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Disruption of transforming growth factor-β signaling through β-spectrin ELF leads to hepatocellular cancer through cyclin D1 activation

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Abstract

Transforming growth factor-β (TGF-β) signaling members, TGF-β receptor type II (TBRII), Smad2, Smad4 and Smad adaptor, embryonic liver fodrin (ELF), are prominent tumor suppressors in gastrointestinal cancers. Here, we show that 40% of $elf^{+/-}$ mice spontaneously develop hepatocellular cancer (HCC) with markedly increased cyclin D1, cyclin-dependent kinase 4 (Cdk4), c-Myc and MDM2 expression. Reduced ELF but not TBRII, or Smad4 was observed in 8 of 9 human HCCs ($P < 0.017$). ELF and TBRII are also markedly decreased in human HCC cell lines SNU-398 and SNU-475. Restoration of ELF and TBRII in SNU-398 cells markedly decreases cyclin D1 as well as hyperphosphorylated-retinoblastoma (hyperphosphorylated-pRb). Thus, we show that TGF-β signaling and Smad adaptor ELF suppress human hepatocarcinogenesis, potentially through cyclin D1 deregulation. Loss of ELF could serve as a

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primary event in progression toward a fully transformed phenotype and could hold promise for new therapeutic approaches in human HCCs.

Keywords

hepatocellular carcinoma; ELF; cyclin D1; transforming growth factor-β; cell cycle

Introduction

Hepatocellular cancer (HCC) is a poor prognosis cancer with a 5-year survival of less than 5%, with a recent rise in incidence in the United States (Parkin *et al.*, 2001). Currently, only 12% of diagnosed HCC patients are eligible for curative surgical therapy, which includes hepatic resection and liver transplantation (El-Serag, 2004; Roberts and Gores, 2005). Development of HCC is a multistep process often beginning with cirrhosis, progressing to adenoma and dysplastic nodule formation.

Risk factors for HCC include viral hepatitis B and viral hepatitis C (HCV) infection that activate JAK/STAT signaling, induce overexpression of insulin-like growth factor-II in 16–40% of HCCs (Breuhahn *et al.*, 2006), and disrupt p53, $p55^{sen}$ and $p21^{WAF1/CIP1/SDI1}$ (Ueda *et al.*, 1995; Feitelson, 1999; Huo *et al.*, 2001). Activation of the Wnt signaling pathway with β-catenin mutations is observed in 17–40% of HCCs and axin-2 inactivation in 3–10% of cases (Taniguchi *et al.*, 2002; Ishizaki *et al.*, 2004). Cyclin D1, Cdk4 and c-Myc levels are raised in approximately 50% of HCCs (Tiniakos *et al.*, 1993; Ito *et al.*, 1999). Concomitantly, pRb is functionally inactivated in a majority of HCCs (Malumbres and Barbacid, 2001; Thorgeirsson and Grisham, 2002; Edamoto *et al.*, 2003). Approximately, 40% of HCCs display microsatellite instability (MSI) or inactivating mutations in the mismatch repair genes, hMSH2 and hMLH1 (Macdonald *et al.*, 1998; Yamamoto *et al.*, 2000). MSI is in turn associated with mutations in TGF-β receptor type II (TBRII), M6P/IGFIIR and BAX genes.

Importantly, the TGF-β signaling pathway plays a key role in regulating endoderm differentiation and suppressing cancers of the foregut. While the TGF-β superfamily is composed of more than 40 members, the basic signaling cascade of TGF-β involves type I and type II transmembrane serine/threonine kinase receptors (TBRI and TBRII). Decreases in TBRI and TBRII occur in 50 and 60% of HCCs respectively (Sue *et al.*, 1995; Kiss *et al.*, 1997; Breuhahn *et al.*, 2006). Intracellular signaling occurs through Smads, which are classified into three functional classes: (i) receptor-activated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5 and Smad8; (ii) co-mediator Smads: Smad4; (iii) inhibitory Smads: Smad6 and Smad7 (Derynck *et al.*, 2001; Edamoto *et al.*, 2003). Activation of Smads by TGF-β results in association of R-Smads with Smad4 followed by nuclear translocation and target gene activation (Attisano and Wrana, 2002).

Smad2 mutations occur in 5% of HCCs (Yakicier *et al.*, 1999; Longerich *et al.*, 2004). Inhibitory Smad7 is upregulated in 60% of advanced HCCs (Park *et al.*, 2004). Smad4 mutations are also seen in 10% of HCCs. Yet to date, none of the mouse mutant models in the Smad family spontaneously develop HCC. Antiproliferative responses of TGF-β occur

primarily by inhibition of G_1–S phase transition through activation of pRb, Cdk inhibitors, p15 and p21, as well as inhibition of c-Myc, Cdk2, Cdk4, cyclin E, cyclin A and cyclin D1 (Sherr, 1996; Siegel *et al.*, 2003; Mishra *et al.*, 2005; Knudsen *et al.*, 2006).

