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miR-221 REGULATES CD44 IN HEPATOCELLULAR CARCINOMA THROUGH THE PI3K-AKT-mTOR PATHWAY

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Abstract

CD44 and miR-221 are upregulated in hepatocellular carcinoma (HCC) cell lines and tumors, however a connection between the two has not been identified. As the expression of miR-221 directly correlated with CD44 in HCC cells, we hypothesized that miR-221 may directly or indirectly regulate CD44 expression. Inhibition of miR-221 with antisense in Sk-Hep-1 or SNU-449 cell lines reduced CD44 protein expression while miR-221 mimic increased CD44 protein levels. miR-221 antisense did not alter the CD44 mRNA levels in Sk-Hep-1 or SNU-449 cells suggesting that regulation of CD44 protein occurs post transcriptionally. To discover miRNAs that may be involved in the miR-221 regulation of CD44, we performed miRNA profiling in SNU-449 cells treated with anti-miR-221. Several miRNAs were increased with miR-221 inhibition including miR-708-5p, a miRNA that targets CD44. As miR-221 targets several regulators of the PI3K-AKT-mTOR pathway and a link between this pathway and CD44 has been previously shown in prostate cancer, we considered miR-221 regulation of CD44 may be through this pathway. Inhibition of miR-221 reduced p-4EBP1, a downstream effector of the PI3K-AKT-mTOR pathway. Likewise, inhibiting the PI3K-AKT-mTOR pathway with the ATP-competitive mTOR inhibitor PP242 reduced CD44 protein in SNU-423 and SNU-449 cells without altering CD44 mRNA levels.

Keywords

cancer; microRNA; mTOR; miR-708-5p

INTRODUCTION

Extensive profiling studies over the past several years have demonstrated that various miRNAs are differentially expressed in HCC [1–7]. Differentially expressed miRNAs in HCC include miR-221/-222, miR-21 and miR-18 (increased expression in HCC) and miR-122a, miR-199a-3p and miR-200 (reduced expression in HCC) (reviewed in [8, 9]).

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These studies led to the conclusion that miR-221/222 are the most deregulated miRNAs in HCC [6, 10]. miR-221 targets a number of key tumor suppressors including CDKN1B/p27 [11–13], CDKN1C/p57 [14, 15], PTEN [10], TIMP3 [10] and the DNA damage inducible transcript 4 (DDIT4), a modulator of the mTOR pathway [6]. For these reasons transgenic models of miR-221/222 overexpression in the liver were developed to validate that dysregulation of these miRNAs lead to hepatocarcinogenesis [16]. Collectively, these studies identify miR-221 as a key onco-miR in HCC.

CD44 is a hyaluronic acid receptor and major cell surface glycoprotein which is involved in cell-cell interaction, cell adhesion, cell migration and invasion [17]. CD44 is one of the important tumor initiating cell markers in several malignancies including HCC [18–21]. CD44 is overexpressed in HCC [22] and patients expressing low amounts of CD44 showed significantly better disease-free survival compared to those patients expressing high amounts of CD44 [23]. CD44 is a complex molecule that exists in a variety of isoforms that stem from alternative splicing of the CD44 pre-mRNA [17]. The standard form of CD44 (CD44s) consists of exons 5 to 15. Interestingly, regulation of splicing of the CD44 variant forms to CD44s by the factor ESRP1 regulates the epithelial mesenchymal transition (EMT) in breast cancer [24].

Ribosome profiling of prostate cancer cells demonstrated that a number of pro-metastatic, invasion and proliferation genes including CD44 act downstream of mTOR signaling [25]. In prostate cancer, CD44 protein was reduced by the mTOR ATP site inhibitor PP242 or the novel mTOR inhibitor INK128 but not with the allosteric inhibitor rapamycin [25]. PP242 and INK128 are mTOR ATP competitive inhibitors with more potent inhibitory activity than rapalogues [26]. Specifically, they block the kinase activity of both mTORC1 and mTORC2. The mTOR kinase inhibitor AZD-8055 induced more potent growth inhibition than rapamycin analogs in sorafenib resistant SK-Hep-1 cells [27].

