Krüppel-like Factor 4 Blocks Hepatocellular Carcinoma Dedifferentiation and Progression through Activation of Hepatocyte Nuclear Factor-6

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Abstract

Purpose: Tumor differentiation is a behavioral index for hepatocellular carcinoma (HCC) and a prognostic factor for patients with HCC who undergo orthotopic liver transplantation (OLT). However, the molecular basis for HCC differentiation and prognostic value of the underlying molecules that regulate HCC differentiation are unclear. In this study, we defined a potential driver pathway for HCC differentiation and prognostication.

Experimental Design: The regulation and function of Krüppel-like factor 4 (KLF4) and hepatocyte nuclear factor-6 (HNF-6) in HCC differentiation was evaluated using human tissues, molecular and cell biology, and animal models, and its prognostic significance was determined according to its impact on patient survival.

Results: There was a direct relationship between the expression levels of KLF4 and HNF6 in HCC. Reduced KLF4 or HNF6 expression correlated with high HCC grade. Poorly differentiated HCC cells had lower expression of KLF4 or HNF6 and differentiation-associated markers than did well-differentiated cells. Elevated KLF4 or HNF6 expression induced differentiation of poorly differentiated hepatoma cells. Mechanistically, KLF4 trans-activated HNF-6 expression. Restored HNF-6 expression upregulated expression of differentiation-associated markers and inhibited HCC cell migration and invasion, whereas HNF-6 knockdown did the opposite. Loss of KLF4 expression in primary HCC correlated with reduced overall survival and shortened relapse-free survival durations after OLT. Combination of KLF4 expression and the Milan criteria improved prognostication for HCC after OLT.

Conclusions: The dysregulated KLF4/HNF-6 pathway drives dedifferentiation and progression of HCC, and KLF4 is a biomarker for accurate prognostication of patients with HCC treated by OLT when integrated with the Milan Criteria. Clin Cancer Res; 22(2); 502–12. ©2015 AACR.

Introduction

Tumor differentiation is a well-recognized morphologic index for assessing the biologic behavior of hepatocellular carcinoma (HCC). Histologic grade increases along with tumor burden (1), and loss of differentiation always parallels early metastasis (2). Tumor grade is widely accepted as a helpful prognostic indicator for HCC after surgery (2, 3) and recommended as a key determinant of liver transplantation for patients with HCC (4, 5). Moreover, differentiation therapy for HCC is proposed as one of the best strategies for treatment of this malignancy because of induction of cell differentiation (6). Nevertheless, the underlying mechanisms of and molecules for regulation of HCC differentiation are poorly understood.

The Krüppel-like factors (KLF), a family of evolutionarily conserved mammalian transcription factors that share a C-terminal three-zinc-finger DNA-binding domain, play critical roles in normal development and carcinogenesis (7). Of the KLF family members, KLF4 is physiologically expressed in terminally differentiated epithelial cells and functions as an imperative regulator of cell differentiation (8–14). However, the causal impact of KLF4 expression and function on cancer differentiation has yet to be...
Translational Relevance

We have used hepatocellular carcinoma (HCC) tissue specimens and molecular biology and animal models to evaluate the activation and function of the KLF4/HNF-6 pathway in human HCC. Our clinical and mechanistic findings indicate that HNF-6 is a direct transcriptional target of KLF4 and that frequently dysregulated KLF4 expression leads to aberrant HNF-6 expression. Moreover, both KLF4 and HNF-6 upregulated expression of differentiation-associated markers and inhibited HCC cell differentiation, whereas knockdown of HNF-6 and KLF4 did the opposite, suggesting a novel molecular basis for the critical role of loss of KLF4 in HCC development and progression. Moreover, loss of KLF4 expression in primary HCC correlated with reduced overall survival and shortened relapse-free survival durations after orthotopic liver transplantation (OLT). Combination of KLF4 expression and the Milan criteria improved prognostication for HCC after OLT. Therefore, our findings may have a significant effect on the clinical management of patients with HCC.

well defined. In gastrointestinal cancers, KLF4 is a tumor suppressor, including HCC (15–19). However, whether dysregulation of KLF4 expression casually impacts HCC differentiation and, if so, the underlying molecular mechanism must be elucidated.

