CD24 isoform a promotes cell proliferation, migration and invasion and is downregulated by EGR1 in hepatocellular carcinoma

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Introduction: CD24 is known as a heavily glycosylated cell surface molecule that is highly expressed in a wide variety of human malignancies. Previous studies have shown that CD24 plays an important role in self-renewal, proliferation, migration, invasion and drug resistance of hepatocellular carcinoma (HCC). However, little is known about the expression and function of CD24 isoform a (CD24A) and CD24 isoform b (CD24B) in HCC.

Materials and methods: Quantitative real-time polymerase chain reaction (qPCR) and Western blotting were performed to detect CD24 and EGR1 expression in HCC cells and tissue. The function of CD24 in cell proliferation was verified with MTT assays, colony formation assays and tumor xenograft models. Wound healing assays and invasion assays were performed to clarify the function of CD24 in the regulation of cell migration and invasion in HCC. A dual luciferase reporter assay and chromatin immunoprecipitation assay were used to analyze the regulation mechanism of CD24A.

Results: CD24A but not CD24B, which was barely detected by qPCR and Western blotting, is significantly upregulated in HCC tissue. Both CD24A and CD24B contribute to HCC cell proliferation, migration and invasion, but CD24A is more effective than CD24B. EGR1 downregulates CD24A and exerts transcription-promoting activity on the CD24A promoter. Furthermore, EGR1 represses HCC cell proliferation via downregulation of CD24A.

Conclusion: CD24A is the predominant CD24 isoform in HCC and plays a major role in cell proliferation, migration, and invasion. EGR1 can exert its antitumor effect through transcriptional downregulation of CD24A in HCC.

Keywords: CD24A, CD24B, EGR1, proliferation, hepatocellular carcinoma

Introduction

Liver cancer is predicted to be the sixth most commonly diagnosed cancer in 2018 and ranks fifth in terms of global cases and second in terms of deaths for males.1,2 Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is a highly therapy-resistant and thus difficult to treat cancer;3 although systemic therapies have clinical benefits, only a few patients with HCC (<10%) are cured.4 Thus, it is of great significance to reveal molecular alterations in HCC and find novel therapy targets for HCC.

CD24 is a highly glycosylated cell surface glycoprotein expressed on the surface of most B lymphocytes5 and is highly expressed in a wide variety of human malignancies.6-14 The CD24 gene is located on chromosome 6q21 and contains five variants; variant 1, variant 2, variant 3 and variant 7 encode CD24A preproprotein, while variant 4 encodes CD24B. CD24B (129aa) has a distinct N-terminus and is longer than...
CD24 (83aa). In HCC, CD24 was found to be a functional liver tumor-initiating cell marker that drives tumor-initiating cell genesis through STAT3-mediated NANOG regulation, and Twist2 augments liver cancer stem-like cell self-renewal in a CD24-STAT3-Nanog-dependent manner. CD24 induces sorafenib resistance by activating autophagy in HCC, and CD24-targeted therapy may be a promising therapeutic strategy for treatment of HCC. Two CD24 isoforms are encoded by different variants and only share approximately 47% amino acid identity; however, previous studies have focused on total CD24, and thus far, very little has been done to investigate the expression and role of the two CD24 isoforms in HCC. Hence, identification of the predominant CD24 isoform may have therapeutic implications for CD24-targeted treatment of HCC.

Our study identified CD24A as a direct target gene of EGR1 (Early growth response protein 1). EGR1, a nuclear transcription factor, binds to the GC enrichment region of DNA sequences to play its role as a transcriptional regulator. Abnormal expression of EGR1 is often correlated with ischemic injury, atherosclerosis, inflammation and tumors. EGR1 plays complicated roles in HCC. Several studies have stated that EGR1 is overexpressed in HCC tissues, enhances drug resistance by promoting hypoxia-induced autophagy and accelerates the progression of HCC; however, data from several independent laboratories have demonstrated that EGR1 inhibits HCC cell motility and invasion.

