Up-regulation of Krüppel-Like Factor 8 Promotes Tumor Invasion and Indicates Poor Prognosis for Hepatocellular Carcinoma

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BACKGROUND & AIMS: The transcription factor Krüppel-like factor 8 (KLF8) has a role in tumor development, growth, and metastasis, but its role in hepatocellular carcinoma (HCC) is not clear. **METHODS:** KLF8 expression in human HCC cell lines and tumor tissues was measured by quantitative real-time polymerase chain reaction, immunoblot, and immunochemical analyses. The effects of KLF8 depletion or overexpression in HCC cells were observed in cultured cells and in mice. Changes in gene expression patterns in HCC cells in which levels of KLF8 were reduced using small interfering RNA were investigated by microarray analysis. The clinical significance of KLF8 expression levels were validated using tissue microarray analysis of surgical samples from 314 HCC patients. **RESULTS:** KLF8 was overexpressed in highly metastatic HCC cell lines and in samples from patients with recurrent HCC. In cultured cells, KLF8 up-regulation promoted cell proliferation and invasion; inhibited apoptosis; down-regulated N-cadherin, vimentin, and fibronectin; and up-regulated E-cadherin. In mice, overexpression of KLF8 increased HCC progression and metastasis. Microarray analysis showed that reduction of KLF8 in HCC cells down-regulated expression of multiple genes involved in tumor progression and metastasis. KLF8 expression was a significant predictor of overall survival ($P = .040$) and time to HCC recurrence ($P = .006$) and was associated with early tumor recurrence ($P = .001$). **CONCLUSIONS: KLF8 promotes HCC cell proliferation and invasion, inhibits apoptosis, and induces the epithelial-to-mesenchymal transition. KLF8 up-regulation might be used to indicate poor prognosis or early recurrence of cancer in patients who have had surgery for HCC.**

Keywords: Small Interfering RNA; siRNA; Liver Cancer; Metastatic Liver Cancer; Tumor Microenvironment.

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death.[1] Although survival of patients with HCC has been improved by advances in surgical techniques and perioperative management, long-term survival following surgical resection remains unsatisfactory because of the high rate of recurrence and metastasis.[2,3] Advances in treatment of this disease are likely to stem from a better understanding of its biology and behavior.[4] Because biologic and clinical behaviors of cancer are affected by multiple molecular pathways that are under the control of transcription factors, improved understanding of how these transcription factors affect cancer biology may lead to an improved ability to predict clinical outcomes and the discovery of novel therapeutic strategies.[5]

Krüppel-like factors (KLFs) constitute a family of nuclear proteins that modulate gene transcription.[6] Emerging evidence suggests that KLFs may be critical factors in tumor development, growth, and metastasis.[7] Several KLF members, such as KLF4,[8] KLF5,[8] KLF6,[9,10] and KLF11[11] have been reported to be associated with oncogenesis in various types of human cancer. As one of these family members, KLF8 was initially identified as a transcription repressor of Krüppel-like C2H2 zinc-finger transcription factor family proteins.[12,13] Recent investigation revealed that KLF8 induces tumor cell epithelial-to-mesenchymal transition (EMT) and maintains the invasive potential of cancer, which seemingly plays a crucial role in metastatic progression of human carcinoma.[13] Together, these results strongly suggest a significant role for KLF8 in tumor progression of human cancer.

Abbreviations used in this paper: AFP, α-fetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; EMT, epithelial-to-mesenchymal transition; FAK, focal adhesion kinase; GGT, γ-glutamyl transpeptidase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IRF7, interferon regulatory factor 7; KLFs, Krüppel-like factors; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; OS, overall survival; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; RNAi, RNA interference; Rsu-1, ras suppressor protein 1; RT-PCR, reverse transcription polymerase chain reaction; shRNA, small hairpin RNA; siRNA, small interference RNA; TACE, transcatheter arterial chemoembolization; TGF-α, transforming growth factor α; TMAs, tissue microarrays; TNM, tumor-node-metastasis; TTR, time to recurrence.

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In our previous study, we find that KLF8 is highly expressed in HCC tissues compared with peritumoral tissues based on transcript profiles, and this result suggested that KLF8 may play an important role in hepatocarcinogenesis. Until now, no studies have reported the clinicopathologic significance of KLF8 in HCC, and the function of KLF8 in cancer remains to be proved. In this study, we demonstrate that KLF8 promotes HCC cell proliferation and invasion, inhibits apoptosis, and induces EMT, and its expression is strongly associated with early tumor recurrence and predicts poor prognosis in HCC patients after surgery.