This body of work led us to hypothesize that the mTOR pathway may regulate CD44 in HCC. It was already shown that miR-221 regulates the PI3K/AKT/mTOR pathway in HCC through targeting of PTEN [10] and DDIT4 [6]. We hypothesized that miR-221 could alter CD44 levels in HCC through the PI3K/AKT/mTOR pathway. Our study reports a novel regulation of CD44 in HCC through miR-221 regulation of the PI3K/AKT/mTOR pathway.

MATERIALS AND METHODS

Cell culture

The HCC cell lines PLC/PRF/5, Huh7, HepG2, SNU-449, SNU-423 and SK-Hep-1 were purchased from American Type Tissue Collection (Manassas, VA). Huh7, HepG2 and SK-Hep-1 cells were grown in MEM medium (Gibco) with 10% FBS (Sigma). PLC/PRF/5, SNU-449 and SNU-423 cells were cultured in RPMI 1640 medium (Gibco) containing 10% FBS.

RNA extraction and Quantitative PCR

Total RNA was isolated from HCC cells using miRNeasy® Mini kit (Qiagen). cDNA was synthesized from 500 ng of total RNA according to the manufacturer's protocol (Invitrogen).

cDNA was analyzed for gene expression using gene specific primers and the Express SYBR® GreenER qPCR super mix (Invitrogen). For the detection of miR-221, 100 ng of total RNA was assayed using the TaqMan® microRNA Assays (Applied Biosystems). Data were normalized to 18S rRNA and the relative gene expression of genes was presented using the comparative CT method. Total RNA from 3p and 3sp HCC cells [28] were obtained from Dr. Wolfgang Mikulits.

Transfection of oligonucleotides

Anti-miR-221, anti-miR-708-5p and scrambled control oligo were synthesized from ThermoFisher (Lafayette, CO). 2'-O-methyl phosphorothioate-modified anti-miR-221 oligo nucleotide with a 5' cholesterol label was used [29]. SK-Hep-1 and SNU-449 were transfected with anti-miR-221, anti-miR-708-5p or scrambled control oligo using Lipofectamine 2000 and Opti-MEM medium (Gibco). For miRNA profiling, SNU-449 was transfected with 100 nM anti-miR-221 or scrambled control oligo using lipofectamine 2000 (Invitrogen) and Opti-MEM medium (Gibco). At 72 h after transfection, total RNA was extracted using Trizol (Invitrogen).

mTOR inhibitor studies

PP242 (Sigma Chemical Company) was dissolved in DMSO and stored at -20° C. SNU-423 and SNU-449 cells were plated at 10⁵ cells per well of a 6-well plate. Cells were treated with the 2.5 and 5 µM of PP242 or an equivalent amount of vehicle control (DMSO in PBS) for up to 48 h. Cells were harvested and evaluated for protein or RNA expression.

miRNA expression profiling

miRNA profiling was conducted to investigate the miRNA expression in anti-miR-221 transfected SNU-449 cells. The expression of over 900 mature miRNAs were profiled using reverse transcription qPCR as described [30]. TaqMan® microRNA Assays (Applied Biosystems) were performed according to the manufacturer's protocol. miRNAs were defined as expressed if the treatment or control group had a mean C_t of ≤ 36.

Luciferase reporter assay

Binding of the miR-708-5p mimic (ThermoFisher) to the 3' UTR of CD44 was determined in HEK293T cells using a luciferase reporter assay as previously described [22].

Western blotting

Total protein was extracted from cells with RIPA buffer (Sigma), protein lysates (15–30 µg) were separated on NuPAGE 4–12% Bis-Tris gels (Novex) and transferred to polyvinylidene difluoride membranes (Roche). Membranes were blocked and incubated overnight with primary antibodies: anti-CD44 (Cell Signaling, #3578 Danvers, MA), anti-vimentin (Abcam, ab92547), anti-phosphorylated S6K (Cell Signaling, #9234), anti-phosphorylated 4EBP-1 (Cell Signaling, #2855), anti-xCT (SLC7A11, Cell Signaling, #12691), anti-α-Tubulin, (Santa Cruz, #sc-8035), anti-β-Actin, (Cell Signaling, #4970 or anti-GAPDH antibodies (Santa Cruz Biotechnology, sc-32233). A secondary anti-rabbit (Cell Signaling) or anti-

mouse immunoglobulin G antibody peroxidase conjugate (GE Healthcare) was detected using ECL Western Blotting Analysis System (Amersham Biosciences).