In our present study, we found that CD24A was the predominant CD24 isoform in HCC and plays a major role in cell proliferation, migration, and invasion. EGR1 regulated CD24A expression directly and exerted its antitumor effect through downregulation of CD24A in HCC.

Materials and methods

Human liver specimens and TCGA cohort

Ninety paired human primary HCC and matched adjacent non-cancerous liver tissue specimens were obtained from Qidong Liver Cancer Institute (Qidong, China). All tissues were frozen at −80°C until mRNA and protein were extracted. This study was approved by the Research Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine. Informed consent was signed by all patients, and all procedures were conducted in accordance with the Declaration of Helsinki. TCGA data (mRNA expression data for 50 paired cancer/non-cancerous tissues were available) were accessed from the website (https://tcga-data.nci.nih.gov/tcga).

Cell lines and cell culture

The human HCC cell lines SMMC-7721 and BEL-7402 and immortalized normal liver L-02 cells were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, People’s Republic of China). Li7 was purchased from SXBIO Corporation (Shanghai, People’s Republic of China). Huh6 and Huh7 cell lines were obtained from Riken Cell Bank (Tsukuba, Japan). The HCC-LY5 and HCC-LY10 cell lines were established in our laboratory. MHCC-LM3, MHCC-97, MHCC-97H, and MHCC-97L cell lines were obtained from Liver Cancer Institute, Zhongshan Hospital of Fudan University (Shanghai, People’s Republic of China). H2P and H2M cell lines were kindly provided by the University of Hong Kong (Hong Kong, People’s Republic of China). Other cell lines not specifically mentioned here were all purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich Co., St Louis, MO, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in 5% CO2. The use of all lines was approved by the Research Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine.

Quantitative real time polymerase chain reaction (qPCR)

Total RNA was extracted from HCC tissues and cells using Trizol (Thermo Fisher Scientific, Waltham, MA, USA), and reverse transcription was performed with a PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara, Dalian, People’s Republic of China). qPCR was performed with SYBR Premix Ex Taq II (Takara, Dalian, People’s Republic of China) according to the manufacturer’s protocol. The expression levels were normalized using human GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The primer sequences are listed in Table S1.

Western blotting

Proteins extracted from HCC tissues and cells were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). Mouse anti-CD24 (SAB-1402713, Sigma-Aldrich Co.), rabbit anti-EGR1 (SC-189, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and mouse anti-β-Actin (A3854, Sigma-Aldrich Co.) antibodies were incubated separately with the membranes at 4°C overnight after blocking with 5% nonfat milk, and the membranes were
then probed with corresponding HRP-conjugated secondary antibody for 1.5 hours at room temperature.

**Plasmid construction, lentivirus production, and cell transfection**

The full-length human CD24A and CD24B open reading frame cDNA sequences were separately amplified and cloned into a pWPXL plasmid (Addgene, Cambridge, MA, USA) using BamHI and EcoRI. The full-length human EGR1 open reading frame cDNA sequence was amplified and cloned into pWPXL using MluI and EcoRI. The CD24A promoter, which spans a 2,100 bp-region (−1,900 bp to +200 bp based on the first ATG), and mutant were separately cloned into PGL3-Enhancer (Promega Corporation, Fitchburg, WI, USA). The primer sequences are listed in Table S1.

For lentivirus production and cell transfection, after co-transfection of HEK 293 T cells with the pWPXL-CD24A vector, pWPXL-CD24B vector or pWPXL-EGR1 vector with psPAX2 and pMD2.G (Addgene) using Lipofectamine 2000 (Thermo Fisher Scientific) for 48 hours, viruses were harvested and used to infected target cells in the presence of 6 µg/mL polybrene (Sigma-Aldrich Co.).

**MTT assays**

A total of 800–1,000 cells per well were seeded into 96-well plates and cultured for 7 days. MTT reagent (5 mg/mL, Sigma-Aldrich Co.) was added and detected according to the manufacturer’s instructions. All of the experiments were performed in triplicate.

