Characterization of \textit{EPO H131S} as a Key Mutation Site in the Hypoxia-adaptive Evolution of \textit{Gymnocypris Dobula}

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Abstract

Erythropoietin (EPO) is a glycoprotein hormone involved in proerythropoiesis, antioxidation and antiapoptosis. It also contributes to cellular immune function in high-altitude species, such as the schizothoracine fish Gymnocypris dobula (G. dobula). Six mutation sites previously identified in EPO from G. dobula (GD-EPO) were injected into zebrafish embryos and their effects were compared with EPO from the low-altitude schizothoracine Schizothorax prenanti (S. prenanti). The key mutation site in GD-EPO was identified as H131S. Under hypoxic conditions, the levels of superoxide dismutase and malondialdehyde were decreased, whereas that of nitric oxide was increased in zebrafish injected with GD-EPO compared with those injected with S. prenanti-EPO (SP-EPO). The results suggest that EPO in high-altitude schizothoracine species is both antioxidative and antiapoptotic, driven by the H131S mutation site. Thus, this enhanced the ability of this species to adapt to the high-altitude hypoxic environment. These results provide a basis for investigating further the hypoxia adaptation mechanisms of teleosts.

Introduction

As the highest plateau in the world, the average altitude of the Tibet Plateau is about 4300 m with a lower oxygen partial pressure (40%) compared with that at sea level (Beall 2007; Frisancho 2013). Local species exhibit physiological and morphological characteristics that have evolved to cope with this relatively extreme hypoxic environment. G. dobula is a highly specialized schizothoracine fish that mainly inhabits shallow lakes and tributaries at an altitude >4500 m (Xu et al. 2016). A recent study reported numerous physiological adaptations in G. dobula compared with another schizothoracine, S. prenanti, a relatively primitive fish distributed at a lower altitude in the upper reaches of the Yangtze River (Luo et al. 2016). These adaptations include relatively higher numbers of red blood cells and a stronger hypoxic respiratory response (Beall 2012). However, the detailed regulatory mechanism of these changes was unclear.

Oxidative stress is caused by a variety of environmental factors, including ultraviolet stress, pathogen invasion, and hypoxia (Blokhina 2003). To resist external changes, the organism has developed antioxidant and apoptosis systems (Radi et al. 2014; He et al. 2017). The antioxidant system is mainly composed of small molecular antioxidants and antioxidant enzymes. The main antioxidant enzymes are superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and so on. (He et al. 2017; Yang et al. 1999). SOD is usually more sensitive than other antioxidant enzymes (Víctor et al. 2009). Oxygen free radicals produced during oxidative stress enable unsaturated fatty acids to form lipid peroxides (MDA). The MDA level does not only reflect the rate and intensity of lipid peroxidation; it also indirectly reflects the degree of tissue peroxidation injury (Tsikas 2017). At physiological levels, nitric oxide (NO) can reduce ROS-induced damage (Wink et al. 2001). As a reactive radical, it is cytotoxic at high concentrations, in mammalian cellular immunity, damage to mitochondria exacerbates cellular lipid peroxidation damage, but at low concentrations, NO can positively and negatively regulate innate and acquire immune responses (Choudhari et al. 2013).
There are two main pathways of apoptosis, namely the mitochondrial pathway (endogenous activation pathway) and the death receptor pathway (exogenous activation pathway) (Morrill and He 2017). These pathways share a set of enzymes termed cysteine-aspartic proteases (Caspases), which degrade cellular proteins. Initiator Caspases include Caspase-8 and Caspase-9 (Riedl and Salvesen 2007; Timmer and Salvesen 2007; Obeng 2021). Following an initial apoptotic signal, these enzymes target scaffold proteins. The main executioner Caspases are Caspase-3, Caspase-6, and Caspase-7 (Morrill and He 2014; Obeng 2021). Upon cleavage of executioner Caspases, a proteolytic Cascade begins that will lead to the lysis of nearly all parts of the cell. Caspase-3 is one of the most important Caspases (Morrill and He 2017). Moreover, the Bcl-2 family of proteins strongly regulate the intrinsic pathway (Antonsson et al. 1997). Bcl-2 is an integral membrane protein (Chen-Levy et al. 1989; Tsujimoto et al. 1987), but it can also serve as an inhibitor of Cytochrome c (Cytc) release from the mitochondria as it binds to Bax and inhibits its oligomerization (Yang et al. 1997).

