Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
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Integrated analysis of hypoxia-induced miR-210 signature as a potential prognostic biomarker of hepatocellular carcinoma: a study based on The Cancer Genome Atlas*#

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https://doi.org/10.1631/jzus.B1900343

Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer and is the second leading cause of cancer mortality with an estimated 745 500 deaths annually (Jemal et al., 2011). Although new therapeutic modalities including novel chemotherapeutic interventions and targeted therapy have been applied, the prognosis of HCC patients remains unsatisfactory due to the high incidence of intrahepatic and distal metastases (Siegel et al., 2018).

Hypoxia or inadequate oxygen supply—a fundamental characteristic of the solid tumor microenvironment—activates adaptive transcriptional programs that promote cell survival, motility, cancer angiogenesis, invasion, metastasis, and dedifferentiation, and enhances glycolytic metabolism (Pugh and Ratcliffe, 2003; Semela and Dufour, 2004; Airley and Mobasheri, 2007; Wang et al., 2017). In HCC, hypoxia induces cell survival by activating growth factor signaling. However, the molecular mechanisms involved in the induction of HCC cell reprogramming, especially under hypoxic conditions, remain largely unknown (Heddleston et al., 2010; Brooks et al., 2016).

Various hypoxia-regulated microRNAs (miRNAs), also termed hypoxamiRs, have been recently identified (Bavelloni et al., 2017; Wan et al., 2017). The master hypoxamiR miR-210 is regulated by hypoxia-inducible factor 1α (HIF1α) in various tissues. Moreover, miR-210 has been reported to regulate responses and tolerate hypoxia-induced stress (Huang et al., 2010; Ivan and Huang, 2014). Despite abundant in vitro evidence on the relationship between miR-210 and hypoxia in HCC (Kai et al., 2016; Yang et al., 2016), in vivo evidence is lacking.

In the present study, we attempted to analyze the correlation between the expression of miR-210 and that of various hypoxia-related genes (*HIF1Al*, *HIF3A*, *PTPN1*, and *BNIP3*) as well as disease prognosis in patients with HCC by extracting a large amount of cases from The Cancer Genome Atlas (TCGA) database. All analyzed miRNA and mRNA data were extracted from TCGA database (https://portal.gdc.cancer.gov). Data including patients' demographic information, primary tumor site, tumor morphology, tumor-node-metastasis (TNM) stage, first course of treatment, and follow-up for prognostic outcome were collected for the HCC tissue and the adjacent non-tumorous tissue samples. The data were processed according to the requirements of the data access policies and the National Institutes of Health (NIH) TCGA human subject protection.

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* Project supported by the Education Department of Zhejiang Province (No. G201432123) and the Public Welfare Technology Application Research Plan Project of Zhejiang Science and Technology Department (No. LGC19H200005), China

Electronic supplementary materials: The online version of this article (https://doi.org/10.1631/jzus.B1900343) contains supplementary materials, which are available to authorized users

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All patients were pathologically diagnosed with HCC from 1995 to 2013 using the morphological code C22.0. Patients, who received pre- or post-operative treatments, died within 30 d post-surgery, or with unknown survival time, were excluded. As shown in Table S1, 424 cases conforming to the inclusion criteria were chosen for the present study. The surgical treatment for HCC in the study included segmental resection, lobectomy, and extended lobectomy.

The results were statistically analyzed using GraphPad Prism, Version 6 (GraphPad Software Inc., CA, USA). The inter-group comparison of the miR-210 expression level was performed using Student's *t*-test. Survival analysis was computed using the Kaplan-Meier method and significance was assessed using the log-rank test. Multivariate analysis with the Cox regression model was used to examine the combined effects. Additionally, the linear regression model was used for discerning the relationship between miR-210 expression and the hypoxia-related genes (*HIF1α*, *HIF3α*, *PTPN1*, and *BNIP3*). For all statistical analyses, *P*-value of <0.05 was considered statistically significant.

During the 20-year study period, a total of 424 patients with HCC, including 141 males and 283 females, were identified. Of these, 169 were less than 60 years old, and 255 aged more than or equal to 60 years. The clinicopathological parameters of the patients are shown in Table S1. To explore the potential role of miR-210 as a hypoxamiR, miR-210 expression in the 424 patients with HCC was characterized. Intriguingly, miR-210 expression was robustly upregulated in the advanced T stages compared to that in the early T stages (T1 vs. T3, *P*=0.002; T1 vs. T4, *P*=0.045; T2 vs. T3, *P*=0.043; Fig. 1a). miR-210 expression also correlated with the pathological grade (G1 vs. G3, *P*=0.012) and the TNM stage (stage I vs. stages III+IV, *P*=0.001; stage II vs. stages III+IV, *P*=0.023; Figs. 1b and 1d). However, miR-210 expression was not associated with metastasis (Fig. 1c), gender, age at diagnosis, year of diagnosis, or fibrosis score (data not shown).