**Colony formation assays**

A total of 500–1,000 cells per well were seeded into 6-well plates and cultured for approximately 2 weeks. Then, the cells were fixed with 10% formaldehyde for 30 minutes and stained with Giemsa solution (Sigma-Aldrich Co.) for 40 minutes at room temperature. All of the experiments were performed in triplicate.

**Wound healing assays**

Cells were seeded into 6-well plates and cultured to approximately 90% confluence in 24 hours. Then, wound healing assays were performed as described previously.

**Transwell invasion assays**

Approximately 1×10⁶ cells in serum-free DMEM were seeded into the top chamber of transwell chambers (8 mm pore, Merck Millipore) precoated with Matrigel (BD Biosciences, San Jose, CA, USA), while 600 µL DMEM containing 10% FBS was added to the bottom chamber. After 24–48 hour of incubation, cells adhering to the lower membrane of the inserts were fixed with 10% formaldehyde for 30 minutes and stained with Giemsa solution. Cells in five randomly selected fields were counted.

**Dual luciferase reporter assay**

Cells were seeded into 96-well plates overnight and co-transfected with the relevant reporter plasmids using Lipofectamine 2000 (Thermo Fisher Scientific). A PRL-TK reporter construct was used as the internal reference. After 48 hours of incubation, firefly luciferase activity and Renilla luciferase activity were detected according to the manufacturer’s instructions (Promega).

**Chromatin immunoprecipitation assay (ChIP)**

ChIP assays were performed with SMMC-7721 and Hep3B cells as previously described. Rabbit anti-EGR1 antibody (SC-189, Santa Cruz Biotechnology) or rabbit IgG were used to immunoprecipitate DNA-containing complexes. The isolated DNA samples were subjected to PCR analyses. The primer sequences are listed in Table S1.

**Tumor xenograft models**

All animal experiments were approved by the Shanghai Cancer Institute Experimental Animal Care Commission prior to commencement of the study and performed following the guidelines and regulations of Shanghai Cancer Institute Experimental Animal Care Commission. The 6–8-week-old male Balb/c (nu/nu) mice were divided randomly into groups. Approximately 2×10⁶ Li7 cells stably expressing CD24A, EGR1 or control (pWPXL) were injected subcutaneously into each mouse. After approximately 7 weeks, all mice were sacrificed. Xenograft tumors were weighed and frozen at −80°C.

**Statistical analyses**

The data are presented as the mean ± standard deviation (SD) and were analyzed using Student’s t-test. Statistical analyses were performed using GraphPad Prism 5 software. P<0.05 was considered statistically significant.

**Results**

**CD24A is overexpressed in human HCC clinical specimens and cell lines**

Amino acid sequence alignment of CD24A with CD24B showed that CD24B has a distinct N-terminus and shares
approximately 47% amino acid identity with CD24A (Figure 1A). We designed different primers to detect the expression of CD24A and CD24B, and the results showed that the CD24A mRNA level in HCC tissues was much higher than that in matched non-cancerous liver tissues (Figure 1B). However, CD24B was barely detected in HCC tissues and their matched non-cancerous liver tissues by qPCR using SYBR Premix Ex Taq II. The same results were obtained in

Figure 1  Expression of CD24 in human HCC clinical specimens and cell lines. 
Notes: (A) Amino acid sequence alignment of CD24A with CD24B; (B) qPCR was carried out to evaluate the mRNA expression of CD24A in 90 pairs of HCC tissues and corresponding adjacent non-cancerous liver tissues; (C) Western blotting was performed to detect the expression of CD24A in 4 pairs of HCC tissues (K) and corresponding adjacent non-cancerous liver tissues (N); (D) mRNA expression levels of CD24A and CD24B in human HCC cell lines were detected by qPCR; (E) the protein expression level of CD24 in human HCC cell lines was determined by Western blotting; (F) the mRNA expression levels of CD24A and CD24B in SK-Hep1 and LI7 cells stably transfected with CD24A, CD24B or control (pWPXL) vectors; (G) Western blot analysis of CD24 protein in SK-Hep1 and LI7 cells stably transfected with CD24A, CD24B or control (pWPXL) vectors. **P<0.01.
HCC and matched non-cancerous liver tissues with Western blotting (Figure 1C). We also detected the expression of CD24 in HCC cell lines via qPCR and Western blotting. As shown in Figure 1D, the mRNA expression of CD24A was much higher than that of CD24B. The Western blotting results showing CD24 protein levels in HCC cell lines were in agreement with the above results (Figure 1E), confirming that only CD24A could be detected. Taken together, these findings indicate that CD24A is the predominant CD24 isoform and is significantly upregulated in HCC tissues.

