Priority Report

mTOR Signal and Hypoxia-Inducible Factor-1α Regulate CD133 Expression in Cancer Cells

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Abstract

The underlying mechanism regulating the expression of the cancer stem cell/tumor-initiating cell marker CD133/prominin-1 in cancer cells remains largely unclear, although knowledge of this mechanism would likely provide important biological information regarding cancer stem cells. Here, we found that the inhibition of mTOR signaling up-regulated CD133 expression at both the mRNA and protein levels in a CD133-overexpressing cancer cell line. This effect was canceled by a rapamycin-competitor, tacrolimus, and was not modified by conventional cytotoxic drugs. We hypothesized that hypoxia-inducible factor-1α (HIF-1α), a downstream molecule in the mTOR signaling pathway, might regulate CD133 expression; we therefore investigated the relation between CD133 and HIF-1α. Hypoxic conditions up-regulated HIF-1α expression and inversely down-regulated CD133 expression at both the mRNA and protein levels. Similarly, the HIF-1α activator deferoxamine mesylate dose-dependently down-regulated CD133 expression, consistent with the effects of hypoxic conditions. Finally, the correlations between CD133 and the expressions of HIF-1α and HIF-1β were examined using clinical gastric cancer samples. A strong inverse correlation (*r* = −0.68) was observed between CD133 and HIF-1α, but not between CD133 and HIF-1β. In conclusion, these results indicate that HIF-1α down-regulates CD133 expression and suggest that mTOR signaling is involved in the expression of CD133 in cancer cells. Our findings provide a novel insight into the regulatory mechanisms of CD133 expression via mTOR signaling and HIF-1α in cancer cells and might lead to insights into the involvement of the mTOR signal and oxygen-sensitive intracellular pathways in the maintenance of stemness in cancer stem cells. [Cancer Res 2009;69(18):7160–4]

Introduction

The CD133/prominin-1 protein is a five-transmembrane molecule expressed on the cell surface that is widely regarded as a stem cell marker. Growing evidence indicates that CD133 can be used as a cell marker for cancer stem cells or tumor-initiating cells in colon cancer, prostate cancer, pancreatic cancer, hepatocellular carcinoma, neural tumors, and renal cancer (1). Strict regulatory mechanisms governing CD133 expression are thought to be deeply related to inherent cancer stemness; however, such mechanisms remain largely unclear, especially in cancer cells. In brain tumors, the Hedgehog (2), bone morphogenetic protein (3), and Notch (4) signaling pathways have been implicated in the control of CD133+ cancer stem cell function.

Some investigators have shown a relation between hypoxia and CD133 expression in brain tissue. The percentage of CD133-expressing cells was found to increase in a glioma cell line cultured under hypoxic conditions (5), and mouse fetal cortical precursors cultured under normoxic conditions exhibited a reduction in CD133(hi)CD24(lo) multipotent precursors and the failure of the remaining CD133(hi)CD24(lo) cells to generate glia (6). With the exception of these studies in brain tissue, however, data on the expression of CD133 and the involvement of hypoxia and other signaling pathways in cancer cells remains limited.

Several reports have indicated that mTOR is a positive regulator of hypoxia-inducible factor (HIF) expression and activity (7), and the inhibition of HIF-mediated gene expression is considered to be related to the antitumor activity of mTOR inhibitors in renal cell carcinoma (8). We found that mTOR signaling was involved in CD133 expression in gastric and colorectal cancer cells. Thus, we investigated the regulatory mechanism of CD133 in cancer cells.

Materials and Methods

Reagents. 5-Fluorouracil, irinotecan (CPT-11), and rapamycin were purchased from Sigma-Aldrich. Gemcitabine was provided by Eli Lilly. Tacrolimus (LKT Laboratories), LY294002 and wortmannin (Cell Signaling Technology), and deferoxamine mesylate (DFO; Sigma-Aldrich) were purchased from the indicated companies.

Cell cultures and hypoxic conditions. All of the 28 cell lines used in this study were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), except for LoVo (F12; Nissui Pharmaceutical), WiDr, IM95, and HEK293 (DMEM; Nissui Pharmaceutical), and Huvec (Humedia; Kurabo). Hypoxic conditions (0.1% O_2) were achieved using the AnaeroPouch-Anaero (Mitsubishi Gas Chemical) with monitoring using an oxygen indicator.