The development and differentiation of the liver are cross regulated by several liver-enriched transcription factors (LETF), including the hepatocyte nuclear factors (HNFs). HNF-6, which belongs to the superclass of cut domain-containing homeoproteins, is expressed at high levels and regulates the transcription of hepatocyte-specific genes in the adult liver (20). HNF-6 functions as an upstream regulator of the LEIF cascade that drives the differentiation of cells of hepatic lineage (21–23). However, the impacts of HNF-6 expression on HCC differentiation and clinical outcome remain unclear.

Orthotopic liver transplantation (OLT) offers a potential cure for HCC and concomitant liver disease and is proposed for patients with tumors limited in size and number (24). The most successful and widely accepted patient selection criteria for OLT are the Milan criteria (25, 26). However, Milan criteria are too conservative, leading to arbitrary exclusion of some patients with tumors at advanced stages but with favorable biology, who would have benefited from OLT (27). Attempts to expand the Milan criteria have been based on pathologic features, such as tumor size and number, histologic grade, and microvascular invasion (27–29). However, the applicability of more liberal indications for OLT than the current Milan criteria based exclusively on morphological criteria in patients with HCC remains controversial. Given that the inherent molecules in rather than the morphology of HCC lesion predicts tumor behavior (30), momentum toward developing effective molecular biomarkers for improving prognostication and selecting candidates for OLT has increased (31, 32). Currently, no pathobiologic markers demonstrating the prognostic significance of HCC after OLT are widely accepted.

In the present study, we found that loss of KLF4 expression was closely correlated with high histologic grade in HCC cases and was an independent negative prognostic factor for patients with HCC after OLT. Restoration of KLF4 expression promoted differentiation of HCC cells. HNF-6, a novel transcriptional target of KLF4, is a pivotal molecular mediator of KLF4-induced differentiation of HCC. Therefore, integration of this novel biomarker into the conventional Milan criteria may increase the accuracy of prognostication for HCC.

Materials and Methods

The Supplementary Methods contains details about the materials and methods regarding immunohistochemistry and tissue microarray (TMA) analysis (16, 33), Western blot analysis (16), RNA extraction and reverse transcriptase PCR (RT-PCR) analysis (Supplementary Table S1), laser capture microdissection (LCM), genotyping and gene expression analyses (10, 34), immunofluorescent cell staining, construction of mutant KLF4 with deletion of zinc finger domain (KLF4AZFD)-expressing vectors, construction of HNF-6 promoter reporter plasmids and mutagenesis, chromatin immunoprecipitation (ChIP) assay (15), cell proliferation assay (15, 16), flow cytometric analysis of the cell cycle (15, 16), scratch assay (15, 16), cell invasion assay (15, 16), and a mouse xenograft tumor study (16).

Patients, clinicopathologic analysis, and tissue specimens

Patient information and follow-up and clinicopathologic data extraction were described previously in detail (33). Tumors in explanted livers were re-evaluated using the Milan criteria on the basis of pathologic data. Matched pairs of HCC and formalin-fixed, paraffin-embedded nontumor tissue blocks for TMA and paired fresh HCC and nontumor tissue specimens were collected as described previously (33). The histologic grade of HCC was classified as well-differentiated (grade 1), moderately differentiated (grade 2), or poorly differentiated (grade 3) according to World Health Organization criteria (35). When the tumor consisted of more than or equal to two grades of histologic differentiation, the most progressive grade was used. The study protocol was approved by the Shanghai jiao tong University Institutional Review Board, and informed written consent for use of the tissue specimens was obtained from each patient or his or her guardian.

TMA construction and immunohistochemistry

The TMA construction was described in detail previously (33). Standard immunohistochemical procedures were performed with human HCC TMA specimens using anti-KLF4 (Santa Cruz Biotechnology) and anti-HNF-6 (Sigma) antibodies, and the staining results were scored by two pathologists blinded to the clinical data as described previously (15, 16) and in Supplementary Methods. Use of archived tissue specimens was approved by the Shanghai jiao tong University and MD Anderson Cancer Center Institutional Review Boards.