CD24A promotes HCC cell proliferation, migration, and invasion in vitro

To better investigate the function of CD24A and CD24B in HCC progression, we selected two CD24 low-expressing cell lines, SK-Hep1 and Li7, to construct stable overexpressing cell lines via lentiviral infection. The efficiency of CD24A and CD24B overexpression was verified by qPCR and Western blotting (Figure 1F and G).

MTT assays and colony formation assays showed that both CD24 isoforms contributed to HCC cell growth, but overexpression of CD24A had a stronger effect on cell proliferation in vitro than overexpression of CD24B (Figure 2A and B). To further clarify the role of CD24A in HCC cell growth in vivo, Li7 cells stably expressing CD24A or the control (pWPXL) were subcutaneously injected into male Balb/c (nu/nu) mice. After 7 weeks, the weight of tumors revealed that overexpression of CD24A significantly promoted the tumorigenicity of Li7 cells in vivo (Figure 2C). Western blotting analysis of CD24A protein levels showed that the
tissues of xenografts overexpressing CD24A maintained a high expression level of CD24A (Figure 2D).

Then, we performed wound healing assays and transwell assays to detect the effect of the CD24 isoforms on cell migration and invasion. The results showed that CD24A enhanced cell migration and invasion more effectively than CD24B in vitro (Figure 3A and B). Therefore, these findings indicate that CD24A but not CD24B effectively facilitates HCC proliferation, migration, and invasion.

**EGR1 represses CD24A expression in human HCC cells**

To understand the regulatory mechanism of CD24A expression, bioinformatics analysis was performed and indicated that there were several functional binding sites for EGR1 on the CD24A promoter region. The mRNA level of EGR1 in 90 pairs of human primary HCC tissues and matched adjacent non-cancerous liver tissues from our lab was analyzed by qPCR. The results showed that EGR1 mRNA expression was significantly downregulated in HCC tissues compared with matched adjacent non-cancerous liver tissues (Figure 4A), which is consistent with the analysis of TCGA data (Figure 4B). Western blotting analyses also showed that EGR1 protein expression was significantly downregulated in HCC tissues (Figure 4C).

To explore whether EGR1 can regulate CD24A expression, we detected the mRNA and protein expression profiles of EGR1 in HCC cell lines (Figure 4D and E) and chose SMMC-7721 and Hep3B cell lines to overexpress EGR1 (Figure 4F). qPCR and Western blotting showed that overexpression of EGR1 repressed CD24A expression (Figure 4F). Considering the complicated role of EGR1 in HCC, we also clarified the effect of EGR1 on cell growth in vivo and found that tumor weights were remarkably decreased in the EGR1-overexpressing group compared with the control group (Figure 4G). EGR1 protein levels in the xenograft tumors

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**Figure 3** CD24A enhances HCC cell migration and invasion in vitro.

**Notes:** (A) Wound healing assays of Li7 and SK-Hep1 cells stably transfected with CD24A, CD24B or control (pWPXl) vectors were performed and analyzed; (B) transwell assays of Li7 and SK-Hep1 cells stably transfected with CD24A, CD24B or control (pWPXl) vectors were performed and analyzed. *P<0.05; **P<0.01.
were analyzed by Western blotting (Figure 4H). Overall, EGR1 is significantly downregulated in HCC tissues and may inhibit HCC cell proliferation by repressing CD24A expression.

