Mechanism and molecular network of RBM8A-mediated regulation of oxaliplatin resistance in hepatocellular carcinoma via EMT

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Abstract

Epithelial-mesenchymal transition (EMT) has been shown to be closely associated with Oxaliplatin (OXA) resistance. Previous study found that RBM8A is highly expressed in HCC and induce EMT, suggesting that it may be involved in the regulation of OXA resistance in HCC. However, the accurate mechanism has not been concluded. In our study, ectopic expression and silencing of RBM8A were performed to explore its function. The OXA resistance potential of RBM8A and its downstream pathway was investigated using in vitro and in vivo models. The results showed that RBM8A overexpression induced EMT in OXA-resistant HCC cells, thereby affecting cell proliferation, apoptosis, migration, and invasion and promoting OXA resistance in vivo and in vitro. Moreover, whole-genome microarrays combined with bioinformatics analysis revealed that RBM8A has a wide range of transcriptional regulatory capabilities in drug-resistant HCC, including the ability to regulate several important tumor-related signaling pathways. Histone deacetylase 9 (HDAC9) is an important mediator of RBM8A activity related to OXA resistance. These data suggest that RBM8A and its related regulatory pathway represent potential markers of OXA resistance and therapeutic targets in HCC.

Background

Hepatocellular carcinoma (HCC) is a highly lethal cancer; although it has the sixth highest incidence of malignant tumors globally, its mortality rate ranks second among all cancers[1]. The advent of the molecularly targeted drug sorafenib has opened the door to advanced HCC drug therapies, but the objective response rate (ORR) and progression-free survival (PFS) for first-line therapies are limited and expensive to administer[2]. HCC is extremely complicated and refractory, and the difficulty in successfully treating it also highlights the necessity and importance of multiple methods and multidisciplinary comprehensive treatments, including systemic chemotherapy.

The EACH trial and subsequent basic experiments and clinical trials have confirmed that OXA-based systemic chemotherapy for Asian patients with advanced HCC can improve the objective efficiency and has good tolerance and safety, a high cost-effectiveness ratio and easy clinical promotion; therefore, OXA is recognized as the most effective chemotherapy for HCC[3, 4]. Even so, the effectiveness of OXA against HCC is still less than 20%, which is unsatisfactory. OXA resistance has become a tremendous obstacle in the treatment of HCC. Identifying key targets affecting oxaliplatin resistance and its related signaling pathways and understanding their mechanism of action have become key issues to be solved urgently.

Epithelial-mesenchymal transition (EMT) refers to the biological process by which epithelial cells gradually lose cell-cell adhesions and undergo a specific transformation to obtain characteristics of a strong mesenchymal phenotype, such as movement and migration; this process is closely related to the occurrence and development of a variety of tumors[5]. Ma *et al.*[6] reported that OXA-resistant HCC cells showed EMT, cell cycle arrest, decreased apoptosis, and significantly enhanced invasion and metastasis

abilities, suggesting that EMT plays a crucial role in the regulation of OXA resistance in HCC cells, but the upstream and downstream molecules within its regulatory network have not been identified.

RNA-binding proteins (RBPs) are a general group of proteins that bind to RNA and regulate the metabolic process via RNA. RBPs, as part of the RNA life cycle, are a powerful and extensive regulatory factor whose main role is to mediate the maturation, translocation, and translation of RNA, all of which play an important role in cell development, differentiation, metabolism, health and disease[7]. Our previous results show that RBM8A was highly expressed in HCC tumor tissues compared to the levels in normal liver tissues[8, 9]. Overexpression of RBM8A was related to the poor overall survival and PFS of patients with HCC. In vitro function experiments further demonstrated that RBM8A promoted proliferation, migration and invasion in HCC via activation of EMT[8]. It can be speculated that RBM8A may be involved in the process of drug resistance of HCC cells by initiating EMT. Currently, the relationship between RBM8A and OXA resistance in HCC has not been clarified. In this study, we sought to delineate whether RBM8A regulates OXA resistance in HCC by EMT and its potential molecular mechanism, aiming to identify more EMT-related regulatory pathways and strategies to reverse tumor drug resistance.

Materials And Methods

Cell lines and cell culture

The human HCC cell lines Bel7404 and MHCC97H were purchased from Stem Cell Bank, Chinese Academy of Sciences, and HCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. All the cells were confirmed to be free from mycoplasma contamination.

Establishment of OXA-resistant HCC cells by concentration-elevation and intermittent induction treatment with oxaliplatin in vitro

Bel7404 cells were suspended at a density of 1×105 cells/mL. After 24 h of culture, an initial induction dose of OXA (8 μM) was added to continue the culture. When cell growth was stabilized, the applied drug concentration was increased by 8 μM, 12 μM, 18 μM, 34 μM, 46 μM, 60 μM, 76 μM, 94 μM, 114 μM, and 136 μM. Similarly, MHCC97H cells were suspended at a density of 1×105 cells/mL. After 24 h of culture, an initial induction dose of OXA (6 μM) was added to continue the culture. When the cell growth was stabilized, the induction dose was increased by 6 μM, 9 μM, 13.5 μM, 20.3 μM, 30.4 μM, 40.5 μM, 55 μM, 70 μM, 86 μM, and 102 μM. Each of the above doses was maintained for 15 days before increasing the concentration again, and by the end of the sixth month, the OXA-resistant cell lines Bel7404/OXA and MHCC97H/OXA were created. Drug-resistant HCC (DR-HCC) cells were maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin. Images in Fig. 1C were taken from an inverted microscope (IX70; Olympus) using an LCA ch 20×/0.60 objective.