Real-time reverse transcription-PCR. The methods were previously described (9). The primers used for the real-time reverse transcription-PCR (RT-PCR) were as follows: CD133, forward 5′-AGT GGC ATC GTG CAA ACC TG-3′ and reverse 5′-CTC CGA ATC CAT TCG ACG ATA GTA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPD), forward 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse 5′-ATG GTG GTG AAG ACG CCA GT-3′. GAPD was used to normalize the expression levels in the subsequent quantitative analyses.

Clinical samples. The mRNA expression levels of CD133, HIF-1α, and HIF-1β in gastric cancer specimens were obtained from previously published microarray data (9).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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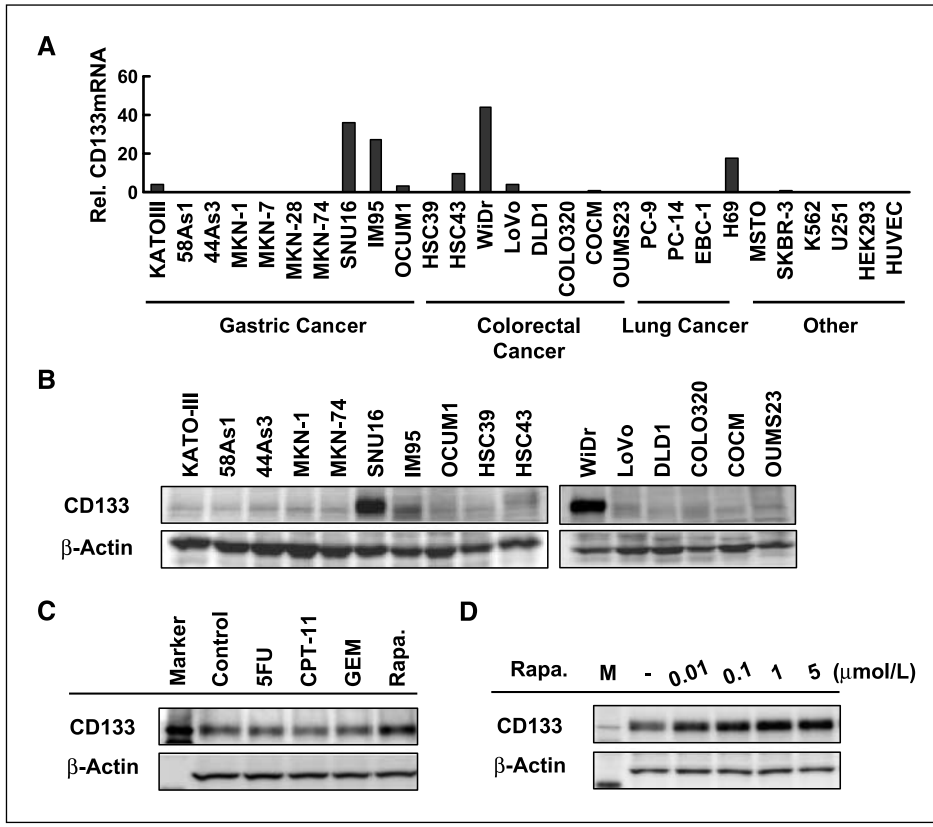

Figure 1. Rapamycin up-regulates CD133 expression. *A,* the mRNA expression levels of CD133 were examined using real-time RT-PCR in 26 cancer cell lines. *B,* the protein expressions of CD133 were determined using Western blotting in 16 gastric and colorectal cancer cell lines. *C,* Western blot of CD133 expression in WiDr cells exposed to cytotoxic drugs [1 μmol/L of 5-fluorouracil (*5-FU*), CPT-11, and gemcitabine (*GEM*)] and rapamycin (1 μmol/L) for 48 h. Note that only rapamycin up-regulates CD133 expression. *D,* WiDr cells were exposed to rapamycin at the indicated concentrations (0, 0.01, 0.1, 1, and 5 μmol/L) for 48 h. Rapamycin dose-dependently up-regulated CD133 expression. *Rel. CD133 mRNA,* normalized mRNA expression levels (CD133/GAPD × 10^4); *Rapa.*, rapamycin.

