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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 HCC patients 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.

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Conflicts of interest

The authors disclose no conflicts.

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 transactivated HNF-6 expression. Restored HNF-6 expression upregulated expression of differentiation-associated markers and inhibited HCC cell migration and invasion, while 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—Dysregulated KLF4/HNF-6 pathway drives dedifferentiation and progression of HCC and KLF4 is a biomarker for accurate prognostication of HCC patients treated by OLT when integrated with the Milan Criteria.

Keywords

Progression; Transcription Factor; Biomarkers; KLF4; HNF6

Introduction

Tumor differentiation is a well-recognized morphological index for assessing the biological behavior of hepatocellular carcinoma (HCC). Histological 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 HCC patients.^{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 (KLFs), 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 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 (LETFs), 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 LETF 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 pathological features, such as tumor size and number, histological grade, and microvascular invasion.^{27–29} However, the applicability of more liberal indications for OLT than in 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 pathobiological 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 histological grade in HCC cases and was an independent negative prognostic factor for HCC patients 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 section contains details about the materials and methods regarding immunohistochemistry and tissue microarray (TMA) analysis,^{16,33} Western blot analysis¹⁶, RNA extraction and reverse-transcriptase polymerase chain reaction (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 (KLF4 ZFD)-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, Clinicopathological Analysis, and Tissue Specimens

Patient information and follow-up and clinicopathological data extraction were described previously in detail.³³ Tumors in explanted livers were re-evaluated using the Milan criteria on the basis of pathological 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 histological 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 histological differentiation, the most progressive grade was used. The study protocol was approved by the Shanghai Jiaotong

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, Santa Cruz, CA) and anti-HNF-6 (Sigma, St. Louis, MO) 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 Jiaotong 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, Armonk, NY). 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 chi-square 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* value less than .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-Figs-S1) and that KLF4 expression in human HCC specimens was drastically lower than that in paired nontumor tissue specimens (Fig-1B-and-1C; Supplementary-Fig-S2; Supplementary-Table-S2). Interestingly, histological grade was the only clinicopathological parameter negatively related to KLF4 expression ($P=.008$) (Fig-1D; Supplementary-Table-S3). These results strongly indicated that KLF4 play 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, SNU423, 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 panel; Fig-2C). 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 (Figs-3A-and-3B) markedly elevated α -AT, α -fetoprotein (AFP), albumin, HNF-6, C/EBP α and E-cadherin mRNA expression (Fig-3A) and upregulated HNF-6, E-cadherin, albumin, and AFP protein expression in these cells (Figs-3B-and-3C). Meanwhile, enforced KLF4 expression in these cells led to a dramatic morphology shift towards 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/EBP α 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 impact 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 (KLF4ZFD) lacking a zinc finger domain (ZFD)³⁸ (Supplementary-Fig-S4A), and we found that KLF4 ZFD protein was mainly distributed in the nucleus, the same as the full-length KLF4 (Supplementary-Fig-S4B). However, KLF4 ZFD lost both its transcriptional activity (Supplementary-Fig-S4C) and its promoting effect on HCC differentiation (Fig-3). These findings strongly suggested that KLF4 promote 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 (Figs-2B-and-2C). Moreover, Treatment of SNU387 cells with sodium butyrate, a chemical inducer of differentiation, upregulated expression of KLF4, HNF-6, and C/EBP α but not HNF-1 α , HNF-4 α , HNF-6 β , or DBP (Supplementary-Fig-S5). Western blot analysis further demonstrated increases in KLF4 and HNF-6 but not C/EBP α 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 (Figs-2B-and-2C). Elevated expression of HNF-6 markedly increased expression of hepatic