Smad function is highly dependent upon adaptor proteins such as embryonic liver fodrin (ELF), SARA and microtubules (Tsukazaki *et al.*, 1998; Tang *et al.*, 2003; Mishra *et al.*, 2005). ELF, a β-spectrin, is first isolated from mouse E11 libraries and is shown to play a crucial role in the propagation of TGF-β signaling (Mishra *et al.*, 1999). Specifically, ELF associates with Smad3 and the TGF-β receptor complex; this interaction is followed by interaction with Smad4 and leads to their translocation to the nucleus. Disruption of *elf* expression by gene knockout was found to result in miscolocalization of Smad3 and Smad4, and disruption of TGF-β signaling (Tang *et al.*, 2003). The epistatic significance of spontaneous and frequent HCC development in $elf^{+/-}$ mutant mice presents an ideal model for further analysis of the role of ELF and TGF-β signaling in hepatocarcinogenesis. We demonstrate that loss of ELF results in cell-cycle disruption with significant increases in Cdk4, cyclin D1 and pRb hyperphosphorylation. Loss of ELF is observed in human HCC specimens ($P < 0.017$) as well as in human HCC cell lines. Restoration of ELF and TBRII results in decreased cyclin D1 and pRb-Pexpress ion in human HCC cell lines, demonstrating a strong tumor suppressor role for ELF in hepatocarcinogenesis.

Results

Elf$^{+/-}$ mutant mice develop HCC

To determine the role of ELF in HCC, we generated $elf^{+/-}$ mice. The presence of mutations was monitored by use of the PCR as described previously (Tang *et al.*, 2003). Out of 40 $elf^{+/-}$ mice, 16 (40%) developed HCC, ranging from well to poorly differentiated carcinoma, as early as 15 months of age; however, none of the wild-type mice developed HCC (Figure 1a and b). Histopathological examination of the $elf^{+/-}$ livers also demonstrated areas of centrilobular steatosis and dysplasia with large nuclei, high-grade atypia, nuclear disarray, stratification and mitosis (Supplementary Figure S1a–d). The expression of caspase3 (a marker for apoptosis) is markedly reduced while expression of phosphorylated-histone H3 (Ser^{10}) (p-histone H3, a marker for active proliferation) is significantly increased in $elf^{+/-}$ HCC compared with wild-type normal liver tissues (Supplementary Figure S2a–h).

Cyclin D1, Cdk4, c-Myc and MDM2 expression markedly increased in *elf*$^{+/-}$ HCC tissues

The occurrence of spontaneous hepatocarcinogenesis in heterozygous *elf* mutants compared with none in the wild type suggests that disruption or inactivation of ELF could lead to malignant transformation and cell-cycle deregulation in $elf^{+/-}$ mutant mice. Immunohistochemical labeling demonstrates a marked increase in cyclin D1, Cdk4, c-Myc and MDM2 in HCCs from mutant mice compared with the normal liver tissue (Figure 1c–j, Supplementary Figure S3).

ELF, TBRII, Smad4 and cyclin D1 expression in human HCC tissues

To determine whether loss of ELF occurs in human HCCs, immunohistochemical analysis was performed in 19 human liver specimens using a peptide-specific anti-ELF antibody

(against residues 2–14; Tang *et al.*, 2003). Nine samples represented HCCs at different grades (Table 1), 10 samples were normal control liver tissues. In normal liver tissues, ELF expression is distributed ubiquitously throughout the liver parenchyma, at the basolateral membrane, cytoplasmic and nuclear compartments of hepatocytes. In all 10 normal liver tissues, hepatocytes display cytoplasmic and greater than 70% nuclear labeling of ELF ($P < 0.26$; Figure 2a and Supplementary Figure S4). Nuclear ELF expression is significantly lower in HCC specimens (Figure 2b and Supplementary Figure S4, HCC Sample 2). Eight out of 9 HCC specimens display a decrease in ELF labeling (Table 1). Using global χ^2 test, the decrease in ELF expression in HCCs is statistically significant ($P < 0.017$; Table 2). Strong expression of TBRII localized to the hepatocyte cell membrane is observed in normal human liver tissues (Figure 2c, Supplementary Figure S5 and Table S1). However, in HCCs, TBRII is predominantly expressed in the cytoplasmic compartment in all nine HCC samples (Figure 2d, Supplementary Figure S5 and Table S1, HCC Sample 6). Smad4 expression tended to decrease in HCC (Figure 2e and f, Supplementary Figure S5 and Table S1, HCC Sample 2). Statistically, TBRII and Smad4 expression do not correlate with cancer progression.

Interestingly, as in the HCCs derived from *elf*$^{+/-}$ mice, human HCCs also display markedly increased cyclin D1 expression (Figure 3a–d) compared with normal liver specimens. This finding is consistent with the overexpression of cyclin D1 in *elf*$^{+/-}$ HCC tissues.

Disruption of TGF-β signaling in human HCC cell lines

The development of HCC in *elf*$^{+/-}$ mice led us to analyse TGF-β signaling pathway members TBRI, TBRII, Smad4 and ELF in three human HCC cell lines (SNU-398, SNU-449 and SNU-475). Expression of ELF and TBRII is significantly reduced in SNU-398 and SNU-475 cells. However, Smad4 and TBRI expression was comparable in all three human HCC cell lines (Figure 3e and f).