Statistical analysis

All the samples between treatments were compared using a student's 2-sample t-tests. A p-value < 0.05 was considered significant.

RESULTS

CD44 and miR-221 expression are increased in mesenchymal like HCC cells

As miR-221 and CD44 are both increased in HCC patients, we wanted to see if a correlation exists between the expression of the two genes in epithelial and mesenchymal HCC cell lines. The expression of both RNAs was measured in the HCC cell lines PLC/PRF/5, Huh7, HepG2, SNU-449, SNU-423 and Sk-Hep-1. A positive correlation exists between the miR-221 expression and the mesenchymal markers vimentin and CD44 while a negative correlation exists between miR-221 and the epithelial marker CDH1 (Fig. 1). To further evaluate the miR-221 expression in epithelial and mesenchymal cells, RNA was obtained from two distinct liver cell lines from an HCC patient previously reported by van Zijl, et al [28]. These authors showed that the cell line 3p is of an epithelial origin while 3sp cells are mesenchymal. From the published gene expression data of those two cell lines (GSE26391), we confirmed that CD44 expression is low in the 3p epithelial cells and high in the 3sp mesenchymal cells (Fig. 1D). miR-221 levels were also significantly lower in 3p, epithelial cells and high in 3sp, mesenchymal cells (Fig. 1E). These findings confirm a direct correlation between CD44 and miR-221 in both continuous HCC cell lines and in primary HCC cells derived from patients and that mesenchymal but not epithelial cells express high levels of CD44 and miR-221.

miR-221 regulates CD44 protein expression post transcriptionally in HCC cells

The direct correlation between CD44 and miR-221 in HCC cells prompted us to determine whether miR-221 directly or indirectly regulates CD44 expression in HCC. Two HCC cell lines with high miR-221 and high CD44 were transfected with anti-miR-221 or control oligo. CD44 protein expression was decreased in the anti-miR-221 treatment compared to the control oligo (Fig. 2A,B). qPCR confirmed that miR-221 levels were reduced by anti-miR-221 (data not shown). In order to see if the inhibition of CD44 protein by anti-miR-221 occurs at the level of transcription, CD44 mRNA was measured in HCC cells transfected with anti-miR-221. Interestingly, CD44 mRNA did not change with anti-miR-221 treatment (Fig. 2C,D). To further investigate the potential regulation of CD44 by miR-221 in HCC, Huh7 cells (which express low levels of miR-221 and CD44) were transfected with 100 nM of miR-221 mimic. Ectopic expression of miR-221 increased CD44 protein expression but not mRNA (Fig. 2E). These results demonstrate that CD44 expression is indirectly regulated by miR-221 and this regulation occurs post-transcriptionally.

miR-708-5p targets CD44 and is indirectly regulated by miR-221

We next wanted to investigate how miR-221 regulates CD44 expression post-transcriptionally. We hypothesized that miR-221 indirectly regulates downstream miRNAs

which target CD44 by translation repression rather than mRNA degradation. To determine which miRNAs are differentially expressed by anti-miR-221 treatment, miRNA profiling in anti-miR-221 treated SNU-449 cells was performed. miR-221 was the most abundantly decreased miRNA while several miRNAs were increased by miR-221 inhibition including miR-708, -1290, -1276 and -146a-5p (Fig. 3A). Interestingly, miR-708-5p has been reported to target CD44 in prostate cancer [31] and miR-1276 is predicted to target CD44.

To confirm these data [31], we evaluated the ability of miR-708-5p to bind to and regulate CD44. miR-708-5p but not control oligo suppressed luciferase expression following binding to the CD44 3' UTR (Fig. 3B). To further implicate the relationship between miR-221 downregulation with upregulation of miR-708-5p, we transfected SNU-449 cells with 100 nM control oligo or with 50 nM of anti-miR-221 or control oligo. To further implicate that the reduced CD44 expression is a result of the effect of increased miR-708-5p, we performed a second transfection of 50 nM anti-miR-221 and anti-miR-708-5p. Addition of the anti-miR-708-5p rescued the ability of anti-miR-221 to reduced CD44 and CD44 levels were restored to that of control treatments (Fig. 3C). Transfection of anti-miR-708-5p and control oligo had no effect on CD44 protein levels, likely the result of very low endogenous levels of miR-708-5p in SNU-449 cells (Fig. 3C). These data confirm that miR-708-5p suppresses CD44 and that increased levels of miR-708-5p following inhibition of miR-221 are responsible in part for the reduction of CD44 protein by miR-221 inhibition.