EPO is an acidic glycoprotein hormone of 166 amino acids (Cai 1992). It stimulates the hematopoietic function of bone marrow, and increases red blood cell numbers to improve the oxygen-carrying capacity of blood (Davis et al. 1987; Browne 1986). Meanwhile, Blixt et al. demonstrated that apoptosis, oxidative stress injury, and inflammation in neurocytes were also repressed by EPO, improving the ability to repair hypoxia-induced brain injuries (Katakura et al. 2013). Previous research confirmed the antiapoptotic and antioxidative effects of EPO on tissues or organs in mammals in response to hypoxia and ischemia (Blixt et al. 2018; Tramontano et al. 2003; Parvin et al. 2014; Wang et al. 2010; Chau et al. 2011). Moreover, most mammals exhibit increased levels of plasma EPO promote erythropoiesis, thus improving the physiological response to hypoxia (Souvenir et al. 2011).

The study of hypoxic adaptation in high-altitude fish is limited. G. dobula, one of the most important fish in Tibet, is a good biomaterial to study the hypoxic adaption mechanism of high-altitude fish. We previously reported (Xu et al. 2016) six mutation sites (L117I, H131S, T133P, S138P, S139T, and L153I) in GD-EPO compared with SP-EPO. We also demonstrated that GD-EPO is involved in improving cell viability in this species. However, it was unclear which mutation site was most important in the adaptation of G. dobula to high-altitude hypoxic conditions.

In the current study, we determined the effects of the six mutation sites on zebrafish embryos. Our results provide additional insight into the regulatory mechanisms involved in the adaptation of G. dobula to their hypoxic environment.

**Materials And Methods**

**Animal sources**

Specimens of G. dobula were collected from Yadong County, Tibet (46°03.371' N, 89°17.831' E, altitude 4506 m; dissolved oxygen 1.9 ± 0.3 mg/L; 11°C), and S. prenanti were collected from Ya’an, Sichuan (altitude 950 m; dissolved oxygen 9.0 ± 0.5 mg/L; 18°C). Wild-type (WT) zebrafish (AB type), procured
from Haisheng Biology, Ltd (Shanghai, China), were used as the model organism and were fed freely at 28°C. The animal experiments were approved by the Ethics Committee of Shanghai Ocean University, and were performed in accordance with the institutional guide for the care and use of laboratory animals.

**Q-PCR and construction of recombinant plasmids**

The cDNAs of *GD-EPO* and *SP-EPO* were cloned with mRNAs extracted from the pronephros of *G. dobula* (GenBank accession NO: KT188754.1) and *S. prenanti* (GenBank accession NO. KT188758.1), respectively. In addition, total RNA from zebrafish tissues was extracted using a Total RNA Extraction Kit (Promega, Madison, WI, USA), followed by synthesizing to cDNA using a First-Strand cDNA Synthesis Kit; the cDNA was amplified with a SYBR Green FAST Mastermix (Qiagen, Dusseldorf, Germany). The sequences of primers used are presented in Table 1. *GD-EPO* and *SP-EPO* were initially cloned into the empty vector Tol2-bactin -2A-EGFP (Fig. 2b; restriction sites *ECOR-I* and *BamH-I*). Meanwhile, six positive-selection amino acid mutation sites (*L117I*, *H131S*, *T133P*, *S138P*, *S139T*, and *L153I*) from *GD-EPO* were replaced with those from *SP-EPO*. The Q5® Site-Directed Mutagenesis Kit was used for PCR in accordance with the manufacturer’s instructions. The constructed plasmids (*pTOL2-bactin-2A-EGFP, GD-EPO, SP-EPO, L117I, H131S, T133P, S138P, S139T, and L153I*) which were insert 6×His Tag. Endotoxin-free plasmids were extracted to determine the concentration and diluted to 50 ng/µL. These plasmids were then injected into fertilized zebrafish embryos. After 24-48 h, green fluorescent zebrafish were observed and screened under a fluorescence microscope.