Restoration of ELF and TBRII leads to a decrease in cyclin D1 and hyperphosphorylated-retinoblastoma expression

Increased expression of cyclin D1 and Cdk4 in HCCs derived from *elf*$^{+/-}$ mice suggest that the loss of ELF protein might result in cell-cycle deregulation. To examine this hypothesis, we transfected an ELF expression vector into the human HCC cell lines and examined cyclin D1, Cdk4 and pRb phosphorylation. In SNU-398, which has a near complete loss of ELF, there is a fourfold reduction of cyclin D1 expression after rescue of ELF. Similarly, reduced cyclin D1 was observed in SNU-475 cells (Figure 3g and h). However, in SNU-449, which has high expression of ELF, there was no significant change in cyclin D1 expression. The most significant reduction of cyclin D1 is observed when both ELF and TBRII expression are restored under TGF-β stimulation. Under these conditions, a nearly sixfold reduction in cyclin D1 expression is observed as compared with naïve SNU-398 cells (Figure 4a–d). Concomitantly with the change in cyclin D1, ELF and TBRII reduce hyperphosphorylated-pRb (3.3-fold) in SNU-398 cells (Figure 4e and f).

Discussion

The TGF-β signaling pathway plays a critical role in diverse cellular functions, including inhibition of cell growth, cell migration, differentiation and matrix stabilization. In the lower gastrointestinal tract, TGF-β serves as a tight regulatory signal controlling cell proliferation through G_1 cell-cycle inhibition that involves pRb activation as well as by sequential inactivation of cyclins and Cdks, such as Cdk4 which associates with one of the D-type cyclins namely D1, D2 or D3 in a context-dependent manner (Quaroni *et al.*, 1979; Hunter and Pines, 1994; Ko *et al.*, 1998). Given its antiproliferative role in various epithelial cells as well as in liver, the TGF-β signaling likely suppresses hepatocellular carcinogenesis. Accordingly, *TGF-β1*$^{+/-}$ mutant mice are significantly more susceptible (3.2-fold) to diethylnitrosamine and phenobarbital after 9 months of treatment compared with wild types (Tang *et al.*, 1998). In addition, transgenic mice overexpressing a dominant negative TBRII exhibit significant increase in incidence, size and multiplicity of preneoplastic lesions and of HCCs (Tang *et al.*, 1998; Kanzler *et al.*, 2001). Also, in chronic hepatitis C infection, HCV-derived core protein associates with the Smad3 MH1 domain inhibiting its DNA-binding activity (Pavio *et al.*, 2005). Smad2 and Smad4 gene mutations have also been identified in 5–10% HCCs (Yakicier *et al.*, 1999; Longerich *et al.*, 2004).

Deficiency in the ELF protein results in mislocalization of Smad3 and Smad4 and consequent disruption of TGF-β-dependent transcription (Tang *et al.*, 2003). In this study, we demonstrate ELF as the strongest liver tumor suppressor in the TGF-β signaling pathway; haploinsufficiency at the *elf* locus results in spontaneous HCC development. In addition, we show that loss of ELF results in cell-cycle deregulation, as evident by an increase in cyclin D1, Cdk4, c-Myc and MDM2 levels in *elf*$^{+/-}$ HCC specimens. Increased expression of protein or mRNA levels of cyclin D1 and Cdks, c-Myc, and MDM2 in chronic hepatitis or HCCs has also been observed by several other groups (Ito *et al.*, 1999; Joo *et al.*, 2001; Sundarrajan *et al.*, 2002; Ueta *et al.*, 2002; Zhang *et al.*, 2002; Masaki *et al.*, 2003).

The importance of ELF in human HCC is further demonstrated by the decrease in ELF expression in the human HCC tissues (89% of the cases). Similar to the findings from the *elf*$^{+/-}$ HCC specimens, there is a marked increase in cyclin D1 expression in the human HCC tissues. Our hypothesis is supported by the fact that restoration of ELF results in a decrease of cyclin D1 levels in human HCC cell lines that display decreased ELF expression. We also show decreased ELF levels in two human HCC cell lines (SNU-398 and SNU-475). In addition, these cell lines also display decreased TBRII. Restoration of both ELF and TBRII suppresses cyclin D1 with TGF-β stimulation. Once TGF-β signaling is re-established, cell-cycle progression is suppressed by a decrease in cyclin D1 synthesis and an increase in active pRb.

Thus, our study has, for the first time, provided a direct correlation between the role of ELF as a tumor suppressor and hepatocarcinogenesis. Loss of ELF could serve as a crucial step in hepatocyte transformation through cyclin D1. Exploration of the mechanisms behind inactivation of TGF-β/ELF signaling and its restoration could hold promise for new therapeutic approaches in human HCC.

Materials and methods

Generation of *elf*$^{+/-}$ mice and analysis

Generation of *elf*$^{+/-}$ knockout in mice has been described previously (Tang *et al.*, 2002). The presence of genetic mutation was monitored by use of the PCR as described previously (Tang *et al.*, 2002). Double homozygous *elf* mutants were embryonic lethal. *Elf*$^{+/-}$ mutant mice were maintained on a mixed 129SvEv/Black Swiss background. Mice were monitored twice a week to detect any abnormal phenotypic changes including tumor formation. All animal procedures were approved by the Institutional Animal Care and Use Committee of Georgetown University Medical Center, Washington, DC.

