Biochemical and Biophysical Research Communications 451 (2014) 334–338

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cluster of differentiation 166 (CD166) regulates cluster of differentiation (CD44) via NF-κB in liver cancer cell line Bel-7402

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ARTICLE INFO

Article history:
Received 21 July 2014
Available online 2 August 2014

Keywords:
Signaling pathway
Transcription factor
UBC3
COP1
Protein degradation

ABSTRACT

Cluster of differentiation 166 (CD166) is critical for liver cancer cell survival. Our previously study demonstrated that CD166 exerts its anti-apoptotic role through interaction with YAP in liver cancer. However, the interaction between CD166 and other cell surface molecules remains unclear in liver cancer cells. In the current study, we found that both mRNA and protein of CD44 expression was significantly inhibited by knocking-down CD166. Moreover, CD166 affected-CD44 expression is dependent of transcription via blocking NF-κB pathway. On the contrary, CD44 promoted up-regulation of CD166 mRNA and protein. And it may be through E3 ubiquitin ligases COP1 and UBC3 to regulate CD166 protein degradation. Collectively, these results suggest that CD166 and CD44 play important roles in liver cancer development. Therefore, CD166 may develop as a potential therapeutic molecule target for the treatment of liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related deaths [1]. Despite it has made some progress in treating liver cancer. There is still existing diagnosis and therapies limitation. The molecular mechanism and etiology of HCC are complicated and contain numerous genetic signaling pathways [2]. Recently, cluster of differentiation 166 (CD166 or Alcam) and Yes-associated protein (YAP) were up-regulated in liver cancer samples and demonstrated that dysfunctional of CD166 and YAP are critical for liver cancer [3]. Furthermore, numerous studies revealed that CD166 was a putative cancer stem cell biomarker in different type of cancers with capacity for proliferation and anti-apoptotic [3,4]. Therefore, the mechanism of CD166 in occurrence and development of hepatocellular carcinoma is needed to be further studied.

CD44 is a cell-surface glycoprotein involved in cell–cell interaction, cell adhesion and migration [5]. It is also a putative cancer stem cell (CSC) marker in colorectal cancer [6], gastric cancer [7] and pancreatic ductal adenocarcinoma [8]. Numerous studies revealed that CD44 is critical for cell survival, migration, invasion, metastasis and epithelial to mesenchymal transition (EMT) [9,10]. However, the role of CD44 in hepatocarcinogenesis is poorly understood.

In the present study, we found that CD166 down-regulated CD44 expression via inhibition NF-κB pathway. On the contrary, CD44 promoted mRNA and protein expression of CD166 independent of transcription. We also discovered that CD44 promoted CD166 protein expression may suppress COP1 and UBC3, which are E3 ubiquitin protein ligase. Thus, this study provides a negative feedback loop in liver tumorigenesis. And suggesting that it could provides a potential effective therapeutic target for blocking the crosstalk between CD166 and CD44 in liver cancer.

2. Materials and methods

2.1. Cell culture and treatment

Bel-7402 and HEK293T were cultured in DMEM medium with 10% fetal bovine serum. Cells were treated by BAY 11-7082 (Calbio-

Abbreviations: HCC, hepatocellular carcinoma; TFs, transcription factors; NF-κB, nuclear factor kappa B; CD166, cluster of differentiation 166; CD44, cluster of differentiation; IF, immunofluorescence; CHX, cycloheximide.

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http://dx.doi.org/10.1016/j.bbrc.2014.07.128
0006-291X/

chem, San Diego, CA, USA) 24 h before harvest to block NF-κB activation. Bel-7402 cell was treated by protein synthesis inhibitor CHX (50 μg/ml, Sigma) before harvest.

2.2. Vectors

CD44-shRNA and CD44-MYC were respectively cloned into PLKO.1 and PLJM1 lentiviral vectors.CD166 expression vectors and lentiviral shRNA against CD166 vector were obtained from our previously study [3]. The sequences of primers were listed in Supplement Table S1.

2.3. Cell proliferation and quantitative RT-PCR

Cell proliferation was determined by a MTT-based proliferation assay and clone formation assay. mRNA levels were measured by quantitative real-time PCR, protocols were performed as previously described [11]. Primers are available in Supplement Table S1.