**Table 1**

Real-time primers for detecting apoptosis-related gene
| Genes          | Primer sequence (5'-3') | Gene Bank     |
|---------------|-------------------------|---------------|
| ZF-β-actin    | F CACTGAGGCTCCCCTGAATC  | NM.131031.2   |
|               | R GGGTCACACCATCACCAAGAG|               |
| ZF-Bcl-2      | F TCTACCGGGTGTTACGGGAT  | NM.001030253.2|
|               | R CGTTGCGCAGAATTTGGGT   |               |
| ZF-Bax        | F CTGGCAGAGCAGTCGGAGTT  | NM.131562.2   |
|               | R GAGGCGGTTTCACCTCTCAA  |               |
| ZF-Caspase-3  | F CCTGACATCCAGATGCTCG   | NM.131877.3   |
|               | R GAGCTCCAGTTCACTGCCAT  |               |
| ZF-Caspase-8  | F ACCAGGAACGAGGAGGCAGAC | NM_131510.2   |
|               | R AATTGTGCCAGGCGAAGAGTT |               |
| ZF-Caspase-9  | F AAGATGGATGCCCAGTCCGTG | NM_001007404.2|
|               | R TTGTCGAGTCGTGATTGGA   |               |
| ZF-Cytochrome C| F GGAGATCGAGGAAACGGCA  | NM_001002068.1|
|               | R TGGCCACGTGTGTTCAAGTTCA|               |

**Hypoxia stress**

The screened zebrafish embryos were transferred into a hypoxia water tank (oxygen concentration 10%). Subsequently, 20 healthy zebrafish (WT) were labeled and added to the same tank. The behavior and mortality of all zebrafish in the tank were observed and recorded at 2, 4, 8, 12, 16, 24, 36, and 48 h.

**O-dianisidine staining**

The constructed plasmids (50 ng/µL) were injected into fertilized zebrafish embryos at the first or second cell stage. After incubation for 48 h, the hemoglobin in the embryos was fixed with 4% paraformaldehyde for 4-6 h. Subsequently, the residual paraformaldehyde and impurities on the surface of embryos were removed by washing with phosphate buffer (1×PBS) and stained with o-dianisidine [o-dianisidine (0.6 mg/mL), sodium acetate (0.01 M, pH 4.5), 0.65% hydrogen peroxide solution, and 40% ethanol] in the dark for 15-20 min. The dye solution was then discarded and glycerin was added. The stained embryos were observed with glycerol under a light microscope.

**Enzyme activity detection**
The levels of superoxide dismutase (SOD), malondialdehyde (MDA), and nitric oxide (NO) were determined in tissue homogenate of WT zebrafish embryos and zebrafish. Zebrafish tissue was homogenized in physiological saline at a mass:volume ratio of 1:9 ratio to obtain the 10% homogenate. SOD, MDA, and NO levels were determined respectively using the specific kits (Jiancheng Science & Technology, Ltd., Nanjing, China), according to the manufacturer's protocol.

**Western blotting**

The embryos underwent hypoxia treatment for 6.5 hours at 48 hours post-birth. After washing the embryos with phosphate buffer saline (PBS) three times, the protein productions were extracted using RIPA buffer containing protease inhibitors. Proteins were then separated by 12% SDS-PAGE gel and transferred onto a PVDF membrane. Blocking was performed using 5% nonfat dried milk. After blocking, membranes were incubated with primary antibodies against Bcl-2 (DIA. An, China), Bax (DIA. An, China), Caspase-3 (DIA. An, China), 6× His (HUABIO, China), and GAPDH (DIA. An, China) for 2.5 h at room temperature. Then, 1 × PBST was used to wash the membranes three times. Subsequently, at room temperature, a secondary antibody (DIA. An, China) was added and incubated for 1 h. The membranes were then washed three times with 1 × PBST. The membranes were developed by Chemiluminescence reagents (Thermo Fisher Scientific, USA) under a Gel-Pro analyzer (version 4.0, USA). Finally, the western blotting images were analyzed using the ImageJ 1.46 software (National institutes of Health, USA).