Immunoblotting. A Western blot analysis was performed as described previously (10). The experiment was performed in triplicate. The following antibodies were used: monoclonal CD133 antibody (W6B3C1; Miltenyi Biotec), rabbit polyclonal HIF-1α antibody (Novus Biologicals, Inc.), β-actin antibody, and HRP-conjugated secondary antibody (Cell Signaling Technology).

Results

Inhibition of the mTOR signal up-regulates CD133 expression in CD133-overexpressing gastrointestinal cancer cells. We examined the mRNA expression levels of CD133 in 26 cancer cell

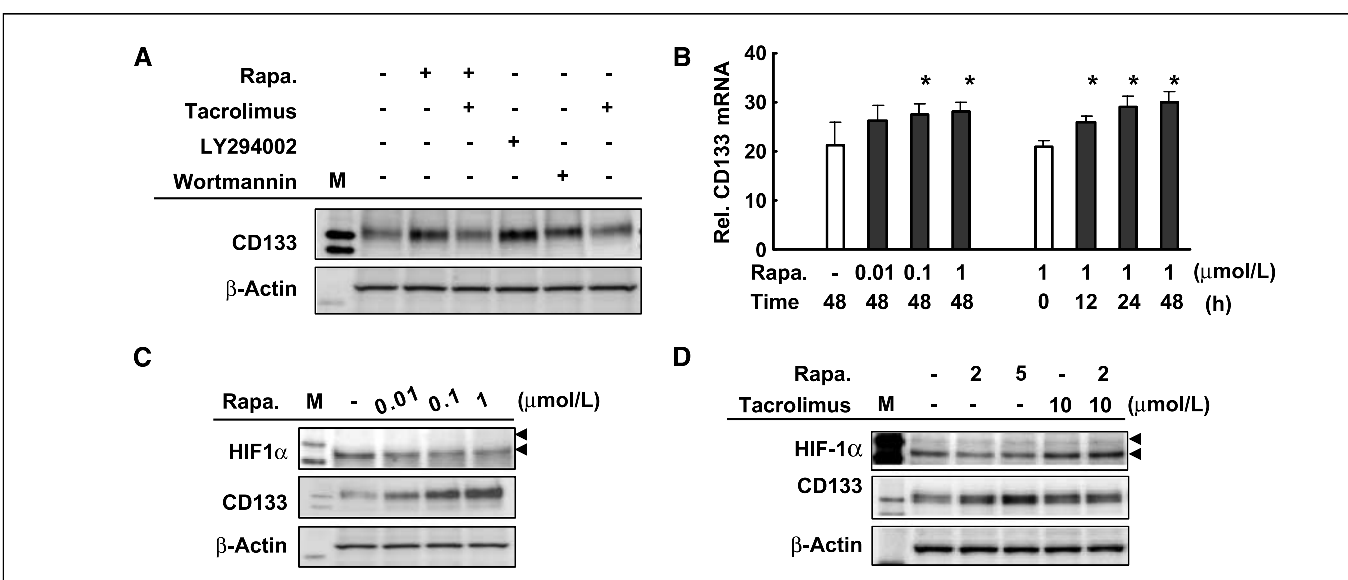

Figure 2. Rapamycin down-regulates HIF-1α expression and up-regulates CD133 expression at the transcriptional level. *A,* WiDr cells were exposed to rapamycin, the rapamycin-competitor tacrolimus, and the phosphoinositide-3-kinase inhibitors LY294002 and wortmannin for 48 h at concentrations of 10 μmol/L. The inhibition of mTOR signaling up-regulated CD133 expression. *B,* rapamycin up-regulated the expression of CD133 mRNA in WiDr cells in a time-dependent and dose-dependent manner. *Columns,* mean determined using real-time RT-PCR; *bars,* SD. *C* and *D,* rapamycin exposure and HIF-1α expression. WiDr cells were exposed to rapamycin with/without tacrolimus at the indicated concentration for 48 h. Rapamycin down-regulated HIF-1α expression and inversely up-regulated CD133 expression; these effects were canceled by tacrolimus. *Rel. CD133 mRNA,* normalized mRNA expression levels (CD133/GAPD × 10^4); *Rapa.*, rapamycin.

lines using real-time RT-PCR. Several gastric, colorectal, and lung cancer cell lines such as SNU16, IM95, HSC43, WiDr, and H69, overexpressed CD133 (Fig. 1*A*). The increased expression of CD133 protein was also confirmed in these cell lines (Fig. 1*B*). The mTOR inhibitor rapamycin, but not cytotoxic drugs (5-fluorouracil, CPT-11, and gemcitabine), increased the expression of CD133 in a dose-dependent manner in CD133-overexpressing WiDr cells (Fig. 1*C* and *D*). These results indicate that mTOR signaling is involved in the expression of CD133 in cancer cells.