Establishment of stable cell lines with RBM8A overexpression or knockdown

According to our previous research, the shRNA sequence 5' AGAGCATTCACAAACTGAA-3' was able to reduce the endogenous levels of RBM8A by more than 80% (8); thus, we selected this sequence for the follow-up study in this experiment. Establishment of the stable cell lines Bel7404-RBM8A-KD, Bel7404/OXA-RBM8A-KD MHCC97H-RBM8A-OE, and MHCC97H/OXA-RBM8A-OE was performed as described previously[8].

Total RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from parental cell lines (PCLs) and DR-HCC cells using TRIzol reagent (Invitrogen). cDNA was reverse transcribed from 1 mg of total RNA using PrimeScript RT Reagent (TaKaRa) following the manufacturer's instructions. qRT-PCR was performed with SYBR Premix Ex Taq (Takara, Dalian, China). The following PCR primers were used: RBM8A forward, GCGTGAGGATTATGACAGCGTG; RBM8A reverse, TTCGGTGGCTTCCTCATGGACT. The primer sequences for other target genes are provided in the supplementary material for Materials and methods. Quantification was normalized to β-actin, which served as the internal control. The relative mRNA expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Protein extracts and western blot analysis

Western blot (WB) analysis was performed as described previously (8). Anti-human RBM8A (Santa Cruz Biotechnology, sc-32312), anti-human actin (Proteintech, HRP-60008), and anti-rabbit IgG (Cell Signaling Technology, 7074) antibodies were used in this study. The reagents used for other proteins are described in the supplementary material for Materials and methods.

Cell counting kit-8 assay

Cell proliferation and the half maximal inhibitory concentration (IC50) were assessed using a Cell Counting Kit-8 (CCK-8) kit (Dojindo, Japan) according to the manufacturer's protocol. To measure the IC50 of OXA against the cells, OXA was added at concentrations of 40 μM, 80 μM, 320 μM, 640 μM, and 1280 μM, and 48 h later, 10 μL of CCK8 was added to the wells (per 100 μL medium). The cells were then incubated at 37°C for another 2 h. The absorbance was finally measured at 450 nm using a microplate reader (5082Grodig, Tecan, Austria).

Flow cytometry analysis

Cells were collected and stained with an ANXA5 (Annexin V)-PE apoptosis detection kit (4A Biotech Co. Ltd., FXP018-100) according to the manufacturer's instructions. Apoptosis was analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA).

Wound-healing assay

PCLs and DR-HCC cells were seeded in a 24-well plate and cultured in an incubator until the confluence reached 100%. A pipette nozzle was scraped from the center of the well to the lower end of the well plate

to form a scratch. After the scraped cells were washed away, the adherent cells were incubated in serum-free medium. An image was taken at 0 h as a control, and then the plate was placed in an incubator with 5% CO_2, after which the plates were removed at 24 h, 48 h, and 72 h after scratching to obtain photographs under a fluorescence microscope (200×).

Cell migration and invasion assays

Cytoskeleton and EMT-related proteins in PCLs and DR-HCC cells were stained by immunofluorescence. PCLs and DR-HCC cells were suspended on a sterilized coverslip, and the rabbits were removed after 24 h of incubation in the incubator. The cells were fixed with 4% paraformaldehyde at room temperature, and 0.5% Triton X-100 (Shanghai Shenggong Biological Co., Ltd., China) was added to permeabilize the cells. Next, 200 μl of TRITC-labeled phalloidin (Sigma), 200 μl of DAPI (concentration: 100 nM) (Gibco, USA) solution (for counterstaining nuclei), and fluorescence mounting medium (Gibco, USA) were added to the slides. Laser confocal microscopy (Olympus) was performed with a TRITC excitation/emission filter (Ex/Em = 545/570 nm) and a DAPI excitation/emission filter (Ex/Em = 364/454 nm). Slides were treated with the same primary antibodies as those used in the WB experiments, and Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L) secondary antibody (1:1000) was purchased from Invitrogen (USA) and incubated with the slides at 37°C for 1 h before DAPI counterstaining as described above.

Xenograft tumorigenesis in nude mice

BALB/C nude mice (5–6 weeks old, 18–22 g) were randomly divided into two groups (each containing eight mice). Bel7404/OXA-RBM8A-KD and Bel7404/OXA-NC cells (2 × 106 cells in 100 μl of serum-free DMEM) were injected subcutaneously into nude mice. OXA at 10 mg/kg was injected around the tumor at 1 week, 2 weeks, 4 weeks and 6 weeks after tumor cell injection. Tumor diameter was measured weekly with calipers, and the tumor volume was recorded. After 6 weeks, the mice were euthanized, and the tumor was removed, weighed and photographed. Mouse studies were conducted according to the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Affiliated Tumor Hospital of Guangxi Medical University. Sample collection was performed according to the ethical guidelines of the Declaration of Helsinki and approved by the ethics and research committee of the Affiliated Tumor Hospital of Guangxi Medical University.