Next, we investigated the correlation between miR-210 expression and disease prognosis in patients with HCC. Disease-free survival (DFS) was calculated along with overall survival (OS). Patients with upregulated miR-210 expression had shorter median DFS than those with low miR-210 expression (Fig. 2a, Table S2). The median DFS time in the patients

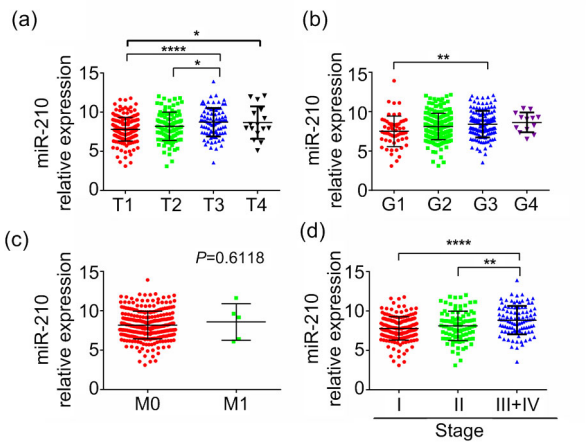

Fig. 1 miR-210 expression in advanced hepatocellular carcinoma (HCC)

(a) miR-210 expression in the advanced T stages was higher than that in the early T stages. (b) miR-210 expression in G3 (poorly differentiated) was higher than that in G1 (well differentiated). (c) The difference in miR-210 expression between the non-metastatic HCC and metastatic HCC tissues was not statistically significant. (d) miR-210 expression in the advanced tumor-node-metastasis (TNM) stage was higher than that in the early TNM stage. * *P*<0.05, ** *P*<0.01, **** *P*<0.0001

with upregulated miR-210 expression was 1560.6 d and in those with low miR-210 expression was 1805.5 d (*P*=0.036; Table S2, Fig. 2a). However, the difference in the median OS time between the patients with low and high miR-210 expression was not significant (*P*=0.449; Table S2, Fig. 2b). Furthermore, previously reported clinicopathological characteristics, such as age at diagnosis (*P*=0.003), pathological grade (*P*=3.218×10^{-45}), fibrosis score (*P*=0.002), T stage (*P*=0.001), M stage (*P*=0.042), and TNM stage (*P*=1.274×10^{-21}), were associated with HCC prognosis (Table S2). However, in the multivariate Cox regression model, the difference in the median DFS time between the patients with low and high miR-210 expression was not significant (*P*=0.957; Table S2).

To further investigate the role of miR-210, the correlation between the expression of miR-210 and the hypoxia-related genes (*HIF1α*, *HIF3α*, *PTPN1*, and *BNIP3*) was investigated. A positive correlation between miR-210 and *HIF1α* expression (*R*=0.078, *P*=0.0068; Fig. 3a) and a negative correlation between miR-210 and *HIF3α* expression (*R*=−0.0102, *P*=0.0291; Fig. 3b) were observed. However, the associations between miR-210 and *PTPN1* (*P*=0.3909; Fig. 3c), and between miR-210 and *BNIP3* (*P*=0.7483; Fig. 3d) expression were not statistically significant.

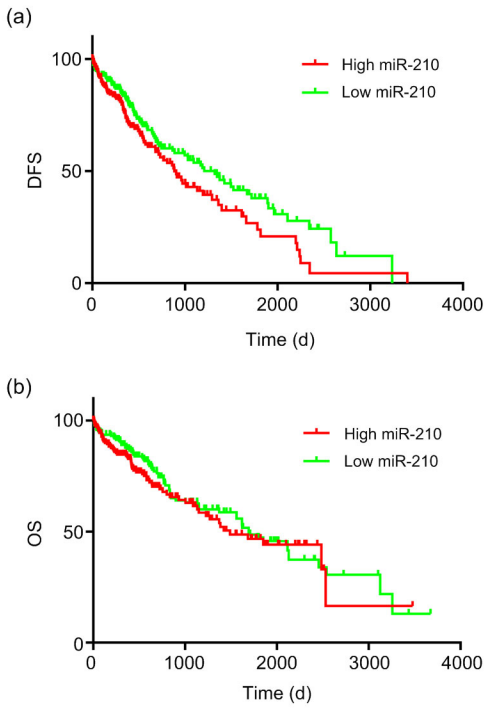

Fig. 2 Correlation between miR-210 expression and the survival of patients with hepatocellular carcinoma (HCC)

(a) Kaplan-Meier curves of disease-free survival (DFS) in patients with HCC according to miR-210 expression. (b) Kaplan-Meier curves of overall survival (OS) in patients with HCC according to miR-210 expression