Statistical analysis

Statistical analyses were performed using the SPSS software program (version 17.0, IBM Corporation). For continuous variables, data were expressed as medians and in the interquartile range and compared using the Kruskal–Wallis test. For categorical variables, data were expressed as numeral counts and percentages and compared using the Pearson χ² test or Fisher exact test. Differences in protein expression between the matched specimens were examined using the marginal homogeneity test. Survival rates were calculated and survival curves were plotted using the Kaplan–Meier method, and differences were compared using the
log-rank test. Univariate Cox regression was used to analyze differences in survival among patient groups. The significant factors in the univariate analyses were included in multivariate Cox proportional hazards models. The significance of the in vitro data in the groups was determined using a two-tailed Student t test. Statistical significance was indicated by a conventional P < 0.05.

Results

Decreased KLF4 expression predicted poor differentiation of human HCC

We first investigated the expression of KLF4 in 99 pairs of primary HCC and matched adjacent nontumor tissue specimens obtained from patients who underwent OLT using a TMA. We found that loss of KLF4 expression was associated with poor tumor differentiation (Fig. 1A; Supplementary Fig. S1) and that KLF4 expression in human HCC specimens was drastically lower than that in paired nontumor tissue specimens (Fig. 1B and C; Supplementary Fig. S2; Supplementary Table S2). Interestingly, histologic grade was the only clinicopathological parameter negatively related to KLF4 expression (P = 0.008; Fig. 1D; Supplementary Table S3). These results strongly indicated that KLF4 plays a role in human HCC differentiation.

In human HCC cell cultures, well-differentiated HCC cells (HepG2, Hep3B, and PLC/PRF/5) exhibited a flattened pleomorphic morphology and enhanced intercellular aggregation (i.e., well-differentiated phenotype), whereas poorly differentiated
HCC cells (SNU387 and SNU423) had spindle-like shapes and appeared to be scattered (Fig. 2A). Consistently, we observed a dramatic loss of a panel of hepatic differentiation–associated markers in poorly differentiated HCC cells or lower expression of them than in the well-differentiated cells (Fig. 2B, left panels); and a similar expression pattern for these markers in well-differentiated (grade 1) and poorly differentiated (grade 3) HCC specimens using LCM (Supplementary Fig. S3). Western blot analysis demonstrated a drastic reduction in the expression of KLF4 and its downstream transcriptional target E-cadherin (36), an epithelial and differentiation marker of HCC (37), in the poorly differentiated HCC cells (Fig. 2B, right and C). These findings indicated that KLF4 expression is critical to the maintenance of HCC differentiation status.

**KLF4 induced differentiation of poorly differentiated HCC cells**

To determine the causal effect of KLF4 expression on HCC differentiation, we transfected the KLF4 expression vector pFLAG-KLF4 or control pcDNA3.1 into poorly differentiated SNU387 and SNU423 cells. Increased expression of KLF4 (Fig. 3A and B) markedly elevated α-AT, α-fetoprotein (AFP), albumin, HNF-6, C/EBPα, and E-cadherin mRNA expression (Fig. 3A) and upregulated HNF-6, E-cadherin, albumin, and AFP protein expression in these cells (Fig. 3B and C). Meanwhile, enforced KLF4 expression in these cells led to a dramatic morphology shift toward well-differentiated phenotype (Fig. 3D). In contrast, we transfected KLF4 siRNA or the control siRNA into PLC/PRF/5 cells, which were well-differentiated and had high levels of KLF4 expression (Fig. 2C). KLF4 knockdown led to a concomitant decrease in α-AT, AFP, albumin, E-cadherin, HNF-6, and C/EBPα mRNA expression (Fig. 3A) and downregulation of E-cadherin and HNF-6 protein expression in PLC/PRF/5 cells (Fig. 3B). These results clearly demonstrated that KLF4 expression positively impacts the differentiation of HCC cells.