Immunohistochemical staining

Hematoxylin & eosin (H&E) staining was performed to observe the histopathology of tumors in nude mice tumors, and the subsequent slides were stained with an HRP kit (UltraTek; Scytek) for further immunohistochemistry. Primary antibodies against RBM8A, E-cadherin, N-cadherin, Snail, ABCG2, ABCB1 and ABCC1 were the same as those used for WB; Ki-67 antibody was obtained from Cell Signaling Technology (9027). Antibodies and working fluid were purchased from Fuzhou Maixin Company, China. The specific steps were carried out according to the kit instructions and as described previously[8].

Whole-genome microarrays

Total RNA was isolated from Bel7404/OXA-RBM8A-KD, Bel7404/OXA-RBM8A-NC, MHCC97H/OXA-RBM8A-OE, and MHCC97H/OXA-RBM8A-NC using an RNeasy micro kit (QIAGEN) following the manufacturer's instructions, and RNA integration was examined using a Bioanalyzer 2100 (Agilent). The microarray analysis was performed using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. The arrays were hybridized, washed, and scanned according to the standard Affymetrix protocol. The raw data were normalized using the MAS 5.0 algorithm in GeneSpring software (version 11.0; Agilent).

Bioinformatics analysis

The differential expression analysis of gene expression profile data in this study was carried out with the limma package of R language[10-12]. Weighted gene coexpression network analysis (WGCNA)[13] was used to analyze the differential expression profile matrix in cell samples, and the gene modules with coexpression were clustered. Furthermore, the R language Clusterprofiler package[14] was used to analyze the Gene Ontology (GO) function (p value cutoff = 0.01, q value cutoff = 0.01) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (p value Cutoff = 0.05, q value Cutoff = 0.2) of these module genes.

Pivot regulators are defined as modulators that have significant regulatory roles for modules in the process of RBM8A-induced resistance, including noncoding RNA (ncRNAs) and transcription factors (TFs). We require that there be more than 2 control connections between each regulator and each module, and the significance of the enriched target in each module calculated based on the hypergeometric test is a p value < 0.01. In the pivot analysis, the background set is based on the mutual opposition of TF-protein in the TRRUST v2 database[15] and the information of lncRNA-protein and miRNA-protein interactions in the RAID v2.0 database[16] for network construction. Finally, the regulatory information of RBM8A on module genes and pivot TFs was obtained by a database search using STRING v10.5[17]. Based on the regulatory information of the pivot regulator to the module and the key KEGG pathway information involved in the module gene, a comprehensive landscape map of RBM8A regulation as it relates to OXA resistance in HCC was obtained.

qRT-PCR and WB analyses confirmed that histone deacetylase 9 (HDAC9) is the pivotal TF that is most closely related to RBM8A-regulated OXA resistance in HCC. The HDAC9-module gene-KEGG signaling pathway was extracted, and a potential mechanism by which the RBM8A-HDAC9 axis regulates drug resistance in HCC was identified.

Statistical analyses

The data were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). All experiments in this study were repeated in triplicate unless otherwise specified. All the results are expressed as the mean ± SD. Student’s t-test was used to analyze the statistical significance of the differences between groups. p values < 0.05 were considered statistically significant.

Results

An OXA-resistant HCC cell line model was established in vitro, and the expression of RBM8A in drug-resistant HCC cells was significantly higher than that in parental HCC cells.

According to the qRT-PCR and WB results, RBM8A had the lowest expression in the human liver cell line HL7702 and was highly expressed in various human HCC cell lines (Fig. 1A). Among them, Bel7404 cells with the highest expression of RBM8A and MHCC97H cells with relatively low RBM8A expression were selected for subsequent experiments. The OXA-resistant Bel7404 (Bel7404/OXA) and MHCC97H (MHCC97H/OXA) cell liens were established by the concentration-elevation and intermittent induction treatment with oxaliplatin in vitro (Fig. 1B). The mesenchymal phenotype of DR-HCC cells was observed under an inverted microscope (Fig. 1C). The following stable cell lines were established: Bel7404-RBM8A-KD, Bel7404/ OXA-RBM8A-KD, MHCC97H-RBM8A-OE, and MHCC97H/OXA-RBM8A-OE. qRT-PCR and WB analysis showed that the expression of RBM8A in Bel7404/OXA and MHCC97H/OXA cells was significantly higher than that in Bel7404 and MHCC97H cells (P<0.05) (Fig. 1D).

High expression of RBM8A promotes proliferation, reduces the proportion of apoptotic parental and OXA-resistant HCC cells, and improves the tolerance of HCC to OXA in vitro.

To explore the effect of RBM8A on the resistance of PCLs and DR-HCC cell lines to OXA, the Cell Counting Kit-8 (CCK8) assay was used to detect and draw the growth curve. In Bel7404 cells with the highest endogenous expression of RBM8A, knocking down RBM8A significantly reduced the proliferation of both the PCLs and DR-HCC cell lines. In MHCC97H cells with relatively low endogenous RBM8A expression, proliferation after overexpression of RBM8A was significantly enhanced in both PCLs and DR-HCC cell lines (Fig. 2A). The IC50 of OXA in the DR-HCC cell lines was significantly higher than that in the PCLs. After overexpression of RBM8A, the IC50 of MHCC97H/ OXA-RBM8A-OE was the highest. After knockdown of RBM8A in Bel7404/OXA cells, the IC50 value was significantly reduced, which was consistent with the proliferation trends (Fig. 2B). Under these conditions, flow cytometry was used to detect cell apoptosis in the control group and groups that received a specific concentration of OXA. The results showed that, regardless of the cell type, the apoptosis level of the DR-HCC cell lines was significantly reduced compared with that of the PCLs (Fig. 2C). In Bel7404 cells with RBM8A knockdown, the apoptosis levels of both the PCLs and DR-HCC cell lines were significantly higher than those in the control group (Fig. 2C and D). In contrast, when RBM8A was overexpressed in MHCC97H cells, the apoptosis levels of both the PCLs and DR-HCC cell lines were significantly reduced compared with those in the control group.

