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# IRE1α PROMOTES HEPATOCELLULAR CARCINOMA METASTASIS

Min Su, Wen-qi Li*
*Department of Oncology, Guangzhou Development District Hospital, Guangzhou 510730, China*
**Corresponding E-mail: Wen-qi Li MS, 294068045@qq.com*

**Abstract:** Objective: Hepatocellular carcinoma (HCC) is one of the common solid tumors of the digestive system with high morbidity and mortality. This work aimed to examine the expression of inositol requiring enzyme 1 alpha (IRE1α) in HCC. Moreover, the role of IRE1α in the HCC cell proliferation and metastasis were also investigated. Methods: We applied quantitative polymerase chain reaction (qRT-PCR) to evaluate the expression levels of IRE1α in HCC cell lines. Furthermore, cell proliferation was evaluated using CCK8 assay, cell invasion was examined by transwell assay with IRE1α silencing or not. Results: This work found that the expression of IRE1α was increased in HCC cell line compared that in normal hepatocyte line. Functional study demonstrated that IRE1α promotes HCC cells proliferating and invading. However, IRE1α silencing obviously inhibited proliferation and invasion of HCC cells. Conclusions: IRE1α played a vital role in the process of HCC progression and enhanced the metastasis capability of HCC cells, which indicated that IRE1α could serve as a new therapeutic target for HCC patients.
**Keywords:** IRE1α, HCC, invasion, metastasis

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common solid tumors of the digestive system with the sixth in terms of morbidity and fourth in terms of mortality [1]. Despite great progress have been made in experimental and clinical therapy, the general prognosis of HCC patients is unfavorable due to the high incidence of tumor metastasis and recurrence. Previous studies had demonstrated that HCC progression might be a multi-step process associated with multi-factorial etiology and numerous genes alternation. However, the specific molecular mechanism of HCC metastasis remains not clear[2]. Therefore, an in-depth understanding of the molecular mechanism of HCC metastasis means importantly for targeted therapy and prognosis of HCC patients.

Increasing evidence suggests that proteostasis control, unfolded protein response (UPR) and endoplasmic reticulum (ER) stress are playing emerging roles in tumor biology, including epithelial-mesenchymal transition (EMT) mediated tumor invasion and metastasis[3]. Both physiological or pathological stimuli can lead to the accumulation of misfolded proteins, resulting in ER stress. To combat ER stress and restore proteostasis, a signaling network called the UPR is activated. The UPR is exacerbated during tumor developing when tumor cells are stimulated by internal and external factors. Three ER-resident transmembrane proteins, inositol requiring enzyme 1 alpha (IRE1α), activating transcription factor 6 (ATF6) and protein kinase RNA-like ER kinase (PERK) are acting as molecular ER stress sensors and controling UPR activation[4]. Among which, IRE1α is considered to be the most evolutionarily conserved one. It has been reported that in most cancers, such as glioblastoma, leukemia, myeloma, breast and renal cancer, IRE1α is important in both tumorigenesis and invasiveness, and is associated with poor prognosis[5]. However, the definite role of IRE1α in HCC metastasis remains not clear.

In this study, researchers explored the expression IRE1α in HCC cell lines and further analyzed the potential role of IRE1α in the HCC cell multiplication, invasion and metastasis, which may provide a new therapeutic target for HCC patients.

## METHODS

*Cell Culture*

The human HCC cells (Huh7, PLC/PRF5, HepG2 and LM3) and normal hepatocyte (LO2) were purchased from Cancer Research Institute of Southern Medical University (Guangzhou, China). All cells were cultured in high-glucose DMEM (Hyclone) containing 10% of fetal bovine serum (FBS, Gibico) with penicillin-streptomycin (100U/mL, Hyclone). A 37°C humidified incubator

with 5% CO2 air was used to cultured cells.