**Statistical analysis**

All results were obtained from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 8.0.2. Data were expressed as the mean ± standard deviation. One-way ANOVA was used to evaluate the differences among multiple groups. The survival rate of zebrafish was obtained by Percent Survival analysis. Differences were significant at $P < 0.05$ or $P < 0.01$.

**Results And Discussion**

**GD-EPO increases the expression level of hemoglobin under hypoxia**

*EPO* is a glycoprotein hormone with a principal regulatory role in erythropoiesis (Spivak 1989; Fried 2009). Various studies on red blood cells and hemoglobin in species from the Tibet plateau and plain areas showed that the oxygen transport and utilization abilities of these species are stronger than those living at lower altitude areas, manifesting as large lung volume, high myoglobin concentration, and strong oxygen uptake ability (Yangzong et al. 2013; Li et al. 2018). In the current study, injection of nine overexpressed plasmids in zebrafish embryos increased the hemoglobin level in the acute hypoxic group compared with the normoxia group, suggesting that hypoxia increases the numbers of red blood cells,
leading to increased levels of hemoglobin (Fig. 1). However, the nine overexpressed plasmids in zebrafish embryos of the normoxia group had no obvious effect on hemoglobin level (Fig. 1b). By contrast, in the hypoxia groups, the expression level of hemoglobin in zebrafish embryos injected with \textit{GD-EPO} was significantly elevated compared with those injected with \textit{SP-EPO} (Fig. 1a). This suggests that, under hypoxia stress, \textit{GD-EPO} may increases the level of hemoglobin to carry more oxygen and overcome the effects of hypoxia. In addition, the hemoglobin level was upregulated by overexpression of \textit{L117I, T133P, S138P, S139T, and L153I} compared with control group and overexpression of \textit{SP-EPO}, whereas there were no obvious changes in the overexpressed \textit{H131S} group (Fig. 1a). Thus, the mutant site \textit{H131S} might have a crucial role in enabling \textit{G. dobula} to adapt to a high-altitude hypoxia environment.

\textbf{H131S is a key mutation site in EPO in the hypoxia-adaptive evolution of \textit{G. dobula}}

Six amino acid mutation sites (\textit{L117I, H131S, T133P, S138P, S139T, H131S, and L153I}) were previously identified in \textit{GD-EPO} compared with \textit{SP-EPO} using a human \textit{EPO} model (Fig. 2a). In the current study, H131S was determined to have a key role in the protective mechanism of \textit{GD-EPO} against a hypoxic environment. To further validate this hypothesis, the percentage survival of zebrafish injected with \textit{GD-EPO, SP-EPO, L117I, H131S, T133P, S138P, S139T, H131S, and L153I} was assessed. The 4 h percentage survival of zebrafish in the \textit{GD-EPO, L117I, H131S, T133P, S138P, S139T, H131S, and L153I} groups was 100%, 100%, 80%, 60%, 70%, and 30%, respectively (Fig. 2c). By contrast, numerous zebrafish in the \textit{H131S} group had died 2 h after hypoxia stimulation, with 0% survival at 4 h. This suggests that H131S overexpression in zebrafish renders them more sensitive to oxygen concentration. Previously, we reported that the \textit{H131S} of \textit{GD-EPO} lacks a CK2 phosphorylation site (serine/threonine protein kinase), which regulates the activity or stability of the substrate via its phosphorylation (Xu et al. 2016). CK2 also participates in the development and function of neurons, and in synaptic information transmission, which controls the development and longevity of synaptic connections. Therefore, the CK2 phosphorylation site might be missing at the 131 amino acid site of \textit{G. dobula}, rendering it insensitive to hypoxia. Thus, mutated \textit{H131S} might have enabled \textit{G. dobula} to adapt to its high-altitude hypoxic environment.