**CD24A is a direct target of EGR1**

To clarify whether EGR1 can transcriptionally regulate CD24A expression directly, we analyzed the binding site for EGR1 on the CD24A promoter using the JASPAR database.
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eg1 downregulates cD24a expression by directly binding to the CD24A promoter. According to the sequence of the potential EGR1 binding site in JASPAR, we constructed a mutant of the CD24A promoter (Figure 5B). The inhibitory effect of EGR1 on the activity of the CD24A promoter was obviously weakened by the mutation (Figure 5C). A ChIP assay further verified that EGR1 can bind the site on the CD24A promoter (Figure 5D). Therefore, CD24A is a direct transcriptional target of EGR1.

EGR1 suppresses HCC cell proliferation by downregulating CD24A

To further investigate whether downregulation of CD24A is responsible for the tumor suppressive function of EGR1 in HCC, CD24A was overexpressed in Li7 and SK-Hep1 cells with stable overexpression of EGR1 (Figure 6A). MTT assays and colony formation assays in vitro showed that overexpression of EGR1 significantly inhibited cell proliferation, and restoration of CD24A expression reversed the EGR1-induced inhibition of cell proliferation (Figure 6B and C). In conclusion, these data indicate that EGR1 suppresses HCC cell proliferation by repressing CD24A.

Discussion

CD24 is a glycosylphosphatidylinositol-anchored membrane protein reported to be overexpressed in many tumor types, including colorectal cancer, ovarian cancer, bladder cancer, prostate cancer, lung cancer, and breast cancer. CD24 plays important roles in tumorigenesis and drug resistance. CD24 is considered to be a negative cancer stem cell marker, specifically in breast cancer. Intriguingly, mounting evidence has shown that CD24-positive tumor cells are tumor-initiating cells for some cancers, such as colon cancer, pancreatic cancer, cholangiocarcinoma, gastric cancer, cervical cancer, and ovarian cancer. Similarly, CD24 promotes HCC

Figure 5 EGR1 downregulates CD24A expression by directly binding to the CD24A promoter.
Notes: (A) Dual luciferase reporter assays were performed to detect the relative activity of the CD24A promoter after transfection of Li7, SK-Hep1 and SMMC-7721 cells with EGR1 or pWPXL; (B) the sequence logo of the potential EGR1 binding site in JASPAR (top panel) and the diagram of mutant sites in the CD24A promoter (bottom panel); (C) relative activities of the CD24A promoter and mutant promoter after transfection with EGR1 or pWPXL; (D) ChIP assays were performed using an antibody against EGR1 and a negative control (IgG) in EGR1-overexpressing SMMC-7721 and Hep3B cells. *P<0.05; **P<0.01.
Figure 6 EGR1 suppresses HCC cell proliferation by repressing CD24A expression.

Notes: (A) qPCR (top panel) and Western blotting (bottom panel) analysis of EGR1 and CD24A in EGR1-overexpressing Li7 and SK-Hep1 cells after restoration of CD24A expression; (B) overexpression of CD24A reversed the inhibitory effect of EGR1 on cell proliferation in vitro based on MTT assays; (C) overexpression of CD24A reversed the inhibitory effect of EGR1 on cell colony formation in vitro. *P<0.05; **P<0.01.
progression and endows HCC cells with stem cell and drug-resistant properties.\textsuperscript{10,16,18} Therefore, increasing evidence suggests that identification of CD24 signaling pathways may provide an attractive therapeutic strategy against HCC. However, these previous studies did not investigate whether CD24 isoforms have distinct expression patterns and roles, especially in HCC.

Two CD24 isoforms are encoded by different variants and share approximately 47% amino acid identity. This study is the first to demonstrate that CD24A is the predominant isoform and significantly upregulated in HCC tissues, while CD24B could barely be detected by qPCR using SYBR Premix Ex Taq II or by Western blotting in HCC tissues and cell lines. Moreover, CD24A had a stronger effect on proliferation, invasion and migration of HCC cells in vitro than CD24B. Therefore, the expression and role of CD24A are consistent with previous reports of CD24 in HCC.\textsuperscript{10} Intermediate and advanced HCC are highly heterogeneous,\textsuperscript{10,54} and thus, whether CD24B is expressed in a very small population of cells in HCC tissues should be further investigated.