differentiation markers such as AFP, α -AT, albumin, and HNF-4 α (Fig-4, left panels), and elicited distinguishable morphology change towards well-differentiated phenotype (Fig-4, right panels). 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 (Figs-2B-and-2C). This transfection inhibited expression of the hepatic differentiation markers (Fig 4A, left panels). Furthermore, the levels of HNF-6 expression in the primary tumors were significantly lower than those in the matched nontumor tissue ($P < .001$) (Fig-1B; Supplementary Fig S6A; Supplementary Table S4). Also, the HNF-6 expression level in HCC specimens was negatively correlated with histological grade ($P=.013$) (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 (Figs-4D), suggesting that altered HNF-6 expression affects certain biological 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 (Figs-3A-and-3B), 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 co-transfected the deletion mutant reporters with the KLF4 or KLF ZFD expression vector or control pcDNA3.1 as indicated into SNU387 and SNU423 cells; we co-transfected pGL3-basic and pFLAG-KLF4 as negative controls. Enforced KLF4 but not KLF4 ZFD expression enhanced the promoter activity of pH6Pro1796 and pH6Pro1002 (Fig-6B), while KLF4 knockdown did the opposite (Fig-6C). Furthermore, ChIP assays showed that KLF4 directly interacted with the HNF-6 promoter (Supplementary-Fig-S9A-&-S9B) and this binding required its ZFD (Fig-6D). These results clearly indicated that KLF4 activated the HNF-6 promoter via KLF4-binding elements.

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 OS duration ($P<.001$) and reduced RFS duration ($P<.001$) 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 live transplantation in HCC patients (Supplementary-Table-S7). To determine whether KLF4 expression improves survival prediction when combined with the Milan criteria, we analyzed the patients with tumors within or beyond the Milan criteria stratified according to KLF4 expression. We could not differentiate the RFS and overall survival (OS) durations in the patients with tumors meeting the Milan criteria according to KLF4 expression ($P=.509$ for RFS, $P=.613$ 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 ($P<.001$ for both RFS and OS) (Supplementary-Fig-S10D). Nearly two thirds (22/34) 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.

DISCUSSION

In the present study, we defined the crucial and mechanistic role 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 HCC patients.

HCC progression is accompanied by a stepwise process of dedifferentiation,^{40,41} while 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 HCC differentiation.¹⁹ This notion is consistent with previous studies linking KLF4 to differentiation of normal

epithelium,^{8–10} and neuroblastoma cells.⁴² Our current study provided the first causal evidence that expression of KLF4 drives differentiation of HCC cells. However, authors previously proposed that KLF4 and other stemness genes—Oct4, Sox2, and c-Myc—are putative targets for human HCC therapy.⁴³ This hypothesis is challenged by our previous and current findings in HCC cases, which demonstrated consistent roles of KLF4 as a potent tumor suppressor and differentiation inducer for HCC.¹⁶ This discrepancy clearly warrants further investigation into the exact role of KLF4 expression in stem cells and cancer stem cells, including those for HCC.

Functionally, KLF4 can act as a protein-binding partner or transcription factor to activate or deactivate target genes and/or pathways.^{8,44} Given the important roles of LETFs in induction of HCC differentiation,^{22,23,45} we determined the regulatory effect of KLF4 on LETFs in HCC cells and identified HNF-6 as the novel, specific downstream target of KLF4. Mechanistically, KLF4 transcriptionally activates HNF-6, which plays a pivotal role in mediation of KLF4-induced HCC differentiation. Interestingly, the role of HNF-6 in HCC cell cycle remains unclear,^{46,47} while we observed no impact of HNF-6 on cell-cycle progression or proliferation in HCC cells. The discrepancy in these findings may be attributed to the distinct biological backgrounds of the cell lines studied among others. However, our data did convincingly demonstrate an inhibitory role for HNF-6 expression in migration and invasion of HCC cells, suggesting that HNF-6 is a tumor suppressor. Our clinical finding that loss of HNF-6 expression correlated with poor histological grade of HCC and unfavorable outcome after OLT further substantiated. Therefore, our study not only expands our knowledge of HNF-6 in the context of cancer but also provides an experimental rationale for designing novel, effective differentiation therapy for cancer, including HCC.

Liver transplantation offers an important opportunity to study the potential impact of molecular signaling on the clinical course of HCC. Accumulating evidence suggests that patients with hepatocellular tumors beyond the Milan criteria may not necessarily suffer posttransplantation tumor recurrence.^{27,48} Therefore, expansion of the criteria for transplantation is warranted so that many more patients can benefit from this procedure.^{27,48–50} In the present study, we demonstrated that the KLF4 expression status can be used to stratify patients with tumors outside the Milan criteria into more homogenous groups with distinct RFS and OS durations after OLT. Indeed, patients with tumors beyond the Milan criteria and having moderate to high KLF4 expression had excellent 5-year RFS and OS. KLF4 thus represents a promising new tool to predict outcomes of HCC after OLT in patients with tumors beyond the Milan criteria, which may be profoundly beneficial in guiding clinical decisions regarding surveillance protocols and therapeutic strategies after OLT. However, our study was retrospective and confined to patients with tumors meeting the Milan criteria based on preoperative imaging. Additionally, we developed the biomarker and analyzed the pathological 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 HCC patients 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 pathological biomarker for HCC prognosis after OLT and helps select HCC patients 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this paper