Rapamycin down-regulated HIF-1α expression and up-regulated CD133 expression at the transcriptional level. To examine the signal transduction of rapamycin-induced CD133 expression, we used the rapamycin-competitor tacrolimus and the phosphoinositide-3-kinase inhibitors LY294002 and wortmannin. Tacrolimus (10 μmol/L) completely canceled the up-regulation of CD133 induced by rapamycin. The inhibition of phosphoinositide-3-kinase by LY294002 (10 μmol/L) and wortmannin (10 μmol/L) also up-regulated CD133 expression (Fig. 2*A*). Rapamycin up-regulated CD133 expression at the transcriptional level in a dose-dependent and time-dependent manner (Fig. 2*B*).

The inhibition of mTOR signaling is likely to lead to the down-regulation of the expression of certain molecules because the mTOR complex positively regulates the general translational machinery. Under the inhibition of mTOR signaling, HIF-1α, among several downstream molecules of mTOR, can activate transcription by acting as a repressor of specific transcription factors such as the MYC-associated protein X homodimer (11). Therefore, we focused on the possible role of HIF-1α in the regulation of CD133 expression. Rapamycin down-regulated HIF-1α expression but up-regulated CD133 expression (Fig. 2*C*). Meanwhile, tacrolimus canceled the effect of rapamycin on the expressions of HIF-1α and CD133 (Fig. 2*D*). These results suggest that the down-regulation of HIF-1α may mediate the up-regulation of CD133 expression in cancer cells. Up-regulation of CD133 expression by rapamycin was reproducibly observed in the CD133 high-expressing cell lines, but not in CD133 low-expressing cell lines (Supplemental Fig. S2).

Induction of HIF-1α down-regulates CD133 expression in cancer cells. Hypoxia mediates the stabilization of HIF-1α protein and enables its escape from rapid degradation, facilitating the up-regulation of HIF-1α expression (12). Hypoxia strongly induced HIF-1α expression, whereas CD133 expression was down-regulated in all three CD133-overexpressing cell lines (Fig. 3*A*). Rapamycin dose-dependently up-regulated CD133 expression under normoxic conditions, but no effect was seen under hypoxic conditions. We speculated that the effect of hypoxia on the induction of HIF-1α is much higher than the effect of rapamycin on the down-regulation of HIF-1α. The expression of CD133 mRNA was also strongly down-regulated under hypoxic conditions in all three cell lines (Fig. 3*B*) and in three additional cell lines (Supplemental Fig. S1).

In addition, DFO, a known HIF-1α activator, induced HIF-1α expression in a dose-dependent manner but down-regulated the expression of CD133 at both the mRNA and protein levels in WiDr cells (Fig. 3*C* and *D*), and in three additional cell lines (Supplemental Fig. S2). These results were consistent with those obtained under hypoxic conditions. Both hypoxia and DFO exposure markedly down-regulated CD133 expression, strongly suggesting that induction of HIF-1α results in the down-regulation of CD133 expression.

Inverse correlation between CD133 and HIF-1α in clinical samples. Finally, to address whether CD133 and HIF-1α expression are inversely correlated in clinical samples of gastric cancer

