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Expression of netrin-1 by hypoxia contributes to the invasion and migration of prostate carcinoma cells by regulating YAP activity

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ABSTRACT

Hypoxia is a hallmark of solid tumor growth microenvironment and appropriates the major contributor for the failure and poor prognosis of clinical tumor treatment, including prostate cancer (PCa). Ectopic expression of netrin-1 is reportedly associated with the progression of several carcinomas. Here, we aimed to investigate the role of netrin-1 in hypoxic metastasis potential of prostate carcinoma. Here, hypoxia induced the up-regulation of netrin-1 mRNA and protein expression in prostate cancer cell lines PC3 and DU145. Importantly, knockdown of netrin-1 dramatically suppressed cell invasion, migration and epithelial-to-mesenchymal transition (EMT) of PC3 and DU145 cells under hypoxia. Furthermore, hypoxia treatment increased the activity of Yes-associated protein (YAP) by increasing YAP expression in the nucleus and inhibiting p-YAP levels. However, YAP activation was notably restrained following netrin-1 down-regulation. Interestingly, interrupting YAP expression attenuated hypoxia-triggered cell invasion, migration and EMT of DU145 cells. More importantly, restoring YAP expression strikingly antagonized the inhibitory effects of netrin-1 decrease on the metastatic potential of prostate cancer cells. Together, these results indicate that netrin-1 may function as a positive regulator of hypoxia-triggered malignant behavior in PCa by activating the YAP signaling. Accordingly, netrin-1 could be a promising therapeutic agent against prostate carcinoma.

1. Introduction

Prostate cancer (PCa) is the second most prevalent diagnosed cancer and as accounts for the major contributor to cancer-related death among men [1]. In recent years, the morbidity and mortality of prostate carcinoma are constantly growing in China [2]. Presently, conventional surgical treatment remains the most effective way for prostate cancer therapy. Although the survival rates among patients with benign PCa have improved, the survival rates beyond 5 years among patients with metastatic PCa is only 28%, representing the leading cause of mortality of patients with PCa [3]. Hence, elucidating the molecular mechanism underlying the metastasis of PCa is an urgent goal in developing novel and effective therapeutic strategies for PCa.

In a variety of solid tumors, including PCa, hypoxic microenvironment (hypoxia) of the tumor tissue is a common biological feature and is widely accepted as a major reason for treatment failures and poor clinical outcomes [4–6]. Under such condition, tumor cells can adapt to hypoxia and acquire the increased potential for cell migration and invasion, which may facilitate tumor metastasis [7]. Abundant preclinical experimental evidences have corroborated the notion that exposure to hypoxia increases the metastatic ability of various cancers, including PCa [8,9]. A growing body of evidence confirms that hypoxia can induce epithelial–mesenchymal transition (EMT), a common process associated with cancer progression. During EMT, tumor cells lose epithelial E-cadherin expression and cellular adhesion, and then acquire the hallmark characteristics related to cancer cell invasion and metastasis [10]. Therefore, understanding the mechanism involved in hypoxia-triggered progression of PCa is urgently required.

Netrin-1 is a laminin-related secreted protein and belongs to the member of the netrin family. It contains approximately 600 amino acid residues and often functions as a multifunctional regulatory molecule for various biological processes, such as cell proliferation, invasion and morphogenesis [11–13]. Recently, the increasing evidences have demonstrated the abnormal expression of netrin-1 in several cancers including PCa [14,15]. However, its function in carcinogenesis is controversial in various types of cancers [11,16]. Netrin-1 ranks as a selective advantage for the progression of several cancers. For example, netrin-1 exerts oncogenic roles by promoting cell proliferation and migration through enhancing Yes-associated protein (YAP) activity in hepatocellular carcinoma (HCC) [16]. Moreover, overexpression of netrin-1 promotes EMT and subsequent invasion of HCC cells [17]. Conversely, up-regulation of netrin-1 effectively arrests the growth of