Histology and immunohistochemical staining

Labeling of tissues was performed as previously described (Tang *et al.*, 2005). The following antibodies were used: cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-246), Cdk4 (Santa Cruz Biotechnology, sc-6246), c-Myc (Santa Cruz Biotechnology, sc-40) and MDM2 (Calbiochem, San Diego, CA, USA, OP115). In addition, immunohistochemical labeling to apoptotic marker, caspase3 and proliferative marker, phosphorylated-histone, was performed (Promega, Madison, WI, USA, G748A and Santa Cruz Biotechnology, sc-8656), All human tissue procedures were approved by Institutional Review Board of Georgetown University Medical Center, Washington, DC.

Tumor specimens

Formalin-fixed and paraffin-embedded human normal livers and HCC specimens were obtained from the Department of Pathology, Lombardi Cancer Center, Georgetown University Medical Center. Nine HCC specimens collected from patients with varying risk factors, representing different grades and stages of HCC and 10 normal liver specimens were analysed for ELF expression. All the specimens were collected after liver resection or autopsy. Cancer grade was determined by histology according to Edmondson criteria (Edmondson and Steiner, 1954). We designated the Edmondson grade I–II as low-grade HCC and Edmondson grade III–IV as high-grade HCC (Table 1).

Labeling was performed as described previously (Tang *et al.*, 2005). The frequency of cells positive for ELF was determined by counting the total number of cells and total positively stained cells in randomly selected × 40 magnification fields to include at least 1000 cells. Average numbers from the field sets were then determined and reported as the percentage of positively stained cells to the total numbers of cells. The percentage of positive stain was categorized in three groups (negative −, 0–15%; 1+, 16–69%; 2+, >70%). Antibodies against TBRII, Smad4 and cyclin D1 (Santa Cruz Biotechnology, sc-220, sc-7966 and sc-246, respectively) were used to determine expression of these proteins. TBRII and Smad4 labeling was measured in three different grades; + +, intense labeling; +, moderate labeling; −, loss of or reduced labeling.

Statistical analysis

Global χ^2 test was used to test the hypothesis that the coefficient of each variable was equal to 0. Results from the immunohistochemical labeling of ELF in HCC samples as compared

with normal liver samples were used to assess the statistical significance. A $P < 0.05$ was considered statistically significant, and all tests were two-sided. All tests were performed with SPSS 10.1 software (SPSS Inc., Chicago, IL, USA).

Cell culture

Human HCC cell lines SNU-398, SNU-448 and SNU-475 (American Type Culture Collection (ATCC), Manassas, VA, USA, CRL-2233, 2234 and 2236, respectively) were grown in RPMI-1640 (ATCC) supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2. Mouse embryo-derived fibroblasts (MEFs) harboring the null allele *elf* as well as wild type were derived as described previously (Tang *et al.*, 2003).

Western blot analysis

Cells were grown in a monolayer up to 70% confluence before harvesting for western blot analysis as described previously (Tang *et al.*, 2003). All experiments were conducted in triplicate. The following antibodies were used: ELF (against residues 2–14; (Tang *et al.*, 2003) and TBRII, TRBI, and Smad4 (Santa Cruz Biotechnology, sc-220, sc-402 and sc-7966, respectively). For protein loading controls, antibody against β-actin (Santa Cruz Biotechnology, sc-1616) was used.

Restoration of ELF and TBRII

Human ELF was cloned by PCR from the reverse transcribed cDNAs of human RNA. The primer sequences were: forward; 5-ACCATGGAATTGCAGAGGACGTCTAG-3, reverse; 5-CAGTCCAGACCATGGCTGGTC-3. The PCR products were cloned into pcDNA3.1/V5-HisTOPO TA plasmid (Invitrogen, Carlsbad, CA, USA). Human TBRII plasmid was a kind gift from Dr Anita Roberts.