Considering the predominant expression and role of CD24A in HCC cells, we further explored the regulation of CD24A in HCC cells. The promoter region of CD24A was analyzed using the JASPAR database, and an EGR1 binding site located in the CD24A promoter region was found. EGR1 plays complex and often contradictory roles in human malignancies.\textsuperscript{55,56} Based on previous reports, EGR1 also displays both facilitating and repressing effects in HCC.\textsuperscript{26–32} For instance, EGR1 functions as an oncogene, promoting HCC growth and migration/invasion\textsuperscript{27,29,31} and enhancing the drug resistance of HCC cells, likely through autophagy.\textsuperscript{26} Conversely, EGR1 can inhibit HCC progression by repressing the EGRF-MAPK/AKT pathway\textsuperscript{32} and promoting PTEN transcription.\textsuperscript{30} In our current study, EGR1 expression in a 90-patient cohort from our laboratory and a 50-patient cohort from TCGA was analyzed, and we found that EGR1 expression was significantly downregulated in HCC tissues compared with matched adjacent non-cancerous liver tissues. The effect of EGR1 on the proliferation of HCC cells in vivo was detected, and the results showed that overexpression of EGR1 remarkably inhibited HCC cell growth. Further investigation into the mechanism showed that EGR1 repressed the activity of CD24A by directly binding to the CD24A promoter, and restoration of CD24A expression reversed the EGR1-induced inhibition of cell proliferation. Therefore, our data support the hypothesis that EGR1 acts as a tumor suppressor in HCC.

Conclusion

Our findings demonstrate that CD24A, the predominant CD24 isoform, plays the main role in cell proliferation, invasion and migration. EGR1 is a key player in transcriptional control of CD24A and inhibits cell proliferation by down-regulating CD24A. These data provide evidence supporting CD24-targeted treatment of HCC.

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Disclosure

The authors report no conflicts of interest in this work.

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# Supplementary material

## Table S1 Primers for qPCR, ChIP and clone

| Name                  | Primer sequence (5′→3′)                                      |
|-----------------------|-------------------------------------------------------------|
| **qPCR**              |                                                             |
| CD24A-Forward         | ATGGGCGAGGCAATGGTG                                          |
| CD24A-Reverse         | ACCCAGAGTTGGAAGTACTCT                                       |
| CD24B-Forward         | GATTCTGTCGCGAGTGCCCG                                       |
| CD24B-Reverse         | CCGGCGGGTCTCGTCTC                                           |
| EGR1-Forward          | TGACCGAGAGTCTCTTTCT                                        |
| EGR1-Reverse          | GCTTGGCTGGGTAACGTGGT                                         |
| **ChIP**              |                                                             |
| ChIP-Forward          | AGGAAGGAGGATCTCTGCC                                         |
| ChIP-Reverse          | TCCCGGGGCTCTGCTC                                            |
| **Clone**             |                                                             |
| CD24A-Forward         | CGGGATCCATGGGCAGAGCAATGGCGCAG                                 |
| CD24A-Reverse         | CGGAAATCTTAAAGATAGAGATCGAGAGAGAGAGGAGG                       |
| CD24B-Forward         | CGGGATCCATGGGCAGAGCAATGGCGCAG                                 |
| CD24B-Reverse         | CGGAAATCTTAAAGATAGAGATCGAGAGAGAGGAGG                       |
| EGR1-Forward          | CGGCCGTATGGGCGGGCGCAGAGAGAGAGG                              |
| EGR1-Reverse          | CGGAATCTTATGCAAATTTGCTTGAGGAGAGGAGGAGG                       |
| **CD24A promoter clone** |                                                           |
| ~1,900-Forward        | CGACCGGTGAGGTTAGGAGGCTTTTACTG                               |
| Mutant-Forward        | CGACCGGTGCGGTCCTCAATAGGCGCCCGG                              |
| Reverse               | GAGATCTGGTTATCTCTCGGCGG                                     |