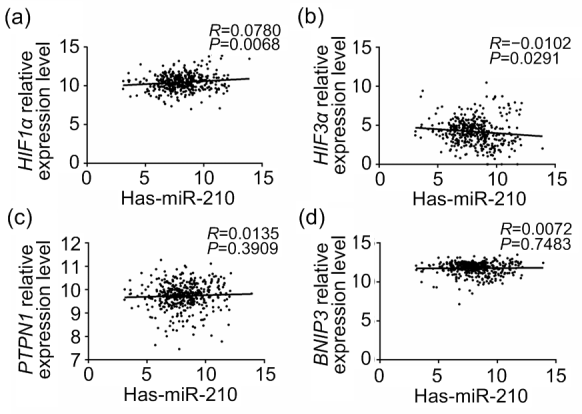

Fig. 3 Linear regression analysis demonstrating the correlation between the expression of miR-210 and the hypoxia-related genes

(a) miR-210 expression positively correlated with *HIF1α* expression. (b) miR-210 expression inversely correlated with *HIF3α* expression. (c, d) The associations between miR-210 and *PTPN1* expression, and between miR-210 and *BNIP3* expression were not significant

Similar to previous studies (Yang et al., 2016; Wang and Zheng, 2018), miR-210 along with other clinicopathological factors, including age, pathological grade, T, M, and TNM stages, was considered prognostic factors for DFS in this study. However, in the multivariate Cox regression model, miR-210 expression did not have a significant impact on DFS. Furthermore, univariate analysis demonstrated that the clinicopathological factors, but not miR-210 expression, affected OS. Based on this result, the multivariate Cox regression model was not applied for further analysis. Thus miR-210 expression is not an independent prognostic factor for DFS or OS.

In vitro evidence showed that miR-210 mediates hypoxia-induced HCC cell metastasis by promoting the migration and invasion of HCC cells (Ying et al., 2011). Several molecular mechanisms are involved in the induction of HCC metastasis, chemotherapeutic resistance, and radiotherapeutic resistance by miR-210 via the hypoxia pathway. miR-210 attenuates hypoxia-induced cell apoptosis by directly targeting *HIF1α* and inhibiting the *HIF1α* pathway in the hypoxic kidney lesions (Liu et al., 2017). It has also been reported that *HIF1α* suppression is regulated by a feedback loop consisting of *HIF1α*/miR-210/*HIF3α* (Kai et al., 2016). However, recent evidences have shown that *HIF3α* suppression by HIF1α-induced miR-210 downregulates *HIF1α* expression and constitutes a feed-forward hypoxic regulatory loop (Silakit et al., 2018). In the present study, a positive correlation between the expression of miR-210 and *HIF1α*, and a negative correlation between the expression of miR-210 and *HIF3α* were observed via linear regression analysis. Thus, the present study provides further evidence on the feed-forward hypoxic regulatory loop. The present evaluation of the correlation between the expression of miR-210 and *BNIP3* and that between miR-210 and *PTPN1* demonstrated no statistical significance. The findings of this study are contrary to those of previous studies. A possible reason may be the different tumor types included in this study. Further verification through in vitro studies in HCC cell lines is warranted.

In conclusion, upregulated miR-210 expression is significantly associated with the advanced TNM stage of HCC and poor DFS of HCC patients. However, miR-210 is not an independent prognostic factor for HCC patients. Moreover, miR-210 expression

positively correlated with *HIF1α* expression and inversely correlated with *HIF3α* expression.

Contributors

Yi DAI performed data analysis, and wrote and edited the manuscript. Ji-liang SHEN, Xue-yong ZHENG, and Tian-yu LIN collected the data. Hai-tao YU contributed to the study design, data analysis, and editing of the manuscript. All authors have read and approved the final manuscript and had full access to all the data in the study. All authors take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yi DAI, Ji-liang SHEN, Xue-yong ZHENG, Tian-yu LIN, and Hai-tao YU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Table S1 Clinicopathological parameters of the patients

Table S2 Univariate and multivariate analyses of survival in 424 patients with HCC according to clinicopathologic factors and miR-210 expression

中文概要

题　目：一项基于肿瘤基因数据库关于综合分析缺氧导致的肝细胞癌的蛋白预测标志物 miR-210 变化的研究

目　的：研究肝癌细胞缺氧微环境导致 miR-210 表达变化与肿瘤进展、预后等相关性。

创新点：首次阐明了 miR-210 表达与肝癌预后及缺氧相关基因的关系。

方　法：选取肿瘤基因数据库（TCGA）中 424 位肝癌患者的 miR-210 表达水平、临床病理参数及缺氧相关基因（*HIF1α*、*HIF3α*、*PTPN1* 和 *BNIP3*）表达量，研究 miR-210 与肝癌预后及缺氧基因之间的相关性。

结　论：miR-210 的表达与肝细胞癌进展分期呈正相关，它的高表达预示更低的无瘤生存率。因此，推测 miR-210 可能与肿瘤细胞缺氧相关性死亡有关。

关键词：miR-210；肿瘤基因数据库（TCGA）；肝细胞癌（HCC）；缺氧；预后意义