High expression of RBM8A promotes the migration and invasion of parental and OXA-resistant HCC cells and promotes the expression of the drug-resistant related proteins ABCG2, ABCB1 and ABCC1 in vitro.

To further validate the effect of RBM8A on migration and invasion in PCL and DR-HCC cells as well as the potential mechanism involved, the following experiment was performed: The results showed that in both Bel7404 and MHCC97H cells, the migration and invasion of the DR-HCC cell lines were significantly enhanced compared with those in the corresponding PCLs (Fig. 3). Compared with the drug-resistant Bel7404/OXA-NC cells, the drug-resistant Bel7404/OXA-RBM8A-KD cells had significantly reduced

migration and invasion (24 h: $P < 0.005$; 72 h: $P < 0.001$). Compared with the MHCC97H/OXA-NC group, the MHCC97H/ OXA-RBM8A-OE group had significantly enhanced migration and invasion (24 h: $P < 0.005$; 72 h: $P < 0.0001$).

Overexpression of the ABC membrane transport pump is one of the most important contributors to multidrug resistance. Exploring the relationship between the expression of RBM8A and that of ATP Binding Cassette Subfamily G Member 2 (ABCG2), ATP Binding Cassette Subfamily B Member 1 (ABCB1) and ATP Binding Cassette Subfamily C Member 1 (ABCC1), which are representative of the ABC family, in PCLs and DR-HCC cells is of great significance for the regulation of drug resistance by RBM8A in HCC. The WB results (Fig. 3D and E) show that the ABCG2, ABCB1 and ABCC1 levels in DR-HCC cells were significantly higher than those in PCL-HCC cells in both the Bel7404 and MHCC97H cell lines ($P < 0.005$). After further altering the expression of RBM8A in DR-HCC cells compared with that in Bel7404/OXA-NC cells, the expression levels of ABCG2, ABCB1 and ABCC1 in Bel7404/OXA-RBM8A-KD cells were significantly lower ($P < 0.005$); furthermore, the ABCG2, ABCB1 and ABCC1 levels were significantly elevated in MHCC97H/OXA-RBM8A-OE cells compared to those in MHCC97H/OXA-NC cells ($P < 0.005$).

High expression of RBM8A induces epithelial-mesenchymal transition of parental and OXA-resistant HCC cells in vitro.

Cells in each group were treated with a rhodamine-labeled fluoropeptide, and changes in the cytoskeleton were observed. The results are shown as follows (Fig. 4A): Compared with the parental Bel7404 or MHCC97H cell lines, the drug-resistant Bel7404 and MHCC97H cells showed slight changes in spindle formation. After overexpression of RBM8A, the cytoskeleton of MHCC97H/OXA-RBM8-OE cells tended to be more spindly, whereas the cytoskeleton of Bel7404/OXA-RBM8-KD cells tended to be round. The expression levels and distribution of the EMT-related factors E-cadherin, N-cadherin and Snail in each group were detected by WB and immunofluorescence (Fig. 4B-F): Compared with the parental Bel7404 or MHCC97H cell lines, drug-resistant Bel7404 or MHCC97H cells had decreased expression of E-cadherin but increased expression of RBM8A, N-cadherin and Snail. The expression level of E-cadherin was significantly reduced in MHCC97H/OXA-RBM8A-OE cells compared to MHCC97H/OXA-NC cells, and the expression levels of N-cadherin and Snail were significantly increased. Compared with Bel7404/OXA-NC cells, Bel7404/OXA-RBM8A-KD cells had increased expression of E-cadherin and significantly decreased expression of N-cadherin and Snail.

RBM8A regulates OXA resistance to HCC via EMT in vivo.

To further evaluate the function of RBM8A in promoting OXA resistance to HCC through EMT in vivo, a nude mouse xenograft model was established using Bel7404/OXA-NC and Bel7404/OXA-RBM8A-KD cells. In brief, 10 mg/kg OXA was injected around the tumor at 1 week, 2 weeks, 4 weeks and 6 weeks after cell injection. The results showed that the tumor size, tumor formation rate and body weight of the Bel7404/OXA-RBM8A-KD group were lower than those of the control group (Fig. 5A and B). Immunohistochemical results of nude mice xenografts showed (Fig. 5C and D) that the expression levels of RBM8A, Ki-67, N-cadherin, Snail, ABCG2, ABCB1, and ABCC1 in the tumors from the Bel7404/OXA-

RBM8A-NC group were higher than those in tumors from the Bel7404/OXA-RBM8A-KD group, which was determined by antigen staining. The expression level of E-cadherin in Bel7404/OXA-RBM8A-NC-derived tumor tissues was lower than that in Bel7404/OXA-RBM8A-KD-derived tumor tissues. This result is essentially consistent with the results in vitro.

Whole-genome microarrays combined with bioinformatics analysis indicated that RBM8A has extensive transcriptional regulation in OXA resistance to HCC, involving several important tumor-associated transcription factors, noncoding RNA and signaling pathways.