To determine whether transcriptional activity of KLF4 is required for the function of KLF4 in HCC differentiation, we constructed a KLF4 vector (KLF4ΔZFD) lacking a zinc finger domain (ZFD; ref. 38; Supplementary Fig. S4A), and we found that KLF4ΔZFD protein was mainly distributed in the nucleus, the same as the full-length KLF4 (Supplementary Fig. S4B). However, KLF4ΔZFD lost both its transcriptional activity (Supplementary Fig. S4C) and its promoting effect on HCC differentiation (Fig. 3). These findings strongly suggested that KLF4 promotes HCC differentiation via its transcriptional activity.
HNF-6-mediated KLF4-induced HCC differentiation

Increasing evidence indicates that LETFs play important roles in inducing differentiation of hepatoma cells (22, 23). In the present study, we observed higher basal levels of expression of LETFs in well-differentiated HCC cells than in poorly differentiated cells (Fig. 2B and C). Moreover, treatment of SNU387 cells with sodium butyrate, a chemical inducer of differentiation, upregulated expression of KLF4, HNF-6, and C/EBPα but not HNF-1α, HNF-4α, HNF-6β, or DBP (Supplementary Fig. S5). Western blot analysis further demonstrated increases in KLF4 and HNF-6 but not C/EBPα protein expression (Fig. 3B), indicating an important role of KLF4 and HNF-6 in the induction of HCC differentiation.

To characterize the effect of HNF-6 expression on differentiation of HCC cells, we transfected the HNF-6 expression vector pHNF-6-GFP or control vector pCMV6-AC-GFP into SNU387 and SNU423 cells, which have low levels of HNF-6 expression (Fig. 2B and C). Elevated expression of HNF-6 markedly increased expression of hepatic differentiation markers such as AFP, α1-AT, albumin, and HNF-4α (Fig. 4, left) and elicited distinguishable morphology change toward well-differentiated phenotype (Fig. 4, right). In comparison, we transfected HNF-6 siRNA or the control siRNA into PLC/PRF/5 cells, which have high basal levels of HNF-6 expression (Fig. 2B and C). This transfection inhibited expression of the hepatic differentiation markers (Fig. 4A, left). Furthermore, the levels of
HNF-6 expression in the primary tumors were significantly lower than those in the matched nontumor tissue (\(P < 0.001\); Fig. 1B; Supplementary Fig. S6A; Supplementary Table S4). Also, the HNF-6 expression level in HCC specimens was negatively correlated with histological grade (\(P = 0.013\); Fig. 1B; Supplementary Fig. S6; Supplementary Table S5). These data indicated that HNF-6 is a critical regulator of HCC differentiation. Moreover, restored HNF-6 expression in SNU387 and SNU423 cells markedly suppressed their invasiveness (Fig. 4B) and migration (Fig. 4C) but not their proliferation or cell-cycle progression (Fig. 4D), suggesting that altered HNF-6 expression affects certain biologic behaviors of HCC cells.

In addition to the similar KLF4 and HNF-6 expression patterns (Fig. 1B), their direct correlation in human HCC samples (Fig. 1C; Supplementary Table S6), and the significant impact of altered KLF4 expression on HNF-6 expression in HCC cell lines (Fig. 3A and B), KLF4 knockdown in sodium butyrate–treated SNU387 cells led to marked downregulation of HNF-6 expression (Fig. 5A). Consistent with these in vitro findings, increased KLF4 expression markedly upregulated HNF-6 expression in SNU398 xenograft tumors (Fig. 5B), whereas conditional deletion of KLF4 led to marked reduction of HNF-6 expression in murine livers (Fig. 5C; Supplementary Fig. S7). Importantly, HNF-6 knockdown markedly reversed KLF4-induced upregulation of expression of differentiation-associated markers in HCC cells (Fig. 5D). These results clearly indicated that KLF4 upregulates HNF-6 expression and that HNF-6 is an important mediator of KLF4-induced HCC differentiation.
KLF4 transactivated HNF-6 transcription