2.4. Western blotting (WB) and immunofluorescence (IF)

Western blot was performed as described previously [12] using primary antibodies: Flag (Cell Signaling technology, Boston, MA, USA, #2368), HA (Cell Signaling technology, #3724), GAPDH (Cell Signaling technology, #5174), CD44 (Epitomics, Burlingame, CA, USA, #1998), CD166 (Epitomics, #3133), CD166 (Santa Cruz Biotechnology, CA, USA, #SC-74557), P50/105 (Epitomics, #1559), P65 (Cell Signaling technology, #8242), RelB (Cell Signaling technology, #4922), BTRC (Cell Signaling technology, #4394S), COP1 (Santa Cruz Biotechnology, #SC-166799) and UBC3 (Cell Signaling technology, #4997).

For IF, primary antibodies: CD44 (Epitomics, #1998), P65 (Cell Signaling technology, #8242), P50/105 (Epitomics, #1559), and RelB (Cell Signaling technology, #4922).

2.5. Luciferase reporter assay

The promoter region of the CD44 gene was subcloned into PGL4/Basic vector. Bel-7402 cell was co-transfected with luciferase reporter vector and CD166 expressing vector or against CD166 expressing vector, and an lng pRLSV40 Renilla luciferase construct was used for normalization. After 24 h, luciferase activities were measured by the dual-luciferase reporter kit (Promega, Madison, WI, USA).

3. Results

3.1. CD166 and CD44 exert opposite roles in liver cancer cells

In our previously study, we demonstrated that CD166 promoted apoptosis and inhibited cell growth in the liver cancer cells. We detected a series of cell surface molecules after down-regulation of CD166, and found that CD44 was up-regulated (Fig. 1A). Similarly, over-expression of CD166 reduced CD44 protein expression (Fig. 1B). Therefore, we mainly focus on CD166 and CD44 in this study. CD166 and CD44 specific shRNA (Fig. 1C) were used to decrease protein expression in Bel-7402. Increasing the expression of CD166 promoted liver cancer cells proliferation by a MTT-based assay (Fig. 1D) and colony formation assay (Fig. 1E). Nevertheless, we found that CD44 had a little impact on cell proliferation (Fig. 1D and E). These data revealed that CD166 and CD44 exert different function in liver cancer cells.