For transfection, cells were seeded in a six-well plate to achieve 70% cell density. Twenty-four hours after cell plating, full-length V5-ELF construct and TBRII plasmids were transfected using Fugene 6 kit (Roche, Indianapolis, IN, USA) and further incubated for 48 h until harvest. To confirm transfection efficiency, primary antibodies to V5 and TBRII were utilized (Invitrogen, 46–0703 and Santa Cruz Biotechnology, sc-220, respectively). Expression of cylin D1 and Cdk4 was detected using antibodies to cyclin D1 (Santa Cruz Biotechnology, sc-246) and Cdk4 (Santa Biotechnology, sc-6246), with and without TGF-β_1 stimulation (1 ng/ml) 1 h before cell harvesting. Expression of hyperphosphorylated-pRL protein was detected using an antibody against the ser780 residue (Cell Signaling Technology, Inc., Danvers, MA, USA, 9307). Expression of α-tubulin (Santa Biotechnology, sc-8035) was used as a loading control. All experiments were conducted in triplicate to assure reproducibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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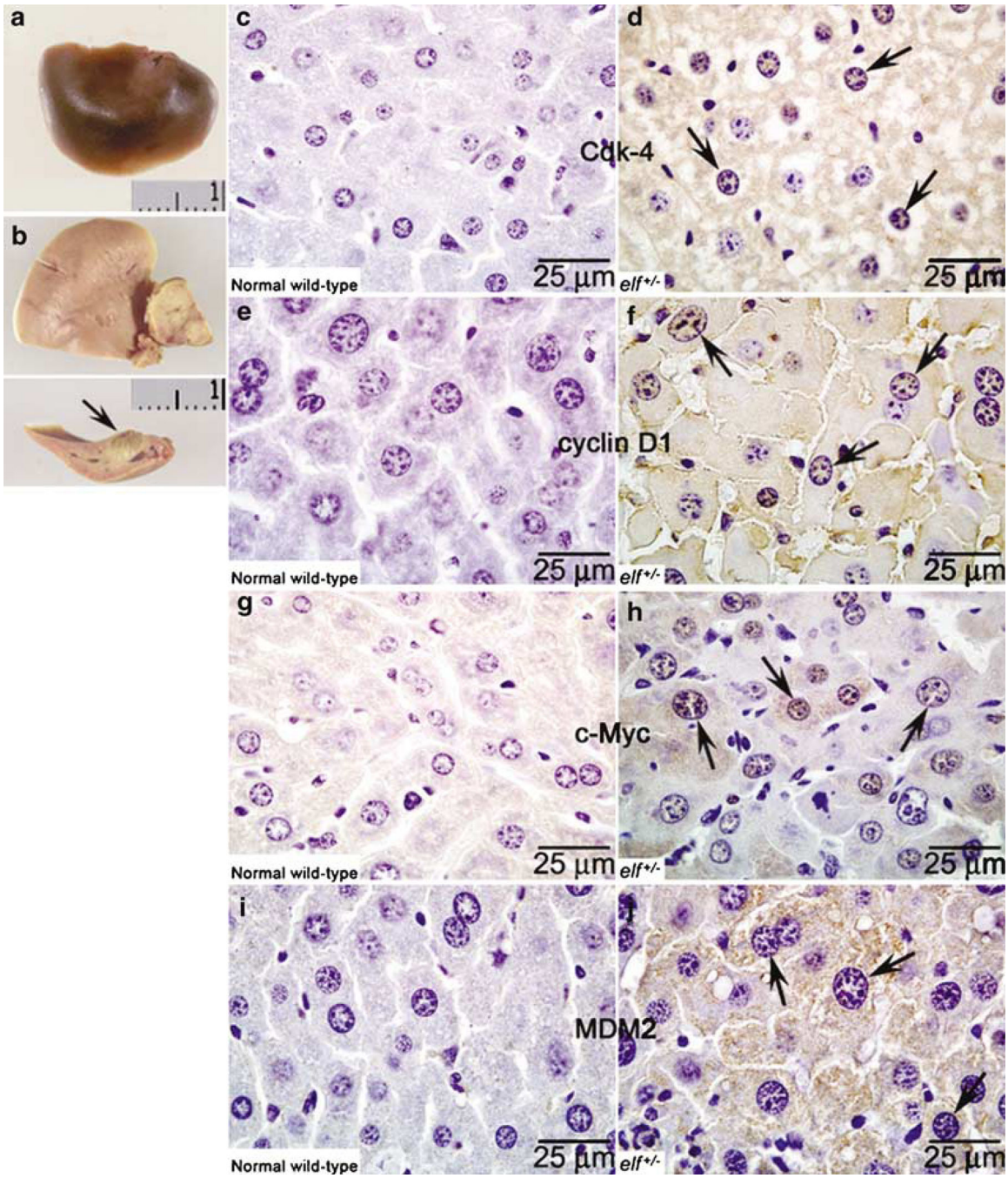

Figure 1.

Elf$^{+/-}$ mutant mice develop HCC with cell-cycle deregulation. Macroscopic picture of wild-type normal liver (**a**) and from *elf*$^{+/-}$ HCC tissues (**b**, arrow); scale bar in centimeter (cm). (**c**–**j**) Immunohistochemical analysis of cell-cycle regulatory protein expression; Cdk4, cyclin D1, c-Myc and MDM2. Increase in Cdk4 expression is seen in HCC sample from *elf*$^{+/-}$ mouse (**d**) as compared with normal liver (**c**). Similarly, labeling of cyclin D1 is significantly increased in *elf*$^{+/-}$ HCC (**f**) as compared with normal sample (**e**). Increased expression of c-Myc and MDM2 are observed in HCC specimens (**h** and **j**), which are

absent in normal counterparts (**g** and **i**). Scale bar is in μm. Please refer to Supplementary Figure S1.