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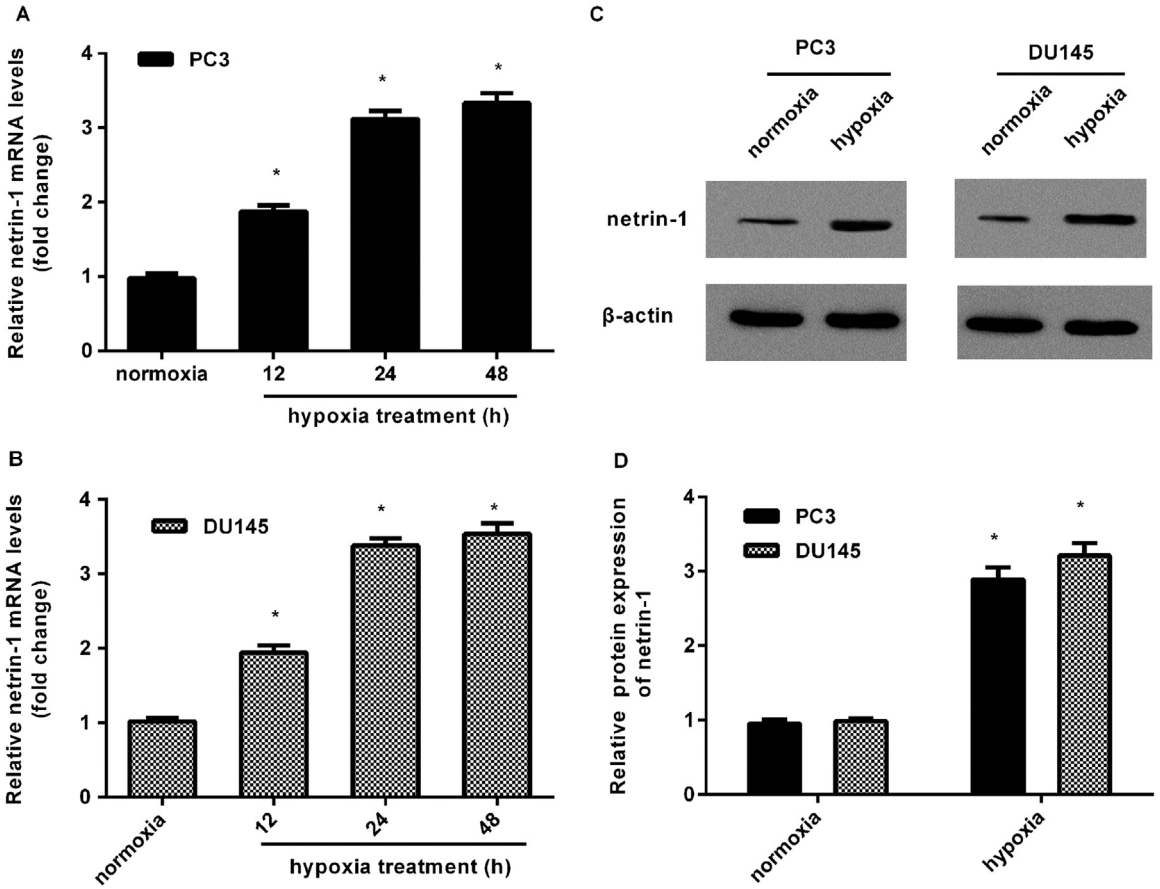

Fig. 1. Hypoxia augmented the expression of netrin-1 in prostate cancer cells. (A, B) Following exposure to normoxia or hypoxia (1% O_2) for 12 h, 24 h and 48 h post incubation, the mRNA levels of netrin-1 in PC3 (A) and DU145 (B) cells were determined by qRT-PCR assay. (C) Western blotting detected the protein levels of netrin-1 in PC3 and DU145 cells. (D) The corresponding quantitative analysis of netrin-1 protein was carried out using Quantity One. *$p < 0.05$ versus normoxia group.

xenografted pancreatic ductal adenocarcinoma (PDAC) cells [11]. Recently, increased netrin-1 levels were detected in the plasma and tissues of prostate cancer and netrin-1 is now recognized as a potential biomarker for predicting the malignancy degree of PCa [15,18]. However, its function and the underlying mechanism of action in the development of PCa remain unclarified.

Emerging researches have validated the elevated release of netrin-1 in response to hypoxia in hepatocellular carcinoma cell and macrophages [17,19]. Therefore, this study aimed to explore the role of netrin-1 in hypoxia-induced invasion, migration and the mechanisms involved.

2. Materials and methods

2.1. Antibodies

Rabbit monoclonal antibodies to netrin-1 (ab126729), E-cadherin (ab40772), N-cadherin (ab76011) and Lamin B (ab133741) were purchase from Abcam (Cambridge, MA, USA). Antibodies against human YAP (#4912), phosphorylated YAP (p-YAP) (#4911) and β-Actin (#8457) were bought from Cell Signaling (Danvers, MA, USA).

2.2. Cell lines and cell culture

Human prostate carcinoma cell lines (DU145 and PC3) were bought from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS), 100 μg/ml penicillin and streptomycin Sulfate. All cells were incubated for the indicated times at 37 °C in a humidified incubator containing 5% (vol/vol) CO_2 and 95% air (normoxia).

2.3. Hypoxia treatment

For hypoxia exposure experiments, cells were cultured in DMEM medium and placed in a mixed-gas incubator containing 5% CO_2, 94% N2, and 1% O_2. At 12 h, 24 h and 48 h post incubation, cells were harvested for subsequent experiments.

2.4. Construction of netrin-1 stable knockdown cells

PC3 and DU145 cells were cultured overnight in DMEM medium in 6-well plates, then were transfected with netrin-1 shRNA plasmids using Lipofectin 2000 reagent (Invitrogen, Carlsbad, CA, USA). The netrin-1 shRNA1, netrin-1 shRNA2 and control shRNA (NC) plasmids were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). About 24 h later, cells were further spread onto 100-mm culture dishes at 1:100 dilutions. For construction of stable transfectants, cells were treated with G418 (600 μg/ml) for 4 weeks to select a stable netrin-1-knockout cell line.

2.5. Transfection of YAP siRNA and plasmid

The scramble siRNA (NC, 5′-CCUACGCCACCAAUUUCGU-3′), siRNA targeting YAP [5′-GGUGAUACUAUCAACCAAATT-3′ (#1) and 5′-GACCAAUAGCUCAGAUCCUUUTT-3′(#2)] were synthesized by GeneChem Co., Ltd. (Shanghai, China). The YAP expression plasmid was purchased from Addgene (Cambridge, MA, USA). For overexpression or knockdown of YAP in DU145 cells, cells were seeded onto 6-well plates the day before transfection. Then, cells were transfected