mTOR inhibitor reduces CD44 expression in HCC cells at the translational level

miR-221 has been reported to regulate the PI3K-AKT-mTOR pathway by targeting PTEN and DDIT4 [6, 10]. In addition, CD44 expression was regulated in an mTOR pathway dependent manner in prostate cancer [25]. We hypothesized, therefore, that miR-221 could regulate CD44 expression through the PI3K-AKT-mTOR pathway in HCC. In order to determine if miR-221 regulates the major downstream effectors of the mTOR pathway, we measured p-4EBP-1, a key regulator of protein synthesis, in SNU-449 cells exposed to anti-miR-221. p-4EBP-1 was reduced by anti-miR-221 treatment and this inhibition correlated with reduction of CD44 protein (Fig. 4A).

To further investigate this relationship, we determined if inhibition of the PI3K/AKT/mTOR pathway using a small molecule inhibitor would reduce CD44 protein levels in HCC cells. SNU-423 and SNU-449 cells were treated with the ATP-competitive mTOR inhibitor PP242. The inhibitor decreased CD44 and vimentin protein levels as well as p-4EBP-1 and p-S6K, downstream effectors of the mTOR pathway (Fig. 4B). Reduction of these proteins was not a result of a general inhibition in protein synthesis by PP242 as GAPDH, α -tubulin and β -actin remained unchanged (Fig. 4B). Since a CD44 variant has been shown to interact with and stabilize the cysteine transporter xCT [32, 33], we asked if xCT levels would correlate with CD44 following treatment with PP242. PP242 reduced both CD44 and xCT protein (Fig 4B). CD44 and xCT mRNA levels were not reduced by PP242 and in fact slightly increased (Fig. 4C) demonstrating that the reduction in CD44 and xCT protein occur at the level of translation. These data confirm the regulation of the PI3K/AKT/mTOR pathway by miR-221 [6, 10] and links CD44 to the PI3K/AKT/mTOR pathway in HCC.

DISCUSSION

miR-221 expression is upregulated in HCC patient specimens [1–7] and transgenic mice overexpressing miR-221 in the liver promotes carcinogenesis [16]. To our knowledge, a direct link between miR-221 and CD44 has not been reported. miR-221 was associated with CD44 in breast cancer, however no details were reported on a mechanism of action linking the two [34]. We report here that miR-221 and CD44 expression directly correlate in HCC continuous cell lines and in primary cells derived from HCC patients. This later point is of particular importance because it demonstrates that phenotypes that are present in continuous cells lines are also apparent in primary cell lines derived from HCC patients.

The correlation between miR-221 and CD44 in HCC led us to investigate whether miR-221 could directly or indirectly regulate CD44 in HCC. Anti-miR-221 reduced CD44 protein expression (Fig. 2A,B) and miR-221 mimic increased CD44 expression (Fig. 2E). As miRNAs typically suppress rather than enhance the expression of their targets, we would not expect CD44 to be a direct target of miR-221. Since expression of CD44 mRNA was unchanged in anti-miR-221 transfected cells (Fig. 2C,D), we hypothesized that one explanation of these phenomena is that anti-miR-221 indirectly regulates miRNAs which represses CD44 at the level of translation and not mRNA degradation. miR-708-5p is one of the most highly upregulated miRNAs following anti-miR-221 transfection (Fig. 3A). We confirm the results of Saini, et al., [31] and show that miR-708-5p binds to the 3' UTR of CD44 (Fig. 3B). Furthermore, reduction in CD44 protein by anti-miR-221 may be rescued by co-treatment with anti-miR-708-5p (Fig. 3C).

We considered the PI3K-AKT-mTOR pathway to further explain miR-221 regulation of CD44 as miR-221 directly targets both PTEN and DDIT4 in HCC, both modulators of PI3K- AKT-mTOR pathway [6, 10]. Moreover, CD44 expression was regulated in an mTOR- dependent manner in prostate cancer [25]. To confirm CD44 regulation through the PI3K/AKT/mTOR signaling pathway, we treated CD44 positive SNU-423 and SNU-449 cells with the ATP-competitive inhibitor of mTOR, PP242. The inhibition of the mTOR pathway indeed decreased CD44 expression at the translational level, which is identical to regulation of CD44 by anti-miR-221 (Fig. 4). Our data demonstrate a novel mechanism for ATP-competitive mTOR inhibitors, regulation of CD44, which will be investigated in future studies.