ChIP	chromatin immunoprecipitation
FFPE	formalin-fixed paraffin-embedded
KLF4	Krüppel-Like Factor 4
HCC	hepatocellular carcinoma
HNF-6	hepatocyte nuclear factor-6
OLT	orthotopic liver transplantation
PBS	phosphate-buffered saline
PBS-T	PBS containing 0.1% Triton X-100
PCR	polymerase chain reaction
qPCR	quantitative PCR
RT	reverse transcription
siRNA	small interfering RNA
TGF	transforming growth factor
TMA	tissue microarray
UTR	untranslated region

References

1. Pawlik TM, Delman KA, Vauthey JN, Nagorney DM, Ng IO, Ikai I, et al. Tumor size predicts vascular invasion and histologic grade: Implications for selection of surgical treatment for hepatocellular carcinoma. *Liver Transpl.* 2005; 11:1086–92. [PubMed: 16123959]
2. Oishi K, Itamoto T, Amano H, Fukuda S, Ohdan H, Tashiro H, et al. Clinicopathologic features of poorly differentiated hepatocellular carcinoma. *J Surg Oncol.* 2007; 95:311–6. [PubMed: 17326126]
3. Zavaglia C, De Carlis L, Alberti AB, Minola E, Belli LS, Slim AO, et al. Predictors of long-term survival after liver transplantation for hepatocellular carcinoma. *Am J Gastroenterol.* 2005; 100:2708–16. [PubMed: 16393224]