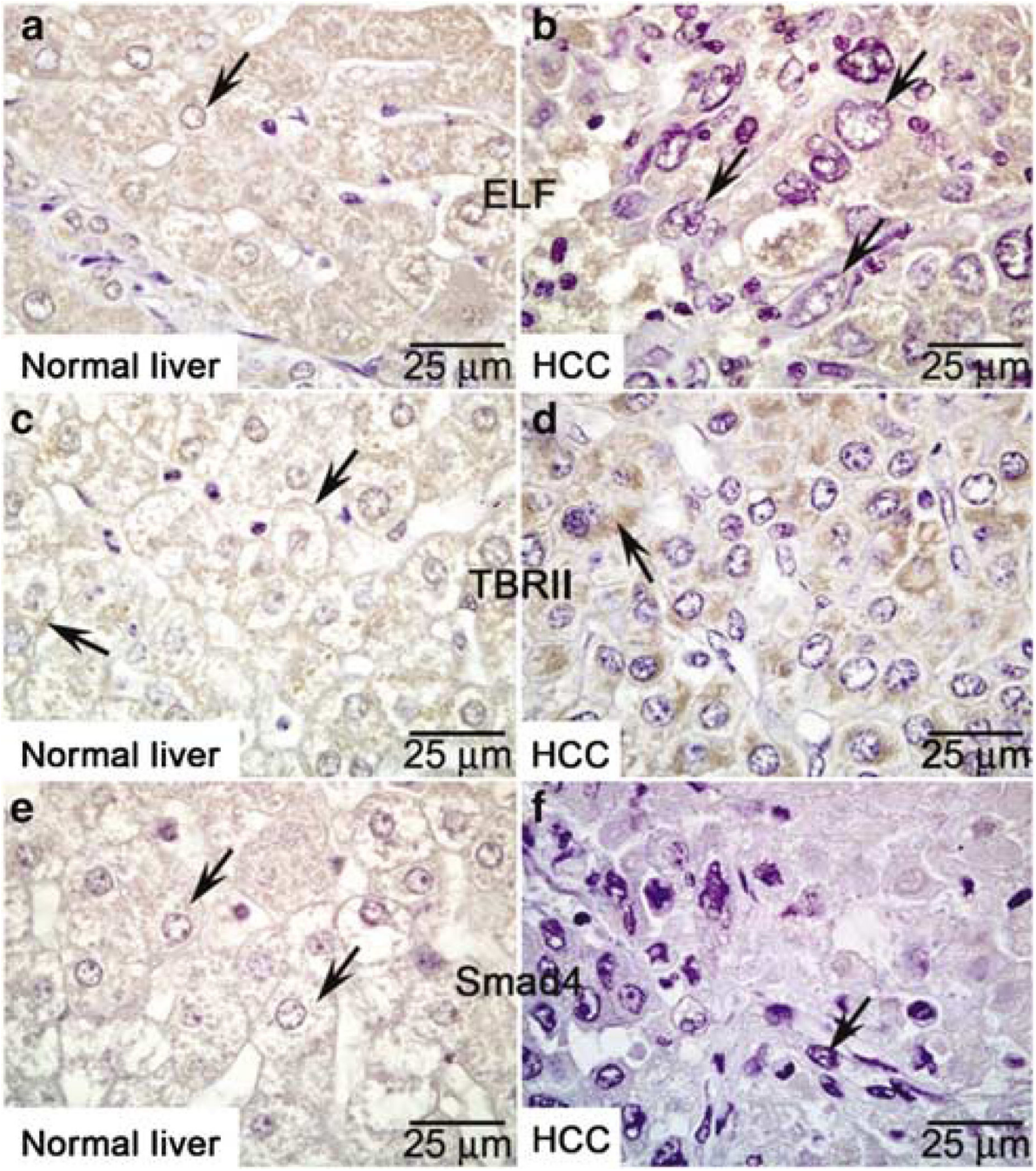

Figure 2.

Expression of TGF-β signaling members: ELF, TBRII and Smad4 in human HCC. In normal liver, there is strong cytoplasmic and nuclear ELF expression (**a**, arrow), whereas there is a significant decrease in nuclear expression of ELF in HCC tissue (**b**, HCC Sample 2, arrows). TBRII is localized in cell membrane in normal liver (**c**, arrows), but ectopically accumulated in cytoplasm in HCC (**d**, HCC Sample 6, arrow), Smad4 expression is decreased in HCC (**f**, HCC Sample 2, arrows) compared with normal liver (**e**, arrows). Scale bar is in μm. Please refer to Table 1, Supplementary Figures S4 and S5.