Our study reports a novel regulation of CD44 in HCC through miR-221. Regulation of both PTEN and DDIT4 by miR-221 will impact the PI3K/AKT/mTOR pathway [6, 10]. Additional regulation of CD44 is possible by miR-708-5p. So miR-708-5p may be added to a growing list of miRNAs that target CD44 including miR-199a-3p [22], miR-34a, [35], miR-143/- 145 [36] and miR-328 [37] among others. As CD44 is involved in regulation of invasion and metastasis of HCC and is a stem cell marker for HCC, our findings suggest alternative means of targeting CD44 in HCC using anti-miR-221 and/or small molecule inhibitors of the PI3K/AKT/mTOR pathway.

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Figure 1. Gene expression correlations in epithelial and mesenchymal HCC cell lines

Gene expression was determined by qPCR in three epithelial (Huh7, HepG2 and PLC/PRF/5) and three mesenchymal (SNU-423, SNU-449 and Sk-Hep-1) HCC cell lines. The expression of miR-221 is compared to (A) vimentin, (B) CD44, and (C) CDH1. The expression of (D) CD44 and (E) miR-221 in both 3p and 3sp HCC cells. CD44 expression was obtained from the data set GSE26391 and miR-221 was determined by qPCR. Mean $\pm$ S.D. from three biological replicates. ** $P < 0.01$; *** $P < 0.001$.

Figure 2. miR-221 regulates CD44 protein expression post transcriptionally

CD44 protein was measured in SNU-449 or Sk-Hep-1 cells transfected with 50 or 100 nM of anti-miR-221 or scrambled control (SC) oligo for 48 (A) or 96 h (B). CD44 mRNA was measured by qPCR in SNU-449 or Sk-Hep-1 cells transfected with 50 or 100 nM of anti-miR-221 or scrambled control (SC-ASO) oligo for 48 (C) or 96 h (D). (E) Huh7 cells were transfected with 100 nM of miR-221 mimic or negative control (NC) oligo for 72 h. CD44 expression was determined by immunoblotting.

Figure 3. miR-708-5p targets CD44 in HCC, anti-miR-708-5p rescues anti-miR-221 inhibition of CD44

(A) The differential expression of miRNAs in SNU-449 cells treated with anti-miR-221 or control oligo are presented as a volcano plot. Data were generated by profiling over 900 miRNAs by qPCR. Only those miRNAs that were expressed as defined in the Methods section are presented. The x-axis represents the $\log_2$ fold change in the anti-miR-221 compared to control treated cells. The y-axis is the P values from the anti-miR-221 to control oligo comparison. The lines represent the fold change = 1.5 and $P = 0.05$ cutoffs. (B) The ability of miR-708-5p to target CD44 is shown using luciferase reporter assay in

HEK293T cells transfected with miR-708-5p along with a vector containing the 3' UTR of CD44 downstream of the luciferase gene. (C) SNU-449 cells were exposed to (left to right on immunoblot) media, media plus lipofectamine, 100 nM control oligo, 50 nM each of anti-miR-221/control oligo, 50 nM each of anti-miR-221/anti-miR-708-5p or 50 nM each of anti-miR-708-5p/control oligo. Total protein was resolved by gel electrophoresis and the immunoblot was probed for CD44 and GAPDH.

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Figure 4. miR-221 regulates CD44 expression through the mTOR pathway

(A) SNU-449 cells were transfected with 50 or 100 nM of anti-miR-221 or control oligo for 48 and 96 h. Phosphorylated 4EBP-1 and other proteins were measured by immunoblotting. (B) SNU-423 and SNU-449 cells were treated with 2.5 or 5 μ M of the ATP-competitive inhibitor of mTOR PP242 for 48 h. (C) The mRNA expression of CD44 and xCT was measured by qPCR in SNU-423 and SNU-449 cells treated with 2.5 or 5 μ M of PP242 or vehicle control for 48 h.