Determining the expression of dysregulated molecules associated with RBM8A in drug-resistant HCC.

The bioinformatics analysis flow chart was shown in Supplemental Fig. 1A. According to the analysis, there were 8365 identical differential genes between Bel7404/OXA-NC VS. Bel7404/OXA-RBM8A-KD cells and MHCC97H/OXA-NC VS. MHCC97H/OXA-RBM8A-OE cells based on the Wayne map (Supplemental Fig. 1B) to review that we believe are associated with RBM8A-mediated OXA resistance in HCC (Supplemental Table S1). We identified the coexpression behavior of these intersection-differentiated genes based on weighted gene coexpression network analysis (WGCNA), showing a significant coexpression phenomenon that systematically revealed changes in RBM8A as well as a subtle relationship between dramatic changes in global gene expression behavior and drug resistance in HCC. We organized these coexpression phenomena as modules and obtained a total of five modular systems for OXA resistance-related genes in HCC (Supplemental Fig. 1C, D, E). Based on the association between gene modules and cells, we found that the fourth module was maximally positively correlated with the phenotype of Bel7404/OXA-RBM8A-KD, while the third module and phenotype of MHCC97H/OXA-RBM8A-OE cells also had a strong positive correlation (Supplemental Fig. 1F, G). These results indicate that the modular gene clustered by coexpression analysis is closely related to the disrupted expression of RBM8A.

Identification of the biological molecular network of RBM8A in OXA resistance to HCC.

Exploring the functions and pathways involved in the relevant modules helps to establish molecular bridges between gene modules and disease pathology and pharmacology in system biology, thereby deepening the potential understanding of the molecular mechanism. Therefore, we performed GO-BP and KEGG pathway enrichment analysis on five modules. From these results, we found that these functions are mainly focused on mRNA splicing, ribonucleoprotein complex biogenesis and ncRNA processing, etc. (Supplemental Table S2). At the same time, the enrichment results of the KEGG pathway reflect that RBM8A-related genes are mainly involved in the PI3K-Akt signaling pathway, MAPK signaling pathway, viral carcinogenesis, mRNA surveillance pathway, cell cycle, etc. (Supplemental Table S3).

We used TF- and ncRNA-targeting regulatory genes as a background set for hypergeometric prediction analysis. The results showed that a total of 1663 ncRNAs and 38 TFs have regulatory effects on the module genes, which are potentially pivotal regulators (Supplementary Tables S4 and S5). Among them, MALAT1, MYCN, HDAC9, FENDRR and other key regulatory nodes have significant regulatory capabilities for more modules and thus were identified as core pivot regulators, which may be driven by RBM8A and

mediate related genes and pathways to exert their effects on OXA resistance in HCC. Based on the gene of the module and the key KEGG signaling pathways involved, we finally obtained a comprehensive landscape map of RBM8A regulation of OXA resistance in HCC (Fig. 6).

Finally, by combining the WGCNA and hypergeometric prediction analysis results, we selected pivotal regulators that had significant effects on the module genes: the lncRNAs MALAT1 and FENDRR and the transcription factors MYC, STAT3, P53, E2F1, YY1, HDAC1, and HDAC9. qRT-PCR and WB were used to further verify the correlation between RBM8A and core pivotal regulator expression in HCC cell lines in vitro. The results (Fig. 7 and Supplementary Fig. 2) showed that HDAC9 expression was significantly higher in DR-HCC cells than in PCLs and was upregulated after RBM8A overexpression in both PCLs and DR-HCC cells. HDAC9 expression decreased after RBM8A knockdown, suggesting that HDAC9 is closely related to RBM8A-regulated OXA resistance in HCC cells. Based on the downstream signaling network involving RBM8A and HDAC9 (Fig. 8), NFKB1 and TP53 are direct target genes of HDAC9. In addition to the NRAS oncogene, several cyclin-dependent kinase and MAPK family genes are involved. Enrichment analysis indicated that the module genes regulated by the RBM8A-HDAC9 axis mainly participate in the PI3K-AKT and MAPK pathways, which mediate pathophysiological processes such as cell proliferation, inflammation, apoptosis and cell cycle. These results are worth further study. In conclusion, this study identified RBM8A as a potential key factor for OXA resistance in HCC cells and provided abundant prediction results and important evidence for the regulation of drug resistance mechanisms.

Discussion

OXA is the most evidence-based chemotherapeutic drug in the treatment of advanced HCC. Drug resistance to OXA is also a profound obstacle in the treatment of HCC. OXA is a third-generation platinum derivative. After entering the nucleus, OXA can bind to DNA and form a variety of cross-linked structures, leading to DNA replication errors and RNA transcriptional damage, ultimately eliciting a cytotoxicity response and antitumor activities[18]. However, the underlying mechanisms of OXA resistance remain largely unknown.

RBM8A (Y 14) is a gene closely related to tumors that was identified within the past ten years. RBM8A has many important biological functions and is involved in the formation, degradation, translation and quality control of mRNA in cells[19, 20]. As a core component in the Exon junction complex (EJC), RBM8A is a protein essential for nonsense-mediated mRNA decay (NMD), a highly conserved mRNA monitoring mechanism in eukaryotes; therefore, its abnormal expression may play an important role in the signal transduction of cell malignancy[19]. Deletion of the RBM8A gene can downregulate the expression levels of Bcl-Xs, Bim and Mcl-1 as well as multiple proapoptotic genes, such as members of the Bcl-2 family, thereby inducing apoptosis[21]; this result is consistent with our findings. In addition, we found that RBM8A expression in DR-HCC cells was significantly higher than that in parental HCC cells. High expression of RBM8A promotes proliferation, reduces the proportion of cells in apoptosis and G1 phase, and increases the chemotherapeutic resistance of HCC cells to OXA.