To determine whether KLF4 transcriptionally upregulates HNF-6 expression, we analyzed the HNF-6 promoter sequence for the presence of potential KLF4-binding sites containing the CACCC motif (39) and identified three putative KLF4-binding elements (referred to as site #1, #2, and #3) in the HNF-6 promoter region and accordingly constructed the deletion mutant reporters pH6Pro-843 (not containing a binding site), pH6Pro1002 (containing #1), and pH6Pro1796 (containing #1, #2, and #3; Fig. 6A; Supplementary Fig. S8). We then cotransfected the deletion mutant reporters with the KLF4 or KLF4DZFD expression vector or control pcDNA3.1 as indicated for 24 hours and then submitted to treatment with or without sodium butyrate (5 mmol/L) for 48 hours. Total RNA and protein lysates were prepared for RT-PCR (left) and Western blot (right) analysis, respectively. B, Immunohistochemical analysis of KLF4 and HNF-6 expression in consecutive sections of HCC xenograft tumors established from adenoviral KLF4 (Ad-KLF4)- and adenoviral enhanced GFP (Ad-GFP)-infected SNU398 cells. C, total RNA from the livers of Alb-Cre<sup>−/−</sup>;Klf4<sup>fl/fl</sup> (1# and 2#) and Alb-Cre<sup>−/−</sup>;Klf4<sup>fl/fl</sup> (3# and 4#) mice at the age of 20 weeks were extracted and subjected to RT-PCR analysis of Klf4 and Hnf-6 expression (left) and Western blot analysis (right). The relative levels of expression were quantified and normalized according to GAPDH expression (the gene expression in the liver of mouse #3 was used as a reference; bottom). D, SNU387 and SNU423 cells were cotransfected with a control pcDNA3.1 and control siRNA (pcDNA3.1 + siCtrl), a pFLAG-KLF4 vector and control siRNA (pKLF4 + siCtrl), or a pFLAG-KLF4 vector and HNF-6 siRNA (pKLF4 + siHNF-6) as indicated. Total RNA was extracted from the cells 48 hours after transfection and subjected to RT-PCR analysis (left). The relative mRNA expression levels of those molecules were quantified and normalized according to GAPDH expression (right).

KLF4 expression predicted good prognosis for HCC patients treated with OLT

Univariate survival analysis demonstrated that loss of KLF4 expression in primary HCC cells was significantly associated with reduced overall survival (OS) duration (P < 0.001) and reduced recurrence-free survival (RFS) duration (P < 0.001) after liver.
transplantation (Supplementary Fig. S10A; Supplementary Table S7), and decreased HNF-6 expression in HCC was shown to correlate with poor OS and RFS after OLT (Supplementary Fig. S10B; Supplementary Table S7). Multivariate analysis further revealed that along with well-established prognostic factors such as macrovascular invasion and the Milan criteria, loss of KLF4 expression but not HNF-6 was an independent biomarker for unfavorable clinical outcome of liver transplantation in patients with HCC (Supplementary Table S7). To determine whether KLF4 expression improves survival prediction when combined with the Milan criteria, we analyzed the patients with tumors meeting the Milan criteria according to KLF4 expression \( P = 0.509 \) for RFS, \( P = 0.613 \) for OS; Supplementary Fig. S10C), whereas the RFS and OS rates in the patients with tumors exceeding the Milan criteria differentiated according to KLF4 expression \( P < 0.001 \) for both RFS and OS; Supplementary Fig. S10D). Nearly two thirds (22 of 34) of the patients with tumors beyond the Milan criteria and exhibiting moderate-to-high KLF4 expression had unexpectedly favorable 5-year RFS (70.5%) and OS (91.7%) rates (Supplementary Fig. S10D). These results indicated that KLF4 is a valuable biomarker that can identify patients with hepatocellular tumors outside the Milan criteria but may have favorable outcomes after OLT.