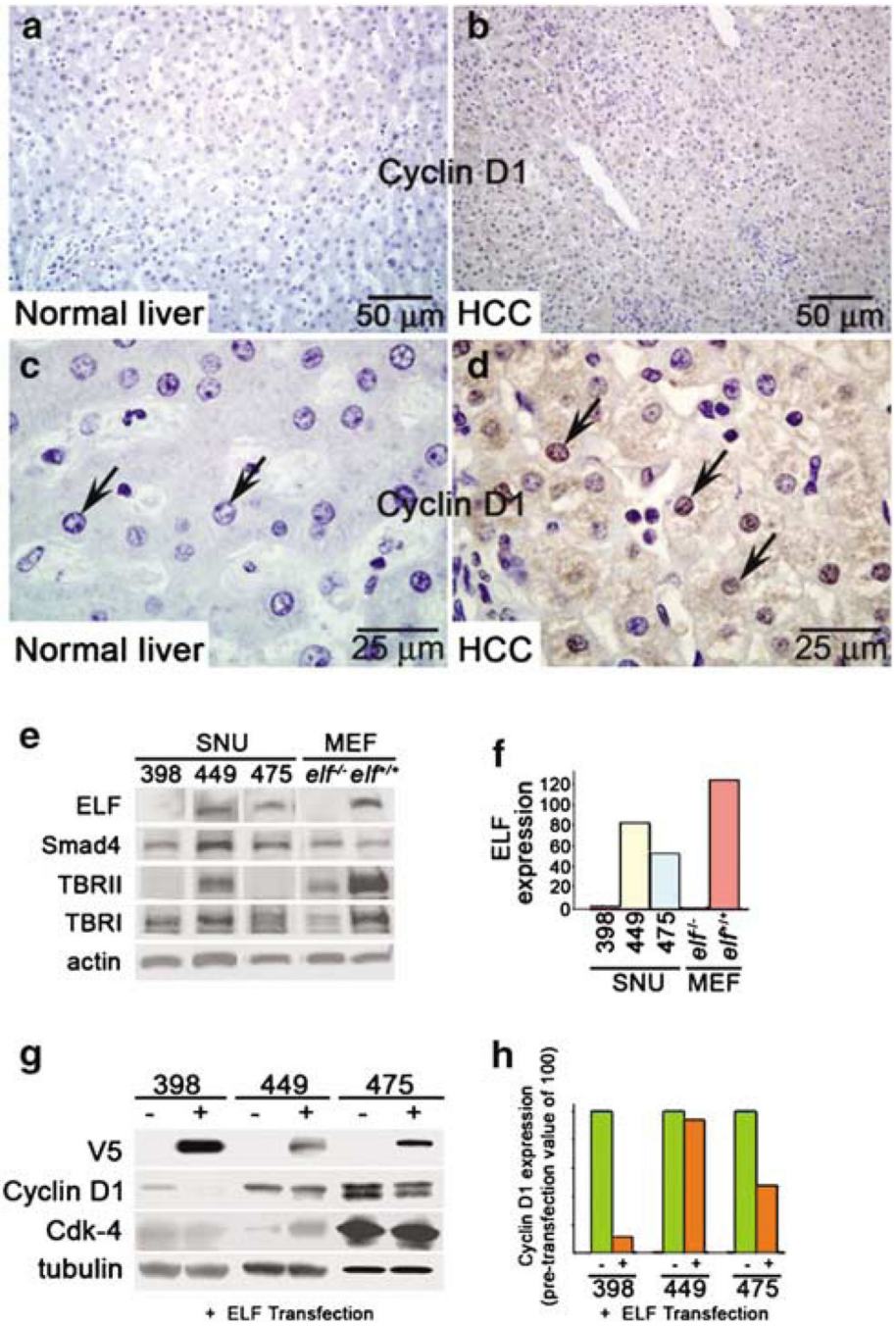

Figure 3.

Increased cyclin D1 expression in human HCC tissue and cell lines and the effect of ectopic ELF expression. Cyclin D1 is markedly increased in human HCC (**b** and **d**) seen here in lower and higher power fields compared with normal liver (**a** and **c**). TGF-β signaling is aberrant in two of three different human HCC cell lines, SNU-398, SNU-449 and SNU-475 (**e**). MEFs derived from *elf*$^{-/-}$ and *elf*$^{+/+}$ embryos are used as controls. ELF expression is significantly decreased in SNU-398 and SNU-475. TBRII expression is decreased in SNU-398 and SNU-475. (**f**) Histogram representation of ELF with integrated density value

(IDV) adjusted by actin-loading control. (**g**) Restoration of ELF by transfecting full-length ELF leads to decrease in cyclin D1 expression in SNU-398 and SNU-475. (**h**) IDV representation of ELF expression adjusted by tubulin loading control. Pre-transfected ELF levels in all cell lines are adjusted to 100 to demonstrate the relative changes after transfection.