4. Cillo U, Vitale A, Bassanello M, Boccagni P, Brolese A, Zanusi G, et al. Liver transplantation for the treatment of moderately or well-differentiated hepatocellular carcinoma. *Ann Surg.* 2004; 239:150–9. [PubMed: 14745321]
5. DuBay D, Sandroussi C, Sandhu L, Cleary S, Guba M, Cattral MS, et al. Liver transplantation for advanced hepatocellular carcinoma using poor tumor differentiation on biopsy as an exclusion criterion. *Ann Surg.* 2011; 253:166–72. [PubMed: 21294289]
6. Yin C, Xie WF. Differentiation Therapy with Transcription Factors Might Present as an Ideal Strategy for the Treatment of Cancer. *Hepatology.* 2009; 50:2046–2047. [PubMed: 19937684]
7. Bureau C, Hanoun N, Torrisani J, Vinel JP, Buscail L, Cordelier P. Expression and Function of Kruppel Like-Factors (KLF) in Carcinogenesis. *Curr Genomics.* 2009; 10:353–60. [PubMed: 20119532]
8. Wei D, Kanai M, Huang S, Xie K. Emerging role of KLF4 in human gastrointestinal cancer. *Carcinogenesis.* 2006; 27:23–31. [PubMed: 16219632]
9. Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet.* 1999; 22:356–60. [PubMed: 10431239]
10. Katz JP, Perreault N, Goldstein BG, Lee CS, Labosky PA, Yang VW, et al. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development.* 2002; 129:2619–28. [PubMed: 12015290]
11. Katz JP, Perreault N, Goldstein BG, Actman L, McNally SR, Silberg DG, et al. Loss of Klf4 in mice causes altered proliferation and differentiation and precancerous changes in the adult stomach. *Gastroenterology.* 2005; 128:935–45. [PubMed: 15825076]
12. Hu R, Zuo Y, Zuo L, Liu C, Zhang S, Wu Q, et al. KLF4 Expression Correlates with the Degree of Differentiation in Colorectal Cancer. *Gut Liver.* 2011; 5:154–9. [PubMed: 21814594]
13. Chen YJ, Wu CY, Chang CC, Ma CJ, Li MC, Chen CM. Nuclear Kruppel-like factor 4 expression is associated with human skin squamous cell carcinoma progression and metastasis. *Cancer Biol Ther.* 2008; 7:777–82. [PubMed: 18376139]
14. Luo A, Kong J, Hu G, Liew CC, Xiong M, Wang X, et al. Discovery of Ca²⁺-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. *Oncogene.* 2004; 23:1291–9. [PubMed: 14647409]
15. Wei D, Kanai M, Jia Z, Le X, Xie K. Kruppel-like factor 4 induces p27Kip1 expression in and suppresses the growth and metastasis of human pancreatic cancer cells. *Cancer Res.* 2008; 68:4631–9. [PubMed: 18559508]
16. Li Q, Gao Y, Jia Z, Mishra L, Guo K, Li Z, et al. Dysregulated Kruppel-like factor 4 and vitamin D receptor signaling contribute to progression of hepatocellular carcinoma. *Gastroenterology.* 2012; 143:799–810. [PubMed: 22677193]
17. Lin ZS, Chu HC, Yen YC, Lewis BC, Chen YW. Krüppel-like factor 4, a tumor suppressor in hepatocellular carcinoma cells reverts epithelial mesenchymal transition by suppressing slug expression. *PLoS One.* 2012; 7:e43593. [PubMed: 22937066]
18. Hsu HT, Wu PR, Chen CJ, Hsu LS, Yeh CM, Hsing MT, Chiang YS, Lai MT, Yeh KT. High cytoplasmic expression of Krüppel-like factor 4 is an independent prognostic factor of better survival in hepatocellular carcinoma. *Int J Mol Sci.* 2014; 15:9894–906. [PubMed: 24897024]
19. Lu XJ, Shi Y, Chen JL, Ma S. Krüppel-like factors in hepatocellular carcinoma. *Tumour Biol.* 2015 Feb; 36(2):533–41. [PubMed: 25652467]
20. Samadani U, Costa RH. The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol Cell Biol.* 1996; 16:6273–84. [PubMed: 8887657]
21. Landry C, Clotman F, Hioki T, Oda H, Picard JJ, Lemaigre FP, et al. HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver-enriched transcription factors. *Dev Biol.* 1997; 192:247–57. [PubMed: 9441665]
22. Zeng X, Lin Y, Yin C, Zhang X, Ning BF, Zhang Q, et al. Recombinant adenovirus carrying the hepatocyte nuclear factor-1alpha gene inhibits hepatocellular carcinoma xenograft growth in mice. *Hepatology.* 2011; 54:2036–47. [PubMed: 21898499]