\textbf{GD-EPO functions as an antioxidant}

The Tibet Plateau is characterized by low temperatures, hypoxia, and strong radiation (Beall 2007). Dramatic hypoxia has significant negative effects on organisms, including metabolic disorders, increasing concentrations of oxygen-derived free radicals, damage to cell membranes and nucleic acid structures, or even death (Fuhrmann and Brune. 2017; Gonzalez et al. 2019). To adapt to hypoxic environments, organisms have evolved endogenous antioxidant enzyme systems and apoptosis, comprising small-molecule antioxidants and antioxidant enzymes. SOD is a cytoplasmic and mitochondrial-based antioxidant that can convert \(O_2^-\) into \(H_2O_2\) and \(O_2\) (Du et al. 2017). Oxygen-derived free radicals produced during oxidative stress can convert unsaturated fatty acids to MDA (Tsikas 2017).
Generally, the MDA level indirectly reflect the degree of tissue peroxidation injury (Yang et al. 1999). In addition, NO as a reactive free radical functions in nerve transmission, the immune response, and apoptosis (Ghasemi 2019). Humans living on the Tibetan Plateau have a relatively high concentration of NO, which suggest that NO has an important role in this hypoxic environment (Beall 2007). In this study, the levels of SOD, NO, and MDA in injected zebrafish embryos under hypoxia conditions were determined. Levels of SOD and MDA in zebrafish embryos injected with GD-EPO were significantly decreased compared with those of WT zebrafish embryos (P <0.05) or those injected with SP-EPO (P <0.05) (Fig. 3b and d). Similarly, Omrani et al. reported that the levels of SOD and MDA in rat tissues were increased significantly after hypoxia stimulation (Omrani et al. 2017). Manor et al. also confirmed that the levels of ROS and lipid oxidation in mice injected with EPO (5000 U/kg) were decreased (Manor et al. 1986). These results suggest that GD-EPO not only alleviated the oxidative damage caused by hypoxia, thus reducing the production of free radicals, but also attenuated the degree of lipid oxidation. In addition, a high level of NO occurred in zebrafish embryos injected with GD-EPO compared with WT zebrafish embryos or those injected with SP-EPO (Fig. 3c). In Tibetans, the utilization of NO is relatively high compared with plain inhabitants. Thus, the antioxidant effect of EPO might be closely associated with the hypoxic environment of the Tibet Plateau.

**GD-EPO functions as a role of antiapoptosis**

Under conditions of ischemia and hypoxia, the mitochondrial membrane potential is decreased and mitochondrial permeability transition pores (mPTP) open, inducing the expression of apoptosis-related genes and proteins (Chen et al. 2018). Cytc is a water-soluble protein located between the inner and outer mitochondrial membranes (Wan et al. 2019). Mitochondria release Cytc into the cytoplasmic matrix to initiate apoptosis once cells are stimulated by apoptosis signals (Wan et al. 2019). Members of the Bcl-2 protein family can interact to induce or prevent apoptosis. Among them, Bcl-2 inhibits the opening of mPTPs, thereby repressing apoptosis. By contrast, Bax promotes the opening of mPTP to accelerate apoptosis (Zhang et al. 2015). In this study, to investigate the role of GD-EPO in apoptosis, the expression levels of Cytc, Caspase-3, Caspase-8, Caspase-9, and the Bcl-2/Bax ratio were determined. Expression levels of Cytc, Caspase-3, Caspase-8, and Caspase-9 in the GD-EPO groups were reduced compared with those in the SP-EPO group (P <0.01) (Fig. 4a and b), whereas the Bcl-2/Bax ratio was higher in the GD-EPO group, as also shown by western blotting (Fig. 4c). EPO interacts with the p53 signaling pathway (Pham et al. 2019) in leukemia cells or the Akt signaling pathway (Tramontano et al. 2003) in cardiac myocytes to suppress apoptosis. Antiapoptotic treatments, such as EPO, are also suggested to improve outcomes in hypoxic brain injury (Jung et al. 2021). EPO and vitamin D3 (VD3) can be used to prevent or treat renal ischemia-reperfusion (I/R) injury, and these beneficial effects are closely related to anti-inflammatory and anti-apoptosis pathways (Qin et al. 2021). Thus, we hypothesize that EPO is also involved in the regulation of apoptosis in *G. dobula* through these pathways. However, the specific mechanisms involved remain to be fully understood.
Conclusions