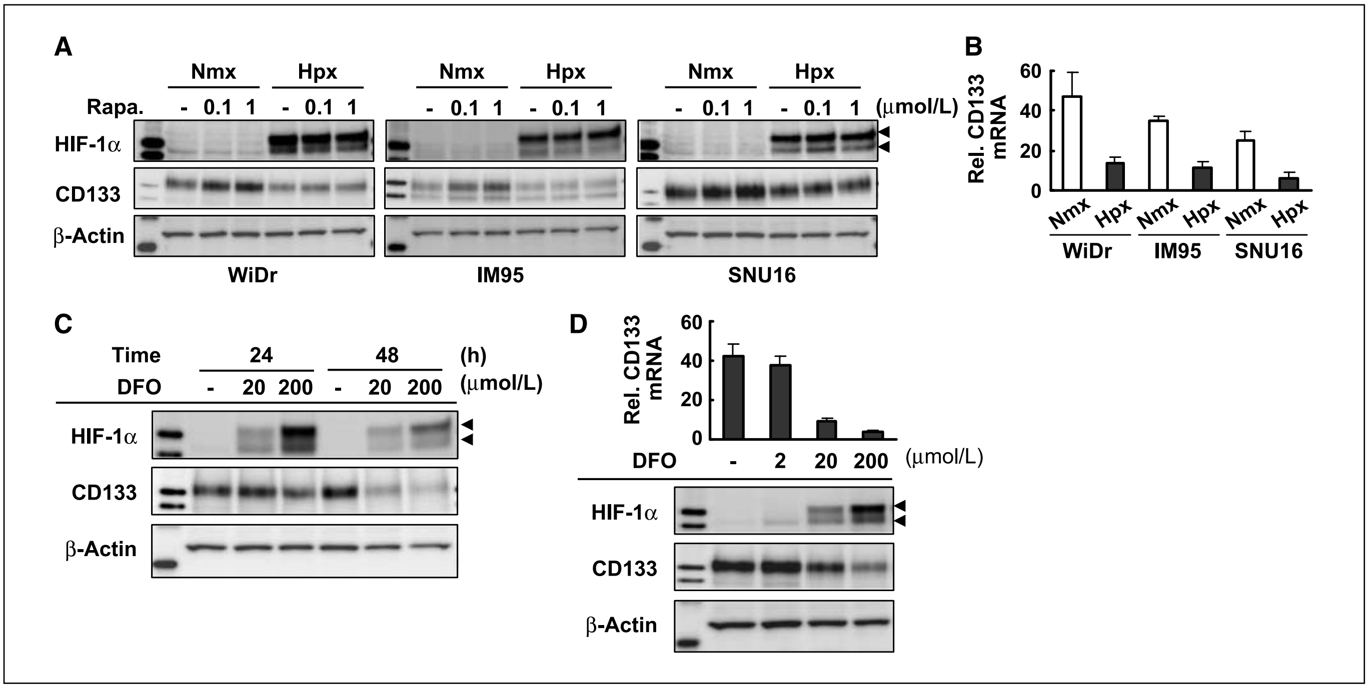

Figure 3. Induction of HIF-1α down-regulates CD133 expression in cancer cells. *A,* three gastrointestinal cancer cell lines were exposed to rapamycin under normoxic or hypoxic conditions for 24 h. Hypoxia induced HIF-1α expression and inversely down-regulated CD133 expression. *B,* hypoxia strongly down-regulated CD133 expression at the mRNA level. *Columns,* mean determined using real-time RT-PCR; *bars,* SD. *C,* DFO, a known HIF-1α activator, induced HIF-1α expression and down-regulated CD133 expression in WiDr cells. *D,* DFO induced these effects at both the mRNA and protein levels. Note that both hypoxia and DFO exposure had similar effects on HIF-1α induction and CD133 down-regulation. *Rel. CD133 mRNA,* normalized mRNA expression levels (CD133/GAPD × 10^4); *Rapa.*, rapamycin.

specimens, we examined the expression of these molecules using previously published microarray data (9). The expressions of CD133 and HIF-1α were inversely correlated in gastric cancer ($r = -0.68$; Fig. 4*A*), whereas the expressions of CD133 and HIF-1β were not ($r = -0.05$; Fig. 4*A*). These results are consistent with the *in vitro* findings in the present study.

Taken together, the present results suggest that an oxygen-sensitive intracellular pathway involving both HIF-1α and mTOR signaling may, at least in part, regulate CD133 expression in cancer cells (shown in the schema in Fig. 4*B*).