*Cell Viability Assay (CCK8 assay)*
Cells were cultured in 96-well plates (5000 cells/well). After cultured respectively for 24 h, 48 h, 72 h or 96 h, each 10ul Cell Counting Kit 8 (CCK8) reagent (Dojindo) was added into the cell medium, and then all plates were cultured in incubator for 1-4 hours. Following cell viability were performed by optical density at 450nm with a microplate reader according to the introductions from manufacturer.

*Small-interfering RNA (siRNA) Transfection*
Small interfering RNAs (siRNAs) against the sequence of IRE1α was synthesized by Cyagen Bioscience and the sequence of the IRE1α siRNA was 5′-GCAAGAACAAGCUCAACUATT-3′. Transient transfection was preformed according to the manufacturer's instruction of Lipofectamine3000 Transfection Reagent (Life technology) to temporarily bring siRNAs into cells when cellular density attained 50%-70%. Suppression of IRE1α expression was operated by si-IRE1α, the si-NC was applied as a negative control.

*Quantitative Polymerase Chain Reaction (qRT-PCR)*
Total RNA extracted from cells used TRizol reagent. NanoDrop-1000 spectrophotometer was used to detect the quality and concentration of RNA. Reverse transcription was performed with ReverTra Ace qPCR RT Master Mix. Quantitative analysis of IRE1α mRNA were performed with qRCR using SYBR® Green Realtime PCR Master Mix with Roche LightCycler 1.1.GAPDH, which were regarded as internal controls for mRNA quantification.

**Table 1. Primer sequences of genes**

| Gene | Forward | Reverse |
|---|---|---|
| IRE1α | CGCTCTCCTACGAAGTTCTGATGTG | CTCTCGGTGAGTCTCCTCCAGTG |
| GAPDH | GTCATCCCTGAGCTGAACGG | CCACCTGGTGCTCAGTGTAG |

*Transwell Invasion Assay*
The transwell chamber, which was called upper chamber, was placed into the 24-well culture plate being called lower chamber. After transfection, cells suspended in serum-free medium were seeded onto the upper chamber, and DMEM with 15% FBS was generally added to the lower chamber. For the transwell invasion assay, matrigel (BD Biosciences) was applied to coat the upper membrane, where the cells were fixed in 4% paraformaldehyde for 15 minutes, and stained by Giemsa(Jiancheng) after 24h of invasion assay.

*Statistical Analysis*
All results involved were analyzed through SPSS 15.0 software (Chicago, IL). The data from more than three repeated experiments were presented as mean ± SD. Comparisons between groups were analyzed by Student's t-test. All experiments were repeated for at least three times. P-Value <0.05 was considered statistically significant.

## RESULTS

*High expression of IRE1α in HCC cell lines*
To explore the potential role of IRE1α in HCC metastasis, researchers analyzed IRE1α expressed in both HCC cells line (huh7, PLC/PRF5, hepG2 and LM3) and normal hepatocyte line (LO2). The mRNA levels of IRE1α were examined by qRT-PCR. The results showed that compared to normal hepatocyte LO2, the mRNA levels of IRE1α in HCC cell lines was obviously upregulated. Furthermore, IRE1α mRNA's expression was higher in LM3 cells, which had higher metastasis potential than that of other HCC cell lines, including HepG2, Huh7 and PLC/PRF5 (Fig. 1).

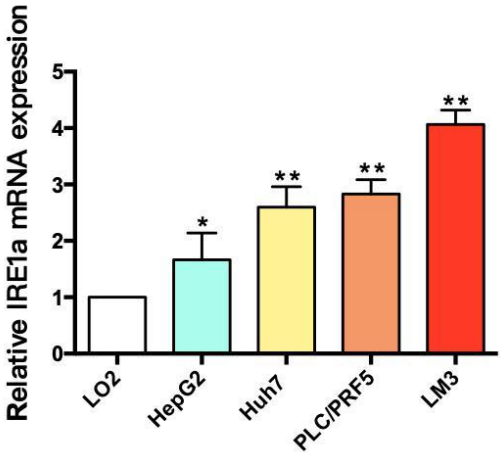


**Fig.1** The expression of IRE1α was significantly upregulated in hepatocellular carcinoma (HCC) cells. The mRNA expression of IRE1α was examined by real-time PCR (*P<0.05, ** P<0.01).