Figure 6.
Regulation and direct binding of the HNF-6 promoter by KLF4. A, schematic structure of the HNF-6 promoter reporters and their putative KLF4-binding sites. B, HNF-6 promoter reporters were transfected in triplicate with pFLAG-KLF4, pFLAG-KLF4ΔZF, or the control vector pcDNA3.1 into SNU387 (left) and SNU423 (right) cells. Cotransfection of pGL3-basic was used as a negative control. The activity of the promoter reporters was measured 36 hours after transfection, and the activities in the negative control and treated groups were expressed as the fold activity in their respective control groups. C, HNF-6 promoter pH6Pro-1796 was transfected in triplicate with KLF4 siRNA (siKLF4) and/or control siRNA (siCtrl) as indicated into PLC/PRF/5 cells. Cotransfection of control siRNA (siCtrl) and pGL3-basic was used as a negative control. The promoter activity was measured and expressed as described in B. , \( P < 0.05 \) and **, \( P < 0.001 \) in a comparison of the control and treated groups. D, chromatin was extracted from SNU387 cells transfected with pFLAG-KLF4 (KLF4 OE), pFLAG-KLF4ΔZF (KLF4ΔZF OE), or the control vector pcDNA3.1 (Ctrl; left) and from PLC/PRF/5 cells treated with KLF4 siRNA (siKLF4) or control siRNA (siCtrl; right). ChIP assays were performed using a specific anti-KLF4 antibody or IgG as a negative control and oligonucleotides flanking the HNF-6 promoter regions containing putative KLF4-binding site #1. Chromatin fragments without IgG or the antibody were used as input controls.
Discussion

In the present study, we defined the crucial and mechanistic roles of KLF4 expression in HCC differentiation and its prognostic value for HCC in patients who undergo OLT. First, decreased expression of KLF4 along with that of differentiation-associated markers correlated closely with poor differentiation of both human HCC cells and tumors. Second, restored KLF4 expression induced differentiation of HCC cells. Third, KLF4 expression causally correlated with HNF-6 expression in human HCC specimens, HCC xenograft tumors, and KLF4-deleted murine livers. KLF4 promoted HCC differentiation by inducing HNF-6 expression. Fourth, KLF4 directly activated HNF-6 expression by binding to its promoter. Fifth, loss of KLF4 expression in primary HCC cells can be used to identify a greatly increased risk of reduced OS and RFS duration after OLT and integration of KLF4 expression into the conventional Milan criteria can identify patients with hepatocellular tumors exceeding the Milan criteria who may have favorable posttransplantation outcomes. Therefore, dysregulation of KLF4/HNF-6 signaling critically contributes to HCC dedifferentiation and progression and KLF4 is a novel, useful biomarker for identification of liver transplantation candidates among patients with HCC.

HCC progression is accompanied by a stepwise process of dedifferentiation (40, 41), whereas the driving force for and mechanistic basis of this process remain unclear. Our recent study has indicated a possible role for KLF4 expression in regulating critically contributes to HCC dedifferentiation and progression outcomes. Therefore, dysregulation of KLF4/HNF-6 signaling critically contributes to HCC dedifferentiation and progression and KLF4 is a novel, useful biomarker for identification of liver transplantation candidates among patients with HCC.

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In addition, we developed the biomarker and analyzed the pathologic data on the patients’ explanted livers. Therefore, in the pretransplantation setting, evaluation of the significance of the KLF4 expression status of tumor biopsy specimens obtained from patients with tumors beyond the Milan criteria at the time of qualification for OLT is critically important. The present study suggests that accurate expansion of the Milan criteria is possible via integration of KLF4 expression.

In summary, loss of KLF4 expression was an independent negative prognostic factor for patients with HCC who underwent OLT and that integration of this novel biomarker of KLF4 expression into the Milan criteria improved the accuracy of prognostication, suggesting that routine evaluation of KLF4 expression in explanted livers is a novel pathologic biomarker for HCC prognosis after OLT and helps select patients with HCC with tumors outside the Milan criteria who could benefit from OLT. The demonstrated role of the KLF4/HNF-6 signaling in HCC dedifferentiation and progression could also be explored for novel therapeutic modalities to reverse HCC dedifferentiation and thus control its progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Sun, L. Mishra, K. Xie
Development of methodology: H. Sun, D. Xie, Z. Jia, K. Xie
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Sun, D. Xie, Z. Jia, Z. Ma, D. Wei, S. Zheng, K. Xie, Z. Peng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Sun, D. Xie, Z. Jia, D. Wei, Y. Gao, S. Zheng, K. Xie
Writing, review, and/or revision of the manuscript: H. Sun, K. Xie
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Tang, L. Mishra, S. Zheng, K. Xie, Z. Peng
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