CD166 plays a pro-carcinogenic role in liver cancer cells via inhibition of FOXO proteins through AKT

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Abstract. Cluster of differentiation 166 (CD166) is a cell surface membrane protein, which is regarded as a valuable prognostic marker in several types of epithelial tumors. We previously reported that CD166 exerts its pro-carcinogenic role by enhancing YAP function in liver cancer cells. However, YAP cannot completely rescue the increased anti-carcinogenic effects by gene silencing of CD166, whose downstream effectors require further investigation. Here, we found that knockdown of CD166 inhibits phosphorylation of anti-carcinogenic FOXO proteins. Overexpression of CD166 led, not only to a faster protein degradation rate, but also a more accumulated ubiquitination of FOXO compared to the control. Moreover, overexpression of CD166 facilitated FOXO protein localization from the nuclear fraction to the cytosolic fraction, suggesting that CD166 modulates FOXO protein stability through alteration of their subcellular localization. In addition, simultaneous overexpression of CD166 partially reversed the evoked anti-carcinogenic effects by overexpression of FOXO both in vitro and in vivo. Furthermore, CD166 knockdown-induced anti-carcinogenic effects and dephosphorylation of FOXO proteins were rescued by overexpression of AKT. In liver cancer tissues, we also observed that higher expression levels of CD166, phospho-AKT, total AKT and phospho-FOXO were correlated with lower expression levels of total FOXO, suggesting that the upregulation of CD166 leads to the activation of AKT, which in turn facilitates phosphorylation and degradation of FOXO. Taken together, our data demonstrate that AKT is an inter-mediator between the upstream regulator, CD166, and downstream effector, FOXO, in liver cancer cells. Disrupting the relationship between CD166 and the AKT/FOXO axis may serve as a novel therapeutic target for liver cancer patients.

Introduction

Cluster of differentiation 166 (CD166) is a cell surface member of the immunoglobulin superfamily (1), which is overexpressed and regarded as a valuable prognostic marker of disease progression and poor survival in several types of epithelial tumors (2-4). Gene silencing of CD166 decreases the concentration of Bcl-2 and increases the level of apoptosis (PARP, active caspase-7) (5). We previously reported that the activation of anti-apoptotic canonical NF-κB signaling greatly induces CD166 expression in liver cancer cells after serum deprivation (6), suggesting its important roles in regulating apoptosis. Most recently, we revealed that CD166 can exert its anti-apoptotic role by enhancing YAP function, demonstrating that CD166 is an upstream regulator of YAP (7). However, overexpression of YAP cannot completely rescue the increased anti-carcinogenic effects evoked by knockdown of CD166 (7). Thus, the downstream regulation of the CD166 pro-carcinogenic function needs to be further explored.

The forkhead box transcription factor superfamily consists of 19 subclasses of FOX genes, FOXA-FOXS (8). The FOX transcription factors that belong to the other (O) subfamily (FOXO) include four members (FOXO1, 3, 4, 6) in mammals (8). Overexpression of FOXO proteins inhibits tumor growth in vitro and tumor size in vivo (9). The nuclear accumulation of FOXO proteins was found to suspend cell cycle progression and promote apoptosis in breast cancers (9,10). Recent research efforts also provide new insights that FOXO...
proteins appear to present antitumor properties in liver cancer, including induction of the expression of pro-apoptotic genes, or interfering with signaling cascades commonly altered in this disease such as Wnt/β-catenin, PI3K/AKT/mTOR or MAPK pathways (11). However, the upstream regulation of FOXO functions, particularly those involving cell membrane proteins are still largely unknown in liver cancer cells.

In the present study, we found that CD166 exerts its pro-carcinogenic role via the inhibition of FOXO proteins, i.e. CD166 facilitates phosphorylation, cytosolic accumulation and instability of FOXO proteins. The anti-carcinogenic function of FOXO proteins can be reversed by CD166. Moreover, our data also demonstrate that AKT is an inter-mediator between the upstream regulator, CD166, and downstream effector, FOXO, in liver cancer cells. Disruption of the relationship between CD166 and the AKT/FOXO axis may serve as a novel therapeutic target for liver cancer patients.

Materials and methods

Cell culture. Bel-7402, SMMC-7721, Chang Liver and HL-7702 cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS). Cells were treated by Wortmannin (50 µM; Cayman, Ann Arbor, MI, USA), LY294002 (20 µM; Cell Signaling Technology (CST), Boston, MA, USA], cycloheximide (CHX, 50 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) or MG132 (50 µM; Cayman) 1-24 h before harvest.