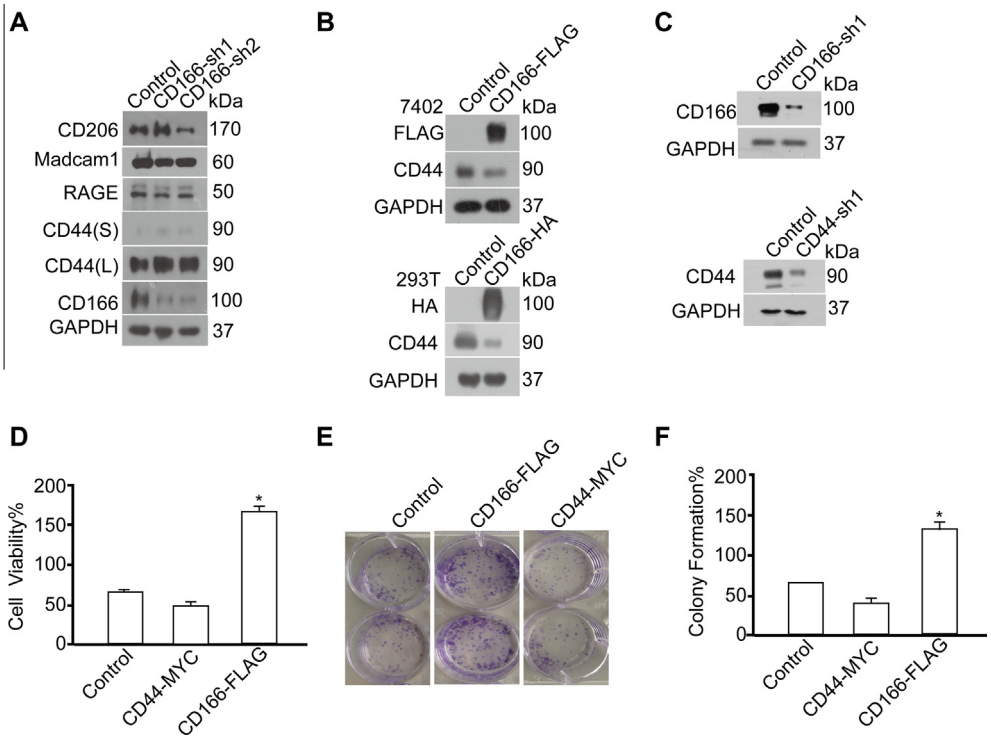

Fig. 1. The roles of CD166 and CD44 in the liver cancer cells. (A and B) CD166 inhibited CD44. (A) Numerous cell surface molecules CD206, Madcam1, RAGE and CD44 were detected by down-regulation CD166. Western blot results showed that CD44 was up-regulated. (B) Over-expression of CD166 (infected by CD166-FLAG in Bel-7402 cell and transient transfection by CD166-HA in HEK293T cell) reduced CD44 protein expression. (C) Validation the knock-down efficiency of shRNAs against CD166 and CD44 in Bel-7402 cell and against GFP in control by Western blot. (D–F) CD166 promoted Bel-7402 cells proliferation. (D) Ectopic expression of CD166-FLAG augmented liver cancer cell proliferation as measured by MTT assays in Bel-7402 cells. $^{*}P < 0.01$ analyzed by Student t test. (E and F) Colon formation assay in Bel-7402 to test liver cancer cell proliferation ability with infected by CD166-FLAG and CD44-MYC. $^{*}P < 0.01$ analyzed by Student t test.

3.2. CD166 mediates CD44 dependent of transcription

Because of CD166 was up-regulated and CD44 was down-regulated in liver cancer cells. We were interested in how CD166 affected the depletion of CD44. Thus, we utilized protein synthesis inhibitor (CHX 50 μg/ml) to block protein expression according to indicate time (Fig. 2A). The results showed that CD44 was a stable protein with a longer half-time in liver cancer cells. Therefore, we tested if CD166 affected CD44 at transcriptional level. And found that knocking down of CD166 largely increased CD44 mRNA levels (Fig. 2B), and vice verse (Fig. 2E). Then after depletion of CD166 we detected a series of transcription factors (Supplementary Fig. 1A). Finally, results showed that NF-κB increased obviously. To confirm this result, we further repeated to detect P50, P65, P-P65, RelB, P105, P100, P-P100, P52 and CD44 after down-regulation and up-regulation of CD166 (Fig. 2C and D). These results indicated that CD166 regulated CD44 dependent of transcription.

3.3. CD166 inhibits CD44 by interdicting NF-κB pathway

It was reported that STAT3 bound CD44 and NF-κB. And CD44 promoted NF-κB to translocate into nucleus and activated hTERT expression. In reverse, activated hTERT could enhance CD44 expression with a positive manner [13]. Thus, we highly hypothesized that NF-κB is a transcription factor of CD44 promoter. Then we next blocked NF-κB pathway by using specific inhibitor BAY11-7082. We found that treatment of BAY11-7082 decreased the protein and mRNA expression of CD44 in Bel-7402, measured by Western blotting (Fig. 3A) and RT-QPCR (Fig. 3B). As well as, we performed a CD44 luciferase reporter system to confirm whether CD166 regulated CD44 activity. We discovered that luciferase activity from the CD44 reporter was largely accelerated by CD166 shRNA and inhibited by CD166-FLAG compared to control (Fig. 3C). To confirm the interaction between CD44 and NF-κB, we performed IF analysis and found co-localization of CD44 and P65, P105/50, RelB respectively in Bel-7402 cells (Fig. 3D). Collectively, these data demonstrated that CD166 inhibited CD44 through blocking NF-κB pathway in Bel-7402 cells.

3.4. CD44 promotes CD166 dependent of transcription and post-transcription

As described above, CD166 inhibited CD44 via inactivated NF-κB pathway. Then we also wanted to know whether CD44 regulated CD166 expression. Inhibition of CD44 by specific shRNA induced CD166 down-regulation measured by Western blotting (Fig. 4A). Similarly, up-regulation of CD44-MYC increased the mRNA (Fig. 4D) and protein (Fig. 4B) expression of CD166 in Bel-7042 cells. We also detected a number of ubiquitin E3-ligases SKP2, C-ABL, COP1, BTRC and UBC3 with over-expression of CD44-MYC in Bel-7402. The results showed that COP1 and UBC3 were down-regulated compared with control (Fig. 4C). We