Malignant tumors have resistance to antitumor drugs, gain unlimited proliferative ability, and eventually progress to local infiltration and distant metastasis. This is the most important biological characteristic of malignant tumors and the main cause of death[22]. Our study confirmed that upon RBM8A overexpression, HCC cells showed further increases in their migration and invasion ability. The first step in invasion and metastasis is initiation of EMT[23, 24]: loss of epithelial cell polarity, less organized structure, cytoskeletal remodeling, and expression of interstitial cell adhesion molecules. Ma et al.[6] observed that in OXA-resistant HCC cell lines, migration and invasion are associated with the increased incidence of the mesenchymal phenotype. Reducing the expression of the EMT transcription factor Snail reverses EMT and makes HCC-resistant cells sensitive to OXA. This is consistent with our results in vitro and in vivo. Elevation of RBM8A can induce EMT in HCC cells: the cytoskeleton in cells with high expression of RBM8A tends to be fusiform, the expression level of E-cadherin is significantly decreased, and the expression levels of N-cadherin and Snail are significantly increased. Reducing RBM8A expression results in the opposite effect. Our data suggest that RBM8A is involved in the resistance of HCC cells to OXA by inducing EMT.

It is currently believed that the drug resistance pattern of OXA in HCC is mainly related to apoptosis escape, autophagy activation, drug excretion, and enhanced epigenetic transformation to HCC stem cells[25-29]. The EMT process is involved in most models[22, 30-32]. Additional studies have shown that the abnormal expression of genes involved in apoptosis and proliferation is related to the inactivation of multiple signaling pathways. Many of the important cytokines are coordinated and controlled by each other, have functional connections, and form a synergistic and interlaced molecular network. Yin et al.[33] used a microarray to detect MHCC97H/OXA expression profiles of OXA-resistant cells and found that most of the genes involved in cell death or apoptosis were altered, and the upregulation of TFs and kinases was the greatest. These genes were associated with tumor-related pathways, such as the Ras, MAPK and p53 pathways, all of which may be involved in OXA resistance. The regulation of noncoding RNAs, such as miR-125[32], miR-31[34], H19[35], and NR2F1[36], has been shown to be involved in the development and progression of HCC and is involved in tumor resistance. The NF-κB, PI3K/Akt, GSK3β/β-catenin and HIF-1α signaling pathways have been reported to be involved in HCC chemoresistance[37-40]. In our study, as shown in Fig. 6 and Supplementary Tables S4 and S5, some TFs and ncRNAs as well as their corresponding metabolic pathways are consistent with the current literature.

It is worth noting that HDAC9 is a TF that is closely related to the RBM8A-mediated regulation of OXA resistance in HCC. Abnormally high HDAC9 expression has been shown to be closely related to malignant biological behaviors such as proliferation, invasion and metastasis in various tumors[41-45]. In recent years, a variety of HDAC inhibitors have been widely used in basic research and clinical anticancer treatment[46, 47], representing a current research hotspot. The specific molecular mechanism is related to the direct regulation of proto-oncogene/tumor suppressor gene and ncRNA expression by HDAC9. The currently known tumor suppressor gene P53[44], deacetylated forkhead box gene O1 (FoxO1)[48], sex determining region Y-box 9 protein (SOX9)[49] and transcriptional coactivator with PDZ-binding motif (TAZ)[50] are potential target genes of HDAC9; changes in HDAC9 expression affect the transcription of target genes, thereby activating downstream signaling pathways, such as those involving the oncogenes

Ras, VEGF, MAPK and EGFR, to promote tumor cell proliferation[51]. HDAC9 has been rarely studied in the context of HCC; most publications have focused on its relationship with miRNA. Zheng et al.[52] found that HDAC9 can catalyze the deacetylation of histone 3 lysine 18 (H3K18), which downregulates the expression of miR-376a and promotes cancer. This study is the first to discover that HDAC9 may be associated with chemotherapy resistance in HCC, and it is worth further exploring its specific molecular mechanism.

Conclusion

In conclusion, our study showed that RBM8A can induce EMT in HCC cells, thereby affecting proliferation, apoptosis, cell cycle distribution, migration, and invasion as well as promoting OXA resistance. Gene chip sequencing combined with bioinformatics analysis revealed that RBM8A has a wide range of transcriptional regulatory capabilities in drug-resistant HCC, including the ability to regulate several important tumor-related signaling pathways. HDAC9 is an important mediator of RBM8A-induced OXA resistance. These data suggest that RBM8A and its related regulatory pathways represent potential markers of OXA resistance and therapeutic targets in HCC.

Abbreviations

EMT: Epithelial-mesenchymal transition

OXA: Oxaliplatin

HDAC9: Histone deacetylase 9

HCC: Hepatocellular carcinoma

ORR: objective response rate

PFS: progression-free survival

RBPs: RNA-binding proteins

qRT-PCR: quantitative real-time PCR

WB: Western blot

CCK-8: Cell Counting Kit-8

WGCNA: Weighted gene coexpression network analysis

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

ncRNA: noncoding RNA

TF: transcription factor

ABCG2: ATP Binding Cassette Subfamily G Member 2

ABCB1: ATP Binding Cassette Subfamily B Member 1

ABCC1: ATP Binding Cassette Subfamily C Member 1

Declarations

Ethical Approval and Consent to participate

Guangxi Medical University Cancer Hospital University Animal Health Committee approved all mouse treatment methods and procedures.