Discussion

Hypoxic conditions promote the proliferation of mammalian ES cells more efficiently than normoxia and are thought to be required for the maintenance of full pluripotency. Hematopoietic stem cells are located in the bone marrow, which is a physiologically hypoxic environment, and the survival and/or self-renewal of hematopoietic stem cells is enhanced *in vitro* if the cells are cultured under hypoxic conditions (13). Thus, accumulating data indicates that oxygen levels influence specific cell fates in several developmental processes; however, the effect of oxygen levels on cell differentiation is thought to be context-dependent (14). Our data on CD133 expression in response to hypoxia were different from the previous study shown in glioma (5). The discrepancy might be explained by (*a*) a different cellular context in glioma from the others, because CD133 expressions of all cell lines including the WiDr, IM95, SNU16, OCUM1, 44As3, and DLD-1 cells were reproducibly down-regulated by hypoxic condition (Supplemental Fig. S1; Fig. 3*B*), whereas the U251 cells failed to exhibit the down-regulation, and by (*b*) the different detection methods in our study (Western blot and quantitative real-time RT-PCR) from the previous report (flow cytometry for CD133-positive cells).

The detailed mechanism responsible for the repressive role of HIF-1α on CD133 expression is not fully understood; one possible explanation is raised by MYC, which is also known as c-Myc. HIF-1α binds to MAX and renders MYC inactive, and HIF-1 (homodimers of HIF-1α and HIF-1β) activates the expression of MXI1 (MAX interactor 1), which binds to MAX and thereby antagonizes MYC function (11). Recent reports have shown that HIF-1α inhibits MYC activity, which is thought to have implications for stem cell function (15, 16). Whether MYC directly activates CD133 transcription remains unclear; our preliminary data indicate that a MYC-inhibitor suppressed CD133 expression in WiDr cells.[4] Because the gene amplification of MYC and MYCN is frequently observed in many cancers, the relations among MYC, HIF-1α, HIF-1β, HIF-2, and CD133 should be investigated in future studies.

In conclusion, we showed that the inhibition of mTOR signaling up-regulated CD133 expression, whereas HIF-1α induction under hypoxic conditions or DFO exposure down-regulated CD133 expression in gastrointestinal cancer cells. Our findings show a novel regulatory mechanism for the expression of CD133 involving mTOR signaling and HIF-1α, and these findings may contribute to our understanding of the stemness character of cancer stem cells.

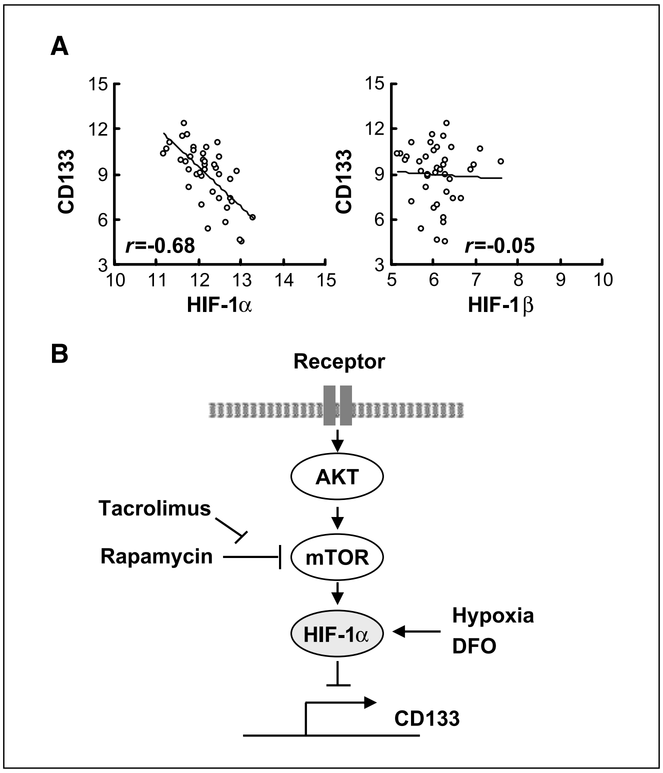

Figure 4. Inverse correlation between CD133 and HIF-1α in clinical samples of gastric cancer. *A,* the correlation between the expressions of CD133 and HIF-1α were analyzed in 40 clinical gastric cancer specimens using previously published microarray data. CD133 and HIF-1α were inversely correlated in gastric cancer ($r = -0.68$), whereas CD133 and HIF-1β were not ($r = -0.05$). *B,* proposed model depicting the involvement of mTOR signaling, HIF-1α, and CD133 expression. HIF-1α, a downstream molecule of mTOR, down-regulates CD133 expression at the transcriptional level in cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[4] Unpublished data.

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