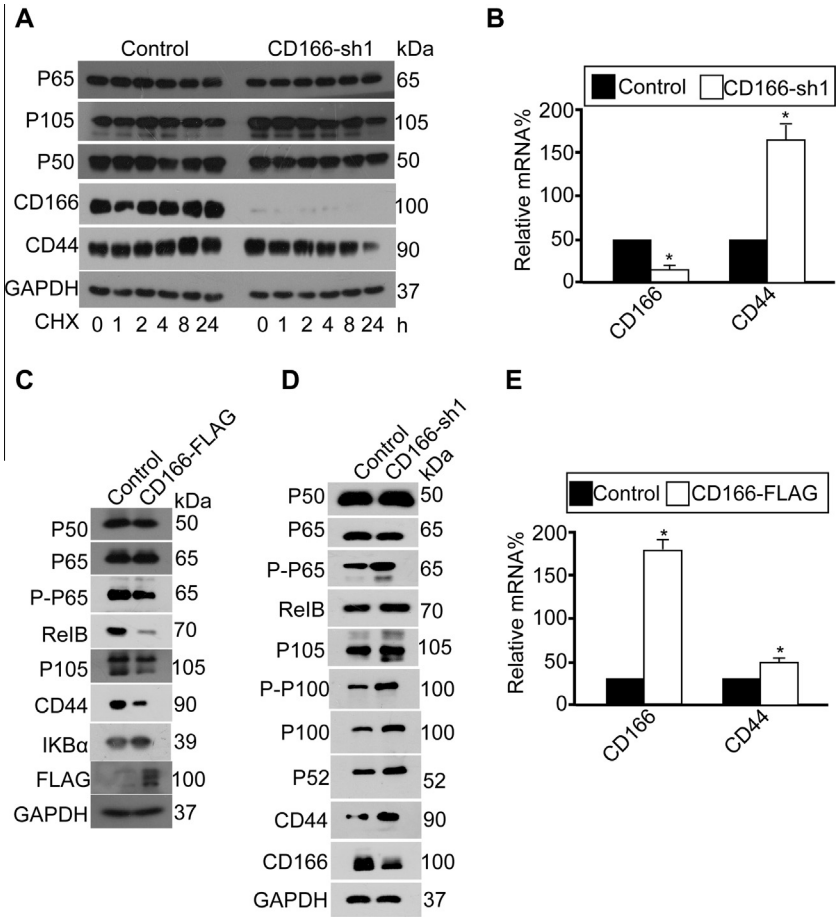

Fig. 2. CD166 regulates CD44 dependent of transcription. (A) CD44 is a stable protein and has a longer half-time. Protein synthesis was inhibited by treatment of CHX (50 μg/ml) for the different time point in control (infected with GFP) and Bel-7402 cells with CD166 knocked down. (B) CD166 inhibited CD44 at transcription level. CD44 mRNA expression was measured by RT-QPCR in control and Bel-7402 cells with CD166 down-regulation. $^{*}P < 0.01$ analyzed by Student *t* test. (C and D) CD166 affects CD44 with a NF-κB pathway dependent manner. Down-regulation of CD166 increased the protein expression of NF-κB (P-P65, RelB, P105, P-P100, P100 and P52) analyzed by Western blot and vice versa. (E) Up-regulation of CD166-FLAG induced the declining of CD44 measured by QPCR. $^{*}P < 0.01$ analyzed by Student *t* test.