Consent for publication

All authors agree to publish the manuscript.

Availability of supporting data

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Contributions

R.L., J.Z. and Z.L. designed and performed all the experiments, analyzed and interpreted data and drafted the manuscript. Z.L., Q.L. and X.L. participated in data analysis and figure preparation., Y.L., J.Y. and Y.L. contributed to the study design, data interpretation and final editing of the manuscript. All authors read and approved the final manuscript.

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Figures

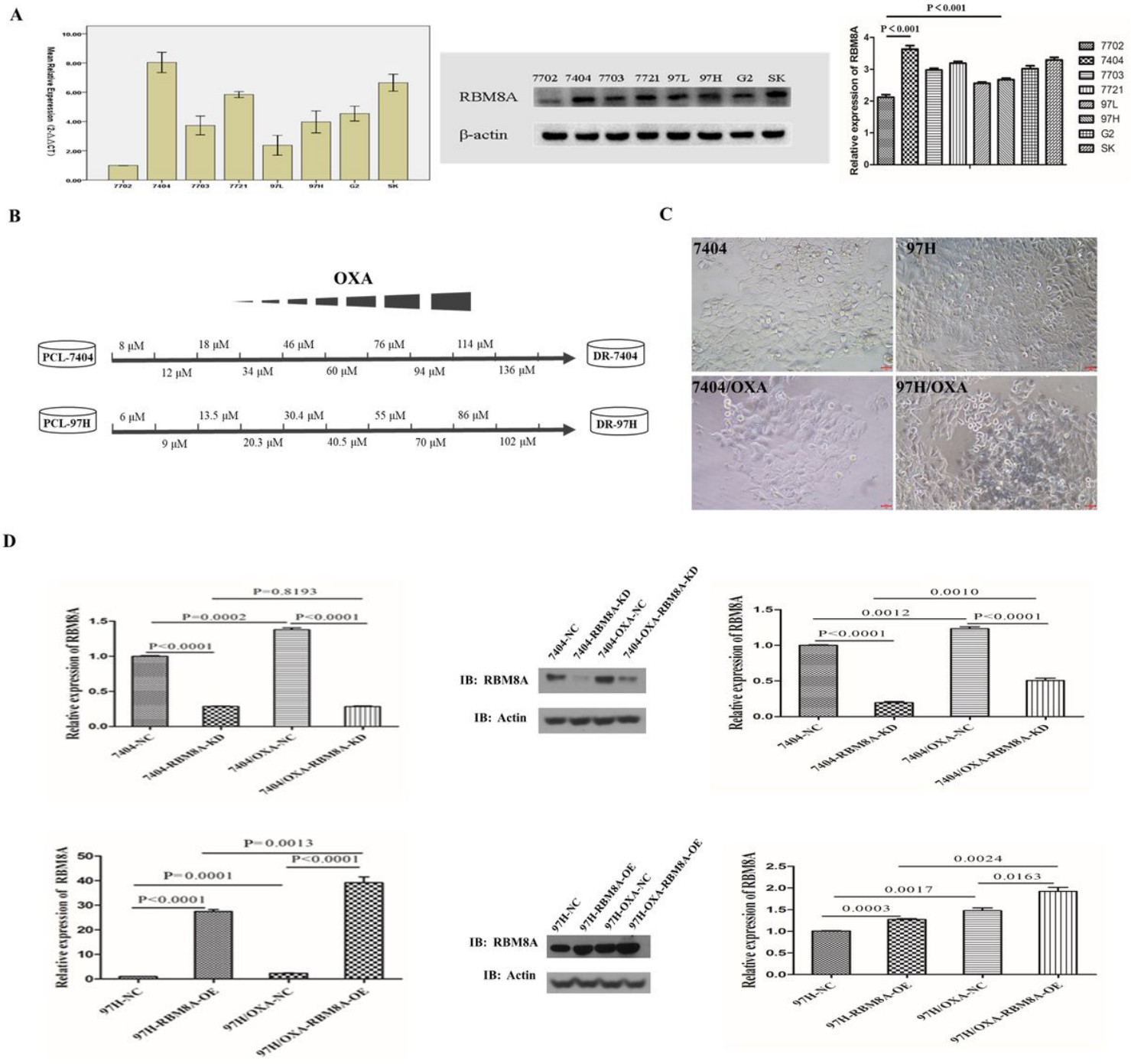

Figure 1

The concentration-elevation and intermittent induction treatment with oxaliplatin selects DR-HCC cells. (A). RT-PCR and Western blot analysis of RBM8A expression in HCC cell lines. Quantitative Western blot data were analyzed. (B). Schematic representation of the protocol used to obtain oxaliplatin (OXA)-induced drug-resistant (DR) HCC cells from the parental cell line (PCL). For the concentration-elevation

and intermittent induction treatment with OXA, each dose was maintained for 15 days, and OXA-resistant cell lines were obtained by the end of 6 months. (C). Representative phase contrast images of PCLs and drug-resistant Bel7404 (left panels) or drug-resistant MHCC97H (right panels) cells (Scale bar = 20 μm, 20× magnification). (D). Knockdown and overexpression efficiency of RBM8A in PCLs and DR-HCC cell lines. RT-PCR and Western blot analysis of the mRNA and protein expression of RBM8A, respectively, in PCL-Bel7404-NC, PCL-Bel7404-RBM8A-KD, DR-Bel7404-NC, DR-Bel7404-RBM8A-KD and PCL-MHCC97H-NC, PCL-MHCC97H-RBM8A-OE, DR-MHCC97H-NC, DR-MHCC97H-RBM8A-OE. Quantitative Western blot data were analyzed (right panels). Data are expressed as the mean±S.D. of three independent experiments or are representative of three independent observations.