23. Yin C, Lin Y, Zhang X, Chen YX, Zeng X, Yue HY, et al. Differentiation therapy of hepatocellular carcinoma in mice with recombinant adenovirus carrying hepatocyte nuclear factor-4 α gene. *Hepatology*. 2008; 48:1528–39. [PubMed: 18925631]
24. Schwartz M, Roayaie S, Konstadoulakis M. Strategies for the management of hepatocellular carcinoma. *Nat Clin Pract Oncol*. 2007; 4:424–32. [PubMed: 17597707]
25. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med*. 1996; 334:693–9. [PubMed: 8594428]
26. Yoo HY, Patt CH, Geschwind JF, Thuluvath PJ. The outcome of liver transplantation in patients with hepatocellular carcinoma in the United States between 1988 and 2001: 5-year survival has improved significantly with time. *J Clin Oncol*. 2003; 21:4329–35. [PubMed: 14581446]
27. Yao FY. Liver transplantation for hepatocellular carcinoma: beyond the Milan criteria. *Am J Transplant*. 2008; 8:1982–9. [PubMed: 18727702]
28. Yao FY, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, et al. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology*. 2001; 33:1394–403. [PubMed: 11391528]
29. Mazzaferro V, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, et al. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. *Lancet Oncol*. 2009; 10:35–43. [PubMed: 19058754]
30. Mann CD, Neal CP, Garcea G, Manson MM, Dennison AR, Berry DP. Prognostic molecular markers in hepatocellular carcinoma: a systematic review. *Eur J Cancer*. 2007; 43:979–92. [PubMed: 17291746]
31. Vauthey JN, Ajani JA. Liver transplantation and hepatocellular carcinoma biology: beginning of the end of the era of educated guesses. *J Clin Oncol*. 2003; 21:4265–7. [PubMed: 14581443]
32. Dai Z, Yu L, Zhou J, Qiu SJ, Fan J. Molecular markers and hepatocellular carcinoma: lending a helping hand in liver transplantation? *Expert Rev Gastroenterol Hepatol*. 2009; 3:211–3. [PubMed: 19485802]
33. Sun H, Teng M, Liu J, Jin D, Wu J, Yan D, et al. FOXM1 expression predicts the prognosis in hepatocellular carcinoma patients after orthotopic liver transplantation combined with the Milan criteria. *Cancer Lett*. 2011; 306:214–22. [PubMed: 21482449]
34. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem*. 1999; 274:305–15. [PubMed: 9867845]
35. Kleihues P, Sobin LH. World Health Organization classification of tumors. *Cancer*. 2000; 88:2887. [PubMed: 10870076]
36. Yori JL, Johnson E, Zhou G, Jain MK, Keri RA. Kruppel-like factor 4 inhibits epithelial-to-mesenchymal transition through regulation of E-cadherin gene expression. *J Biol Chem*. 2010; 285:16854–63. [PubMed: 20356845]
37. Shimoyama Y, Hirohashi S. Cadherin intercellular adhesion molecule in hepatocellular carcinomas: loss of E-cadherin expression in an undifferentiated carcinoma. *Cancer Lett*. 1991; 57:131–5. [PubMed: 1851053]
38. Schuetz A, Nana D, Rose C, Zocher G, Milanovic M, Koenigsmann J, et al. The structure of the Klf4 DNA-binding domain links to self-renewal and macrophage differentiation. *Cell Mol Life Sci*. 2011; 68:3121–31. [PubMed: 21290164]
39. Yet SF, McANulty MM, Folta SC, Yen HW, Yoshizumi M, Hsieh CM, et al. Human EZF, a Kruppel-like zinc finger protein, is expressed in vascular endothelial cells and contains transcriptional activation and repression domains. *J Biol Chem*. 1998; 273:1026–31. [PubMed: 9422764]
40. Kojiro M. Histopathology of liver cancers. *Best Pract Res Clin Gastroenterol*. 2005; 19:39–62. [PubMed: 15757804]
41. An FQ, Matsuda M, Fujii H, Tang RF, Amemiya H, Dai YM, et al. Tumor heterogeneity in small hepatocellular carcinoma: analysis of tumor cell proliferation, expression and mutation of p53 and beta-catenin. *Int J Cancer*. 2001; 93:468–74. [PubMed: 11477549]

42. Shum CK, Lau ST, Tsoi LL, Chan LK, Yam JW, Ohira M, et al. Kruppel-like factor 4 (KLF4) suppresses neuroblastoma cell growth and determines non-tumorigenic lineage differentiation. *Oncogene*. 2013; 32(35):4086–99. [PubMed: 23045286]
43. Moriguchi H. The reprogramming factors of human somatic cells are novel targets for human hepatocellular carcinoma therapy. *Hepatology*. 2009; 50:2046. [PubMed: 19937684]
44. Zhang W, Chen X, Kato Y, Evans PM, Yuan S, Yang J, et al. Novel cross talk of Kruppel-like factor 4 and beta-catenin regulates normal intestinal homeostasis and tumor repression. *Mol Cell Biol*. 2006; 26:2055–64. [PubMed: 16507986]
45. Ishiyama T, Kano J, Minami Y, Iijima T, Morishita Y, Noguchi M. Expression of HNFs and C/EBP alpha is correlated with immunocytochemical differentiation of cell lines derived from human hepatocellular carcinomas, hepatoblastomas and immortalized hepatocytes. *Cancer Sci*. 2003; 94:757–63. [PubMed: 12967472]
46. Tan Y, Yoshida Y, Hughes DE, Costa RH. Increased expression of hepatocyte nuclear factor 6 stimulates hepatocyte proliferation during mouse liver regeneration. *Gastroenterology*. 2006; 130:1283–300. [PubMed: 16618419]
47. Lehner F, Kulik U, Klempnauer J, Borlak J. Inhibition of the liver enriched protein FOXA2 recovers HNF6 activity in human colon carcinoma and liver hepatoma cells. *PLoS One*. 2010; 5:e13344. [PubMed: 20967225]
48. Hiatt JR, Carmody IC, Busuttil RW. Should we expand the criteria for hepatocellular carcinoma with living-donor liver transplantation?--no, never. *J Hepatol*. 2005; 43:573–7. [PubMed: 16112768]
49. Yao FY, Roberts JP. Applying expanded criteria to liver transplantation for hepatocellular carcinoma: too much too soon, or is now the time? *Liver Transpl*. 2004; 10:919–21. [PubMed: 15237378]
50. Fan ST. Hepatocellular carcinoma--resection or transplant? *Nat Rev Gastroenterol Hepatol*. 2012; 9:732–7. [PubMed: 22965432]