Materials and Methods

Cell Lines

HCC cell lines with stepwise metastatic potential (MHCC97L, MHCC97H, and HCCLM3, which are hepatitis B virus [HBV]-positive cell lines with the same genetic background but different lung metastatic potentials) were established at our institute.[14–16] The normal liver cell line L-02 and the HCC cell lines HepG2 and SMMC7721 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. SF/SMMC7721 (SMMC7721 transfected by a c-Met expression vector) is established in our experimental center. These 3 cell lines were HBV negative.

Patients and Specimens

Fifty fresh tumor samples used in quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assays were randomly collected from HCC patients who underwent curative resection between 2000 and 2002 at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China. Ten frozen tissues used in Western blot analysis were randomly chosen from these 50 cases. All tissues were collected immediately upon tumor resection in the operating theater, transported in liquid nitrogen, and then stored at −80°C until use. Tumor specimens used in tissue microarrays (TMAs) were obtained from 314 consecutive HCC patients who underwent curative resection between 1997 and 2000 at our institute. The inclusion criteria or clinical status of all patients was described in a previous report.[17,18] Patient follow-up was completed on March 15, 2008. The median follow-up period was 67 months (range, 2–133 months). Postsurgical patient surveillance was performed as previously described.[19,20] Overall survival (OS) was defined as the interval between surgery and death or between surgery and the last observation point. For surviving patients, the data were censored at the last follow-up. Time to recurrence (TTR)[21] was defined as the interval between the date of surgery and the date of diagnosis of any type of relapse (intrahepatic recurrence and extrahepatic metastasis). Tumor recurrence was classified as early recurrence and late recurrence using 2 years as the cutoff. Institutional Review Board approval was obtained prior to experimentation, and written informed consent was obtained from all patients. The clinicopathologic characteristics of the 314 patients are summarized in Supplementary Table 1.

qRT-PCR, Western Blot, and Immunocytochemistry Analysis

Total RNA was prepared using Trizol (Invitrogen), and then complementary DNA (cDNA) was synthesized using the Superscript First-Strand Synthesis system (TOYOBO, Osaka, Japan). The cDNA was used for qRT-PCR analysis using an SYBR-Green PCR master mix and an ABI7300 instrument (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions.

For Western blot analysis, nuclear protein or total protein was prepared from the cell lines and HCC tissue samples. Immunoblotting experiments were performed according to standard procedures with polyclonal rabbit anti-human KLF8 (1:1000; Aviva Systems Biology, San Diego, CA), rabbit monoclonal anti-human glyceraldehyde-3-phosphate dehydrogenase (1:1000), and polyclonal rabbit anti-human histone1 (1:100). KLF8 (1:150) and E-cadherin (1:500; Abcam, Cambridge, UK) antibody were used in immunocytochemistry analysis.

Cell Transfection and Clone Selection

For small interfering RNA (siRNA)-mediated KLF8 silencing, the following target siRNA sequences of KLF8 (NM_007250) were used: sense CGAUAUGGAUAAACUCAUATT and antisense UAUGAGUUUAUCCAUAUCGAC. The RNA duplexes were synthesized by Genepharma Company (Shanghai, China). Transfection of the siRNAs into HCCLM3 was performed using Lipofectamine 2000 (Invitrogen).

pGCSIL-KLF8 small hairpin RNA (shRNA), a KLF8-RNA interference (RNAi) lentiviral vector was constructed (Shanghai GeneChem Co, Ltd, Shanghai, China). Double-stranded oligonucleotides encoding human KLF8-vshRNA (NM_007250; CCGGCTAGCATGCTACAAGCTCCAATTCAAGAGATTGGAGCTTGTAGCATGCTAGTTTTTG) were annealed and inserted into the small hairpin RNA (shRNA) expression vector pGCSIL-green fluorescent protein (GFP). GFP-lentiviral vector (pGCSIL-GFP) was used as a negative control.

The cDNA encoding KLF8 was amplified by reverse transcription polymerase chain reaction (RT-PCR) from HCCLM3 cells. The forward primer is 5′-ATTAGAATTCGCCACCATGGTCGATATGGATAAACTCATA-3′, and reverse primer is ACCTCTCGAGTCACATGGTGTCATGGCGAC-3′. KLF8 was cloned into pcDNA3.1 vector and generated expression vectors of KLF8. KLF8 or mock vectors were transfected into SMMC7721 cells using Lipofectamine 2000 reagent (Invitrogen). The transfected cells were screened under 800 μg/mL G418 (Calbiochem, Darmstadt, Germany) for 3–5 weeks. HCC cell