shRNA and protein expressing plasmids. Lentiviral CD166-shRNA1 was purchased from Open Biosystems (Huntsville, AL, USA; catalog no. TRCN0000150706). CD166-shRNA2 and AKT-shRNA were cloned into pLKO.1 lentiviral plasmid using the following primers: CD166-shRNA-F, GTACGGATCCCACCAGAATGATGGCACTCGAGTGCCATCATTCTTGAGGATTTTTTTTG and CD166-shRNA-R, AATTCAAAAAAACTCAACCATCTAAACCTGCTCGAGCAGGTTAGATGGTTGCTTGA; AKT-shRNA-F, CCGGAACTCCTCAAGCAACCATCTAAACCTGCTCGAGCAGGTTAGATGGTTGCTTGA and AKT-shRNA-R, AATTCAAAAAAACTCAACCATCTAAACCTGCTCGAGCAGGTTAGATGGTTGCTTGA; AKT-Myc-F, GTATCCTGCAGGTTACAGATCTTCTTCAAGTTAAGCGTAGTCTGGGACGTCGTATGGGTAGGCT and AKT-Myc-R, GTATCCTGCAGGTTACAGATCTTCTTCAAGTTAAGCGTAGTCTGGGACGTCGTATGGGTAGGCT. The pTEN-HA-expressing plasmid was a gift from Dr Xuqian Fang (Shanghai Jiaotong University, Shanghai, China), and the FOXO1 and FOXO3a protein-expressing plasmids were purchased from Dr Xuqian Fang (Shanghai Jiaotong University, Shanghai, China).

Results

CD166 regulates phosphorylation of FOXO proteins. We previously reported that silencing of CD166 can induce apoptosis (7). Notably, activation of FOXO proteins can also induce apoptosis (13). In addition, FOXO protein activity can be inactivated through phosphorylation (8). Thereby, we tested whether CD166 has a role in the regulation of phosphorylation of FOXO proteins. Compared to the control, dephosphorylation of both FOXO1 and FOXO3a was detected in Bel-7402 cells following CD166 knockdown (Fig. 1A). Similar results were also obtained in SMMC-7721 cells (Fig. 1B). Moreover, it
was demonstrated that both FOXO1 and FOXO3a had relative higher levels in normal hepatic cell lines (HL-7702 and Chang Liver) compared to levels in the liver cancer cell lines (Bel-7402 and SMMC-7721) (Fig. 1C), suggesting that lower FOXO proteins levels in liver cancer cells may lead to tumorigenesis. Collectively, the above data revealed that CD166 exerts its pro-carcinogenetic role through inhibition of FOXO proteins.

CD166 reverses the anti-carcinogenetic effects induced by FOXO proteins. We examined whether CD166 plays a negative role on FOXO proteins in liver cancer cells. We found that simultaneous overexpression of FOXO1 and FOXO3a decreased cell proliferation compared to the control, as measured by an MTT-based assay and Ki-67 immunostaining (Fig. 1E and data not shown). Furthermore, we found that overexpression of FOXO proteins impaired the ability of these cells to form colonies in soft agar (Fig. 1F), whereas markedly increased apoptosis was noted, as shown by increased caspase-3/7 activity and caspase-3 cleavage by immunostaining (Fig. 1D and data not shown). In addition, we observed that the reduced cell survival and transformative phenotype induced by overexpression of FOXO proteins could be partially rescued by simultaneous ectopic expression of CD166 (Fig. 1D-F). These data indicate that the inhibition of FOXO by CD166 is important for human liver cancer cell growth and survival.

CD166 controls stability and subcellular localization of FOXO proteins. Protein degradation is initialized by target protein modification, such as phosphorylation (14,15). Since CD166 regulated the phosphorylation of FOXO proteins (Fig. 1A and B), we hypothesized that CD166 also modulates degradation of FOXO proteins. It was found that when Bel-7402 cells were treated with the protein synthesis inhibitor, cycloheximide (CHX), the half-life time of FOXO1 and FOXO3a...
was >8 h. However, FOXO1 and FOXO3a degraded much more rapidly with a half-life time of ~8 h after overexpression of CD166 (Fig. 2A, upper panel). Compared to FOXO1, the effects of CD166 on FOXO3a were much more obvious (Fig. 2A, lower panel). In addition, in Bel-7402 cells with long exposure (72 h) to CD166, reduced expression accompanied by a greater accumulation of ubiquitinated FOXO1 and FOXO3a was detected compared to the control (for FOXO1, lane 2 vs. lane 1 and for FOXO3a, lane 4 vs. 3) (Fig. 2B), suggesting that CD166 is a regulator of ubiquitination and degradation of FOXO proteins. As known, inactivation of FOXO proteins leads to their accumulation in the cytoplasm (8). To ascertain whether CD166 affects the subcellular localization of FOXO, Bel-7402 cells were transfected with CD166-HA-expressing plasmids. It was detected that nuclear FOXO3a expression was significantly reduced in the Bel-7402 cells with CD166-HA overexpression compared to the control (Fig. 2C). This observation was confirmed by fractionation studies, which revealed that overexpression of CD166 facilitated FOXO protein localization from the nuclear fraction to the cytosolic fraction (Fig. 2D). Taken together, the data demonstrate that CD166 modulates FOXO protein stability through alteration of their subcellular localization.