*Effects of IRE1α on HCC cells proliferation in vitro*

These results showed that higher IRE1α expression was observed in HCC cell line LM3 and lower IRE1α expression was exhibited in normal hepatocyte line LO2. To investigate the involvement of IRE1α in HCC cell proliferation, the researchers knocked down IRE1α in LM3 cells using specific siRNA. After transfection of siRNA against IRE1α, the transfection efficiency was first analyzed by qRT-PCR. The expression of IRE1α was lower in si-IRE1α group than negative control (si-NC) group (**P<0.01; Fig. 2A). Through CCK8 assay, after knocking down the expression of IRE1α by si-IRE1α, the cell proliferation ability of LM3 cells was significantly impaired (*P<0.05; Fig. 2B).

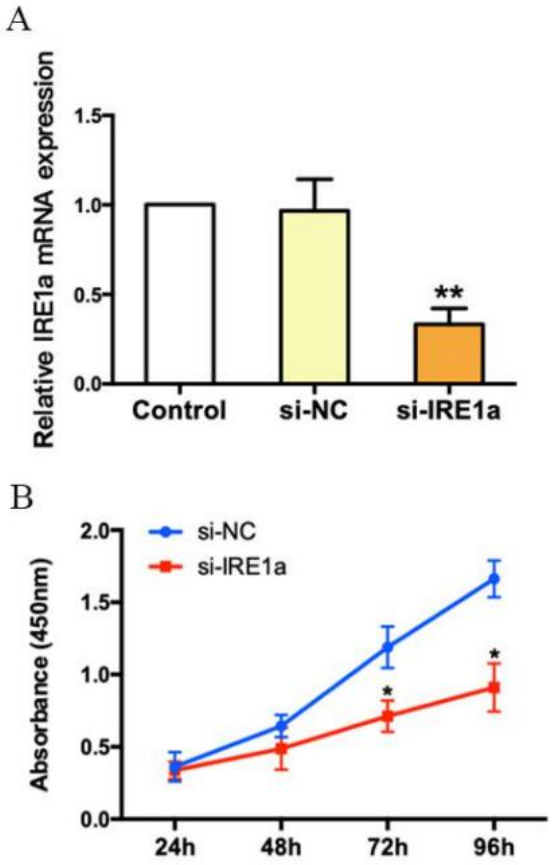


**Fig.2** Effects of IRE1α on HCC cells proliferation in vitro. A. The interfere RNA designed against the sequence of IRE1α was verified by qRT-PCR to be efficient (**P < 0.01). B. Compared to si-NC group, the growth rate of HCC cells in si-IRE1α group was slower and slower with time (*P <0.05).

*Acceleration of IRE1α for HCC cells invasion in vitro*

We further evaluated the invasion ability of HCC cells by transwell invasion assay. Transwell invasion assay showed that silencing of IRE1α significantly inhibited the invasion behavior of HCC cells (**P<0.01; Fig. 3A-B). The data demonstrated that IRE1α could enhance HCC cells invasive ability.