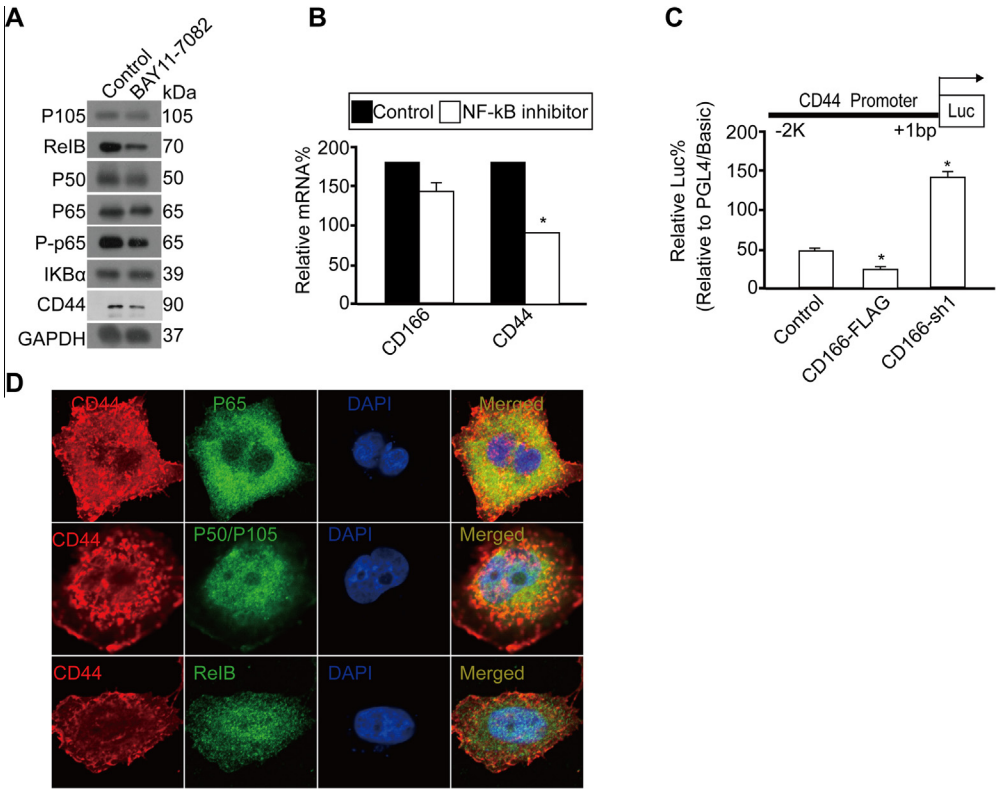

Fig. 3. CD166 inhibits CD44 by inactivity NF-κB pathway. (A and B) NF-κB promotes CD44. Bel-7402 cells treated with DMSO or NF-κB inhibitor BAY11-7082 (30 μM) measured by Western blot and QPCR. (C) CD166 inhibited CD44 promoter activity. CD44 promoter containing −2 kb to +1 bp luciferase activities were measured in Bel-7402 cells with control, CD166-FLAG or CD44-MYC. $^{*}P < 0.01$ versus control measured by Student *t* test. (D) Co-localization of CD44 and NF-κB in Bel-7402 cells. Cells were fixed for IF analysis by anti-CD44, anti-P65, anti-P50/P105 and anti-RelB. Scale bar, 15 μM.

hypothesized that COP1 and UBC3 may regulate CD166 protein degradation. In conclusion, there exist the underling mechanism between CD166 and CD44 in Bel-7402 cells (Fig. 4D).

4. Discussion

In our previously study, we revealed that the mutual interaction between CD166 and YAP promote hepatocarcinogenesis [3]. The relationship between CD166 and other cell surface molecules are still pool understood. We screened a series of cell surface molecules after down-regulation CD166, and found that CD44 was significantly up-regulated (Fig. 1A). Over-expression of CD166 leads to down-regulate CD44 (Fig. 1B). Here, we showed that CD166 and CD44 interaction is crucial for liver cancer cells with a negative feedback loop. It was reported that CD44 is a specific biomarker of small hepatocytes and is up-regulated at the small hepatocytes. And CD44 is critical for hepatic progenitor cells proliferation and/or differentiation [14]. We performed a MTT-based assay (Fig. 1D) and clone formation assay (Fig. 1E and F) to detect liver cancer cell proliferation. And found that CD44 has a little role in Bel-7402 cell proliferation.

We attempted to explore how CD166 and CD44 interact with each other. Firstly, we found that CD44 protein was quite stable (Fig. 2A) with a longer half-time. And CD166 regulated CD44 dependent of transcriptional (Fig. 2B and E). Gene transcription needs RNA polymerase and transcription factors (TFS), which binding to promoter to promote mRNA expression [15,16]. Thus, CD166 regulated CD44 at transcription level. We screened numerous TFS (Supplementary Fig. 1A), including SP1, C-Jun, Fos, C-Raf, Egr1, CREB and NF-κB. Over-expression of CD166 induced NF-κB and CD44 down-regulation (Fig. 2C). Similarly, knockdown of CD166 attenuated the expression of NF-κB and CD44 (Fig. 2D). It was reported that NF-κB is a TF, which is a downstream target of several cell signaling pathways [17]. It is also very important for the pathogenesis of inflammatory, autoimmune and neoplastic diseases [18]. However, the role of NF-κB pathway in the liver cancer is poorly understood. Thus, we thought that NF-κB may be a candidate TF of CD44 promoter.

In this study, we mainly discussed that CD166 inhibited CD44 via blocking up NF-κB pathway. Specific NF-κB inhibitor BAY11-7082 decreased the protein and mRNA expression of CD44 (Fig. 3A and B). Moreover, CD44 interact with NF-κB (P65, P50, P105 and RelB) in Bel-7402 cell (Fig. 3D). Therefore, we constructed a CD44 luciferase reporter vector and discovered that CD166 affected the promoter activity of CD44 (Fig. 3C). These experiments demonstrated that NF-κB is a transcription factor of CD44 promoter. And CD44 is positively regulated by both canonical and non-canonical NF-κB pathway.