CD166 promotes liver cancer cell growth through inhibition of FOXO in vivo. On the basis of the evidence that CD166 reduces protein stability and expression (Fig. 2), we investigated the growth of Bel-7402 clones after injection into athymic mice. Compared to the control (transfected with the empty plasmid), Bel-7402 cells with CD166 overexpression exhibited a relatively higher tumor growth rate (Fig. 3A). In comparison, Bel-7402 cells with FOXO1 and FOXO3a overexpression effectively prevented tumor growth, yet this effect was rescued by simultaneous overexpression of CD166 (Fig. 3A),
thereby confirming the close relationship between FOXO and CD166 in vivo. To ascertain whether CD166 contributes to the inhibition of FOXO in vivo, we stained sections from the xenografts using anti-FOXO3a antibodies. Similar to the data shown in Fig. 2B and C, the protein expression of FOXO3a, particularly the nuclear fraction of FOXO3a was significantly downregulated in xenograft tissue with CD166 overexpression compared to the control (transfected with the empty plasmids) (Fig. 3B), suggesting that long-term exposure to the overexpression of CD166 leads to translocation from the nucleus to the cytoplasm and instability of FOXO proteins.

AKT regulates FOXO proteins in liver cancer cells. Emerging evidence suggests that AKT controls the activity of FOXO proteins (8,16,17). However, to the best of our knowledge, there is no direct evidence to support the conclusion that AKT regulates FOXO proteins in liver cancer cells. Thus, we investigated the effect of AKT on FOXO in Bel-7402 cells. Compare to the control, we found that phosphorylation of FOXO1 and FOXO3a was greatly induced by overexpression of AKT (Fig. 4A), whereas it was reduced after knockdown of AKT (Fig. 4B). By using chemical PI3K/AKT inhibitors, LY294002 and Wortmannin, respectively, we found that phosphorylation of both FOXO1 and FOXO3a was markedly reduced (Fig. 4C). Furthermore, when endogenous PI3K/AKT inhibitor, pTEN, was overexpressed, phosphorylation of FOXO1 and FOXO3a was also inhibited in a dose-dependent manner (Fig. 4D), suggesting that FOXO proteins are also regulated by AKT in liver cancer cells. As shown in Fig. 4E, we confirmed that AKT activity could be downregulated after knockdown of CD166, as the phosphorylation of AKT substrates was markedly reduced in the Bel-7402 cells with CD166 knockdown compared to the control suggesting that the regulation of FOXO by CD166 may be AKT dependent.

CD166 regulates FOXO proteins via AKT. We then investigated whether the phosphorylation of FOXO proteins controlled by CD166 is AKT dependent. It was found that knockdown of CD166-induced dephosphorylation of FOXO proteins could be rescued by simultaneous overexpression of AKT (Fig. 5A). Moreover, the induced caspase-3/7 activity, reduced cell proliferation and soft agar colony formation by depletion of CD166 was partially reversed after overexpression of AKT (Fig. 5B-D). Thus, we proposed that AKT may act as an inter-mediator between the upstream regulator, CD166, and the downstream effector, FOXO. Next, we tested the protein expression patterns of FOXO, AKT and CD166 in liver cancers and their adjacent normal liver tissues. Compared to the adjacent normal liver tissues, higher expression levels of CD166, p-AKT, total AKT and p-FOXO1/3a were correlated with lower expression levels of total FOXO1/3a in liver cancer tissues (Fig. 5E), suggesting that upregulation of CD166 leads to induction of AKT, which in turn facilitates phosphorylation and degradation of FOXO in liver cancer.
Discussion

In the present study, we describe a close relationship between CD166 and FOXO proteins (Fig. 5F). These two types of proteins play opposing roles in the regulation of tumorigenesis in liver cancer (6,7,11,18,19). Nuclear localization of FOXO proteins induces expression of anti-carcinogenic genes (11), whereas CD166 facilitates translocation of FOXO proteins from the nucleus to the cytoplasm (Fig. 2C and D). CD166 maintains anti-apoptotic Bcl-2 protein expression, suggesting that the anti-apoptotic function of CD166 is partially dependent on Bcl-2 (7). Notably, overexpression of Bcl-2 also diminishes death induced by expression of FOXO proteins (20). We previously reported that the anti-apoptotic function of CD166 is via enhancement of both expression and activity of onco-protein, YAP (7). The degradation of YAP can be protected by TRIB2 (21-22), a protein that is also capable of inhibiting and reducing nuclear FOXO proteins (23). Thus, we consider that CD166 plays a similar role to that of TRIB2. YAP is a pro-carcinogenic protein (7,12), while FOXO proteins are anti-carcinogenic in liver cancer. However, whether and how YAP antagonizes FOXO still remains unknown and needs further exploration.
It has been reported that AKT phosphorylates FOXO3a at T32 and S253 and FOXO1 at T24 and S256, respectively, which are conserved from Caenorhabditis elegans to mammals (24). Notably, these phosphorylation sites can also be regulated by CD166 (Fig. 1A and B). Furthermore, the anti-carcinogenic effects and the regulation of phosphorylation of FOXO proteins by knockdown of CD166 can be reversed by AKT (Fig. 5A-D), providing another evidence that CD166 can regulate AKT.

In summary, our data indicate that the CD166/AKT axis modulates tumorigenesis via promotion of phosphorylation and facilitation of degradation, ubiquitination and cytosolic accumulation of FOXO proteins in liver cancer cells. Further exploration of the interplay among these important signaling pathways may lead to more effective therapeutic strategies for liver cancer.

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