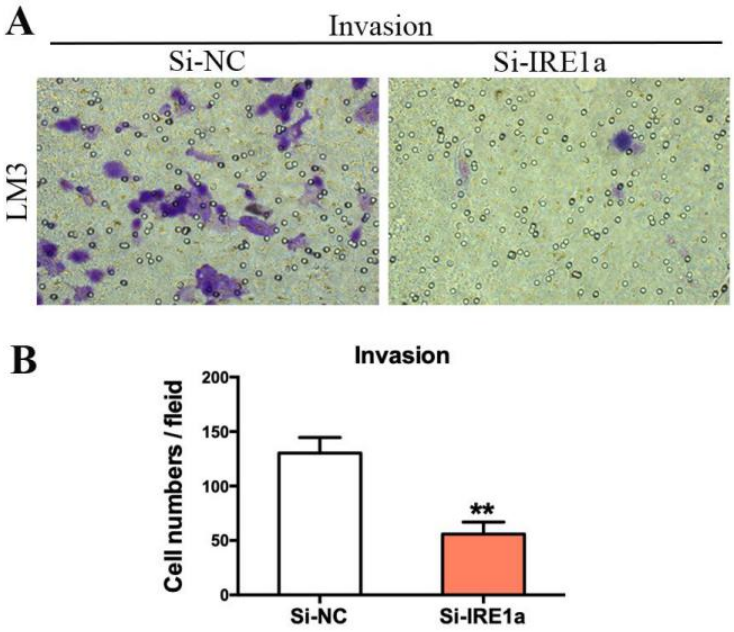


**Fig.3** Acceleration of IRE1α for HCC cells invasion in vitro. A-B. The ability of HCC cells for invasion was eliminated after transfected with si-IRE1α (**P <0.01).

## DISCUSSION

HCC is one of the lethal malignant cancers in digestive system with high morbidity and mortality. HCC has a poor prognosis and causes nearly 800,000 deaths worldwide in 2018[6]. The high mortality rate of HCC patients mainly stems from their high recurrence and metastasis rate[1]. However, the detailed mechanisms underly the high metastasis rate of HCC still needs to be further clarified.

The development of solid tumors is featured by the uncontrolled growth, invasion and the metastasis of malignant cells, leading to cells dense and hypoxic tumor micro-environment, both of which are well-characterized ER-stress inducers[7]. Therefore, there is no doubt that the activation of UPR is a major marker of several solid tumors, such as colon cancer[8], breast cancer[9], and even HCC[10]. Tumor progression can be divided into different stages beginning with transformation followed by uncontrolled cell division, invasion and metabolism, remarkably, among which the UPR is associated with each stage[11]. There is growing evidence that UPR activation and ER stress play an important role hepatic inflammation, chronic liver disease as well as HCC[7, 12]. The progression of HCC is often accompanied by hypoxia, reduce glucose supplement, and active UPR in the tumor microenvironment. According to reports, UPR contributes to the progression, chemoresistance and prognosis of HCC[13]. However, the specific mechanism by which UPR affects the development of HCC and its relationship with HCC metastasis is still unclear.

IRE1α is the most evolutionally conserved UPR-related protein, and its increased activity has been confirmed in some cancers[14]. IRE1α activity is important in the development and invasion of lots of cancers, which can regulate the adhesion of glioblastoma multiform (GBM) cells[15], enhance the metastasis of triple-negative breast cancer (TNBC) tumors to the lung[16], and is associated with chemoresistance in a number of cancers, including prostate cancer, breast cancer, leukemia and glioblastoma[5]. Liu and the group recently reported that IRE1α played an important role in controlling hepatocyte proliferation and regeneration[17]. However, the role that IRE1α plays in HCC proliferation and metastases is still unclear and studying the exact effect of IRE1α in HCC is critical for relevant therapeutic interventions.

In present study, the researchers demonstrated that IRE1α expression was increased in HCC cells line by qRT-PCR. To investigate the possible biological function of IRE1α in HCC progression, researchers

detected the effect of IRE1α on cell proliferation in LM3 cells using CCK8 assay, which found that the silencing of IRE1α resulted in a significant HCC cells growth rate decrease. The data revealed that IRE1α has a pro-proliferative effect in HCC cells. Correlation analysis showed that IRE1α expression was closely related to the invasion and metastasis of HCC cells line. These studies showed that the silencing of IRE1α markedly decreased the migration and invasion abilities in LM3 cells.

The results mentioned above indicated that IRE1α may work as a new regulator of HCC metastasis. For the first time, IRE1α was also shown to obviously promote the invasion and metastasis of HCC. Therefore, IRE1α may play a role as an oncogene in HCC, and may be a novel therapeutic target for HCC patients.

## ACKNOWLEDGMENTS

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