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Disclosure Statement
The authors have no conflict of interest.

Supporting Information
Additional supporting information may be found in the online version of this article:

Doc S1. Supporting materials and methods.