Translational Relevance

We have used HCC tissue specimens and molecular biology and animal models to evaluate the activation and function of 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, while 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 OLT. Combination of KLF4 expression and the Milan criteria improved prognostication for HCC after OLT. Therefore, our findings may have a significant effect on clinical management of patients with HCC.

Fig. 1. KLF4 and HNF-6 protein expression in human HCC specimens and their association with tumor grade

Immunostaining of HCC and matched adjacent nontumor tissue specimens in a TMA was conducted using a specific anti-KLF4 or anti-HNF-6 antibody. (A) Representative photos of KLF4 protein expression in grade 1, 2, and 3 HCC samples ($\times 200$). Note that the percentage and intensity of positively stained tumor cells decreased gradually as tumor grade increased. (B) Representative images of similar expression patterns for KLF4 (upper panels, reddish-brown staining) and HNF-6 (lower panels, gray-black staining) in the tumor border ($\times 100$), a grade 2 tumor ($\times 200$), and a grade 3 tumor ($\times 200$). T, tumor tissue; N, nontumor tissue; red dotted line, border. (C) Western blot analysis of KLF4 and HNF-6 protein expression in grade 1 and 3 HCC (T) and nontumor tissue (N) specimens (left panel). The expression of KLF4 and HNF-6 protein (normalized according to expression of GAPDH) in tumors was quantified and expressed as the ratio to that in nontumor tissue (right panel). Note that loss of KLF4 and HNF-6 protein was more prominent in grade 3 than grade 1 tumors. (D) Left panel: Loss of KLF4 expression was correlated with poor differentiation ($P=.008$ [Fisher exact test] in a comparison of grade 3 tumors with grade 1 and 2 tumors). Middle panel: Decreased HNF-6 expression was correlated with poor tumor differentiation ($P=.013$ [Fisher

exact test] in a comparison of grade 3 tumors with grade 1 and 2 tumors). Right panel:
Direct correlation between the expression levels of KLF4 and HNF6 (n=99). N/W, negative/
weak; M, moderate; S, strong.

Fig. 2. Different morphologies of and biomarker expression in HCC cell lines with different grades of differentiation

(A) Different morphologies of HCC cell line cultures derived from well-differentiated and poorly differentiated tumors ($\times 200$). (B) Distinct patterns of expression of hepatic differentiation markers in HCC cell lines established from tumors with different histological grades as measured using RT-PCR. (C) The relative mRNA expression levels of those molecules were quantified and normalized according to GAPDH expression.

Fig. 3. Genetically engineered expression of KLF4 and its impact on expression of differentiation-associated biomarkers in and morphology of HCC cells

(A) SNU387 cells were transfected with 4 μ g of a control pcDNA3.1 vector (Ctrl), 1 μ g of a pFLAG-KLF4 vector plus 3 μ g of a pcDNA3.1 vector (pFLAG-KLF4 1 μ g), 4 μ g of a pFLAG-KLF4 vector (pFLAG-KLF4 4 μ g), and 4 μ g of a pFLAG-KLF4 ZFD vector (pFLAG-KLF4 ZFD 4 μ g) in six-well plates. PLC/PRF/5 cells were transfected with control siRNA (siCtrl) or KLF4 siRNA (siKLF4) in six-well plates at the indicated doses. Total RNA was harvested from the cultures 48 hours after transfection and reverse-transcribed into cDNA. The expression of KLF4 and differentiation-associated markers was measured using Real-time PCR. (B) Left panel: SNU387 and PLC/PRF/5 Cells were transfected as in A, total protein lysates were prepared 48 hours after transfection and subjected to Western blot analysis. Right panel: Western blot analysis of KLF4, E-cadherin, HNF-6, and C/EBP α protein expression in SNU387 cells treated with sodium butyrate (NaBu). (C) SNU387 cells were transfected with control pcDNA3.1 (Ctrl) or a pFLAG-KLF4 expression vector (pKLF4) or pFLAG-KLF4 ZFD expression vector (pKLF4 δ ZFD) and submitted to double