Then, we want to know whether CD44 also regulates CD166 expression. Knock-down of CD44 obviously decreased CD166 protein expression compared to control (Fig. 4A). The over-expression of CD44-MYC increased the protein and mRNA expression of CD166 in the Bel-7402 cell (Fig. 4B and D). CD44 regulates CD166 at transcription level as well as post- transcription level. As we all know, E3 ligases is critical for protein degradation [19]. We tested which E3 ligase mediates CD166 protein degradation. Numerous E3 ligases, including SKP2, C-Abl, COP1, BTRC, and UBC3 were detected in Bel-7402 cell with up-regulation CD44 (Fig. 4C). The results

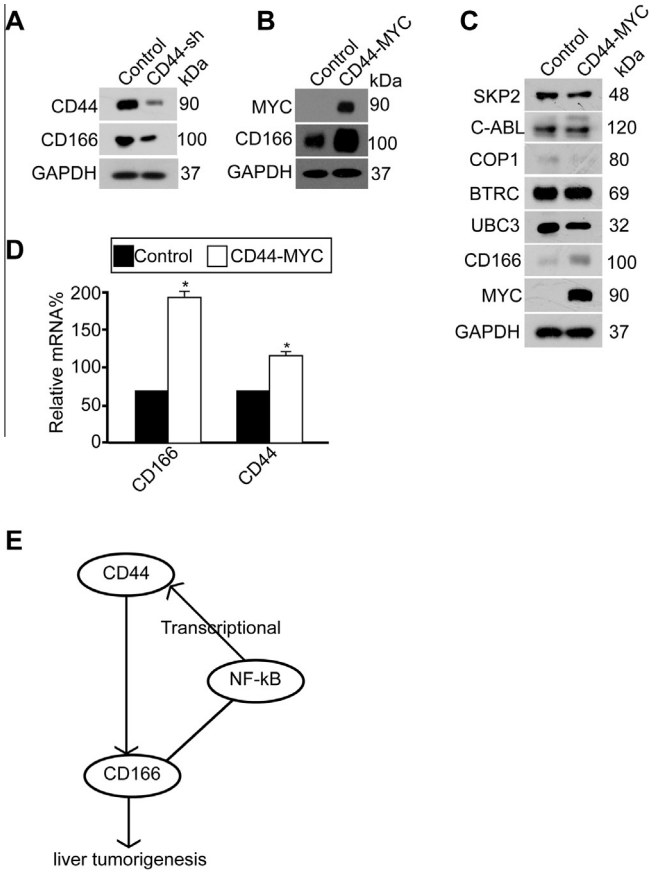

Fig. 4. CD44 promotes CD166 at both transcription and post-transcription. (A and B) CD44 promotes CD166 in Bel-7402. (A) Western blot of CD166 and CD44 in control (infected with shRNA against GFP) and Bel-7402 with knock down of CD44. (B) And over-expression of CD44 induced CD166 up-regulation analyzed by Western bolt. (C) The correlation between CD166 and ubiquitin E3 ligases. Western blot of SKP2, C-ABL, COP1, BTRC, UBC3 and CD166 in Bel-7402 with over-expressed of CD44-MYC. (D) CD44 regulates CD166 partially in transcription level. Up-regulation of CD44-MYC increased the expression of CD166 mRNA. $^{*}P < 0.01$ versus control measured by Student *t* test. (E) The potential molecule mechanism between CD44 and CD166 in Bel-7402 cells.

showed that COP1 and UBC3 were down-regulated. Therefore, we speculate that COP1 and UBC3 may regulate CD166 protein degradation. Taken together, these experiments revealed that there exists a negative feedback loop in the Bel-7402, and the interaction between CD166 and CD44 promote liver cell tumorigenesis (Fig. 4E). Significantly, it could also provide therapy strategies in liver cancer.

Acknowledgments

This study was supported by Natural Science Foundation of China (#81301689 and #81201884), Yangfan Project of Shanghai Committee of Science and Technology (#14YF1412300, to J. Wang), Outstanding Youth Training Program from Tongji University (to J. Wang, #1501219080) and the Shanghai Tenth People's Hospital Climbing Training Program (to J. Wang, #04.01.13024).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.128.

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