In summary, the $H131S$ mutation was identified as a key mutation affecting the cytoprotective function of $G. dobula EPO$ during the process of adaptation to long-term hypoxia. Our results showed that $EPO$ from high-altitude fish could increase the hemoglobin level. Furthermore, the antioxidative and antiapoptotic functions of $G. dobula EPO$ were enhanced during evolution. Although the detailed regulatory mechanism remains unclear, these results provide a research direction to elucidate the mechanisms utilized by schizothoracine fish under long-term hypoxia conditions.

Declarations

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Author contribution
Qin Zhang carried out most of the experiments. Congcong Wang designed the methods and experiments, interpreted the results and finished the discussion. Yang Liu prepared the materials and partly worked on the experiments. Qianghua Xu was responsible for overall supervision, and participated in coordination. All authors have read and approved the final manuscript.

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Data availability
The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval
Experimental protocols involving live animals were approved by the Ethics Committee for the Use of Animal Subjects of Shanghai Ocean University.

Consent for publication
All authors consent to participate in this publication.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures
Figure 1

GD-EPO increases the expression level of hemoglobin under hypoxia. (a) Overexpression of GD-EPO, L117I, T133P, S138P, S139T and L153I significantly enhanced the expression of hemoglobin, and in contrast to overexpression of SP-EPO and H131S based on O-dianisidine staining at 10% oxygen, the blue arrow indicate hemoglobin expression. (b) Overexpression of eight EPO genes were similar with control on expression of hemoglobin under normoxia. The scale is 40 μm.
Figure 2

H131S is a key mutation site in EPO in the hypoxia-adaptive evolution of G. dobula. (a) Eight EPO sequences, including GD-EPO, SP-EPO, L117I, H131S, T133P, S138P, S139T, and L153I, six positive selection mutation sites for GD and SP are marked in red label and highlighted with blue rectangle. (b) The schematic drawing of the recombinant pTOL2-bactin-EPO-2A-EGFP plasmid. The coding sequences of GD-EPO or SP-EPO were subcloned into the pTOL2-bactin-2A-EGFP vector digested with ECOR-I and BamH-I. EPO was co-expressed with pTOL2-bactin-2A-EGFP. (c) Survival rate of zebrafish under hypoxia and the relative viability (%) was estimated as the percentage. (d) Western blotting analysis fusion of EPO-6xHis-EGFP and GAPDH.
Figure 3

GD-EPO functions as a role of antioxidant. (a) The flow chart for obtaining GD-EPO and SP-EPO zebrafish. The recombinant plasmid (pTOL2-bactin-GD-EPO-2A-EGFP, pTOL2-bactin-SP-EPO-2A-EGFP) were injected into the zebrafish embryo, and the green fluorescent zebrafish was detected and screened after 24 h. (b-d) The SOD, NO, MDA activities in the 4 dpf zebrafish under hypoxia. “+” yes, “-” none, All data were expressed as the mean ± SD of three independent experiments. * p < 0.05, ** p < 0.01.
Figure 4

GD-EPO can inhibit the expression of apoptosis-related genes. (a-b) The mRNA level of Caspase-3, Caspase-8, Caspase-9, Cytc, Bax and Bcl-2. (c) Expression level of Bcl-2, Bax, Caspase-3 by Western blotting. The results were expressed as the mean ± SD of three independent experiments. * p < 0.05 and ** p < 0.01.