immunofluorescent staining for KLF4 and albumin (left panels) or KLF4 and AFP (right panels) 48 hours after transfection. Nuclei were stained with DAPI ($\times 200$). (**D**) Different morphologies of HCC cells transfected with pcDNA3.1 (Ctrl) or pFLAG-KLF4 (pKLF4) plasmids ($\times 200$).

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Fig. 4. Influence of HNF-6 expression on differentiation and biological behavior of HCC cells (A) SNU387 cells were transfected with a pHNF-6-GFP expression vector (pHNF-6) or control vector pCMV6-AC-GFP (Ctrl), and PLC/PRF/5 cells were transfected with HNF-6 siRNA (siHNF-6) or control siRNA (siCtrl). The cultures were incubated for 48 hours. Total RNA was harvested, the expression of HNF-6 and differentiation-associated markers was measured using Real-time PCR (left panels). Different morphologies of HCC cells transfected with pCMV6-AC-GFP (Ctrl) or pHNF-6-GFP (pHNF-6) plasmids ($\times 200$, right panels). (B) SNU387 and SNU423 cells were treated as described above, and cell invasion was assessed. Representative photos of cell invasion are shown (left panels), and the control cell groups (Ctrl) were given an arbitrary invasion percentage of 100% (right panels). * $P < 0.05$ in a comparison of the pHNF-6-treated group with the control group. (C) SNU387 and SNU423 cells were treated as described above for a scratch assay. Shown are photos taken at the indicated time points after the cultures were wounded by scratching (left panels). Quantification of cell migration is shown on the right measured by ImageJ2x software (right panels). (D) SNU387 and SNU423 cells were treated as described above and subjected to

cell proliferation assay (left panels). SNU387 cells were prepared as described in *A* and subjected to cell-cycle analysis (right panels).

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Fig. 5. Regulation of HNF-6 expression by KLF4 and the influence of HNF-6 expression on KLF4-induced HCC differentiation

(A) SNU387 cells were transfected with control siRNA (siCtrl) or KLF4 siRNA (siKLF4) as indicated for 24 hours and then submitted to treatment with or without sodium butyrate (5mM) for 48 hours. Total RNA and protein lysates were prepared for RT-PCR (left panel) and Western blot (right panel) analysis, respectively. (B) Immunohistochemical analysis of KLF4 and HNF-6 protein 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^{-/-}; Klf4^{fl/fl}* (1# and 2#) and *Alb-Cre^{+/+}; Klf4^{fl/fl}* (3# and 4#) mice at the age of 20 weeks were extracted and subjected to RT-PCR analysis of *Klf4* and *Hnf-6* expression (right panel) and Western blotting analysis (left panel). 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; lower panel). (D) SNU387 and SNU423 cells were co-transfected 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 real-time PCR analysis (left panels). The relative mRNA expression levels of those molecules were quantified and normalized according to GAPDH expression (right panels).

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Fig. 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) The HNF-6 promoter reporters were transfected in triplicate with pFLAG-KLF4, pFLAG-KLF4 ZFD, or the control vector pcDNA3.1 into SNU387 (left panel) and SNU423 (right panel) cells. Co-transfection of pFLAG-KLF4 and 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) The 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. Co-transfection of control siRNA (siCtrl) and pGL3-basic

was used as a negative control. The promoter activity was measured and expressed as described in B. $*P<.05$ and $**P<.001$ in a comparison of the control and treated groups. (D) Chromatin was extracted from SNU387 cells transfected with pFLAG-KLF4 (KLF4 OE), pFLAG-KLF4 ZFD (KLF4 ZFD OE), or the control vector pcDNA3.1 (Ctrl; left panel) and from PLC/PRF/5 cells treated with KLF4 siRNA (siKLF4) or control siRNA (siCtrl; right panel). 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.