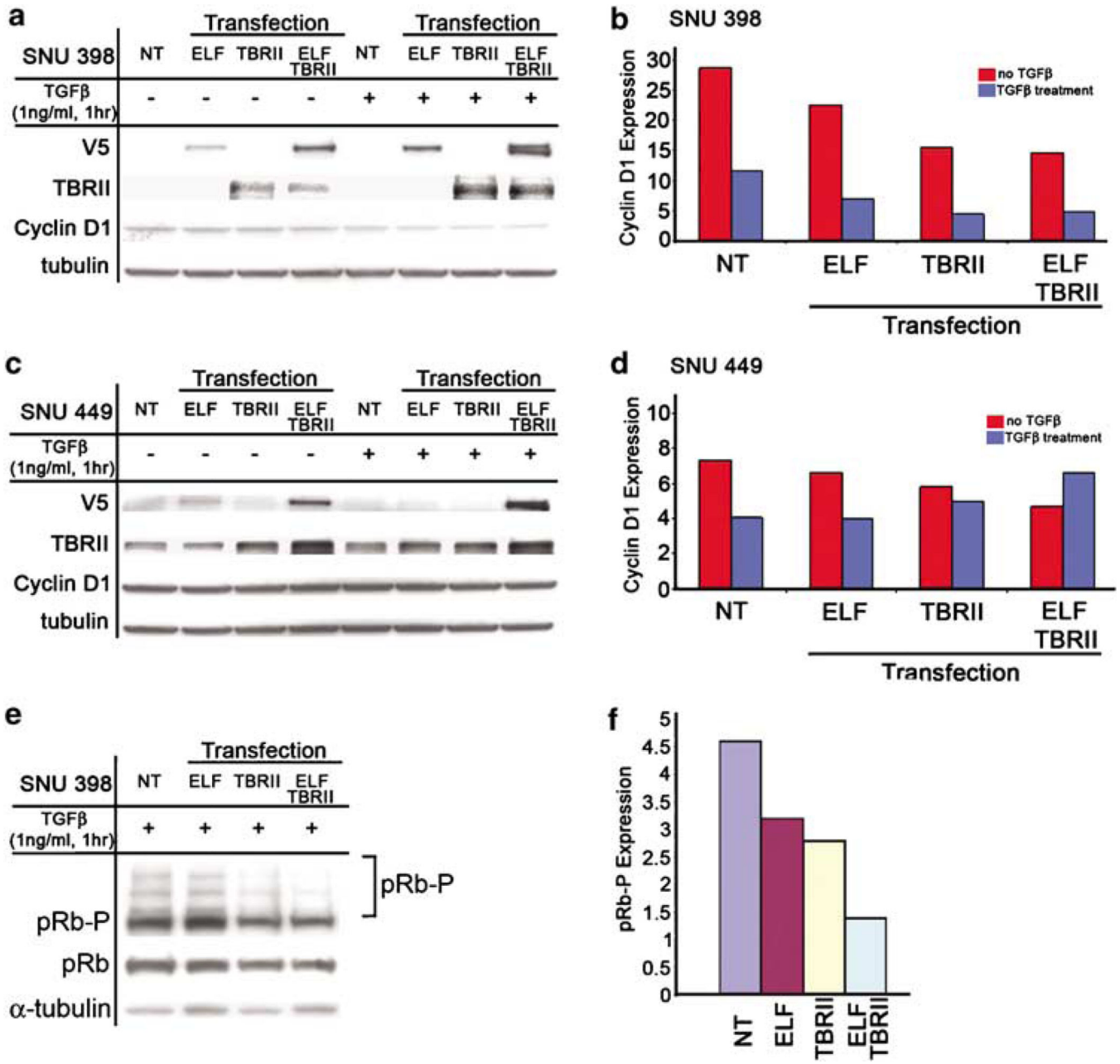

Figure 4.

Restoration of ELF and TBRII results in decreased cyclin D1 and hyperphosphorylated-retinoblastoma (pRb-P) expression. Restoration of ELF and TBRII suppresses cyclin D1 with TGF-β stimulation in SNU-398 cells (**a**). No significant change in cyclin D1 is observed in SNU-449 cells (**c**). Histogram of cyclin D1 expression with IDV adjusted by tubulin (**b** and **d**). Concomitant alteration in pRb-Pis observed in SNU-398 cells that lack ELF and TBRII (**e**). An additive decrease in pRb-Pis demonstrated following co-transfection of ELF and TBRII. Histogram of pRb-Pwith IDV-adjusted value by tubulin loading control (**f**).

Table 1

Histologic classification of 19 hepatocellular cancers and normal liver tissues in the study and percentage of ELF staining

Sample (HCC)	Histology	Nuclear ELF labeling	Sample (NL)	Histology	Nuclear ELF labeling
1	Moderately to poorly differentiated HCC with focal fibrosis (III–IV)	−	1	Normal	+ +
2	Moderately differentiated HCC (II)	−	2	Normal	+ +
3	Moderately to poorly differentiated HCC with focal fibrosis (III–IV)	+	3	Normal	+ +
4	Moderately to poorly differentiated HCC with cirrhosis (III)	+ +	4	Normal	+ +
5	Moderately differentiated HCC (II)	+	5	Normal	+ +
6	Moderately to poorly differentiated HCC with cirrhosis (III)	−	6	Normal	+ +
7	Moderately differentiated HCC with bridging fibrosis (III)	+	7	Normal	+ +
8	Moderately to poorly differentiated HCC with cirrhosis (III)	−	8	Normal	+ +
9	Moderately differentiated HCC with mild septal fibrosis (II)	−	9	Normal	+ +
			10	Normal	+ +

Abbreviations: ELF, embryonic liver fodrin; HCC, hepatocellular cancers; NL, normal liver. + +, >70% nuclear labeling; +15–70% nuclear labeling; +, <15% nuclear labeling.

Table 2

Immunohistochemical labeling results of liver tissues

Tissue type	*Labeling for*														
	ELF					*TBRII*					*Smad4*				
	+ +	+	−	*NA*	**P**	+ +	+	−	*NA*	**P**	+ +	+	−	*NA*	**P**
Normal	10	0	0	−	0.026*	3	0	0	6	NS	1	2	0	7	NS
Carcinoma	1	3	5	−	0.017*	5	3	1	−	NS	0	3	2	4	NS

Abbreviations: ELF, embryonic liver fodrin; NA, not available; TBRII, transforming growth factor-β receptor type II. Data obtained were statistically significant at $P < 0.05$. For ELF, + +, >70% nuclear labeling; +, 15–70% nuclear labeling; −, <15% nuclear labeling; for TBRII and Smad4, + +, intense labeling; +, moderate labeling; −, loss of or reduced labeling.

*Statistically significant.

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