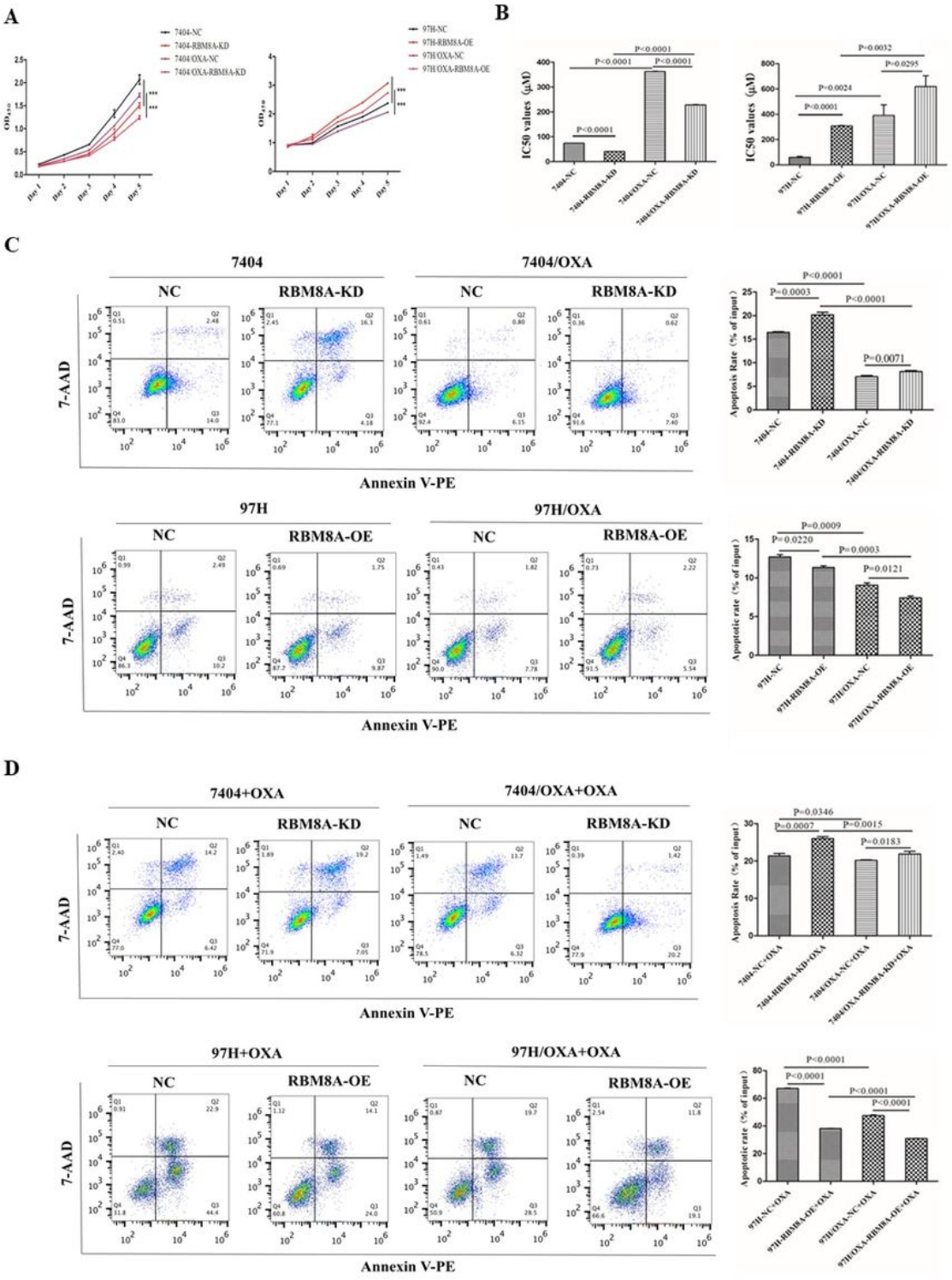

Figure 2

Modulation of RBM8A expression affects proliferation, apoptosis and cell cycle progression in PCL and DR-HCC cells. (A). Cell proliferation was measured using Cell Counting Kit (CCK-8) assays. The number of cells at each indicated time point was determined as the absorbance at 450 nm. ***<0.001. (B). The half maximal inhibitory concentration (IC50) values were measured using CCK-8 assays. The cells were treated with various concentrations of OXA for 48 h. (C). Apoptosis was determined by flow cytometry.

Representative quadrant figures are presented on the left. The summaries of apoptosis in PCLs and DR-HCC cells are expressed as the rate of apoptotic cells (right panels). (D). Apoptosis after OXA treatment was determined by flow cytometry. The PCLs and DR-HCC cells subjected to OXA treatment were harvested at 48 h.

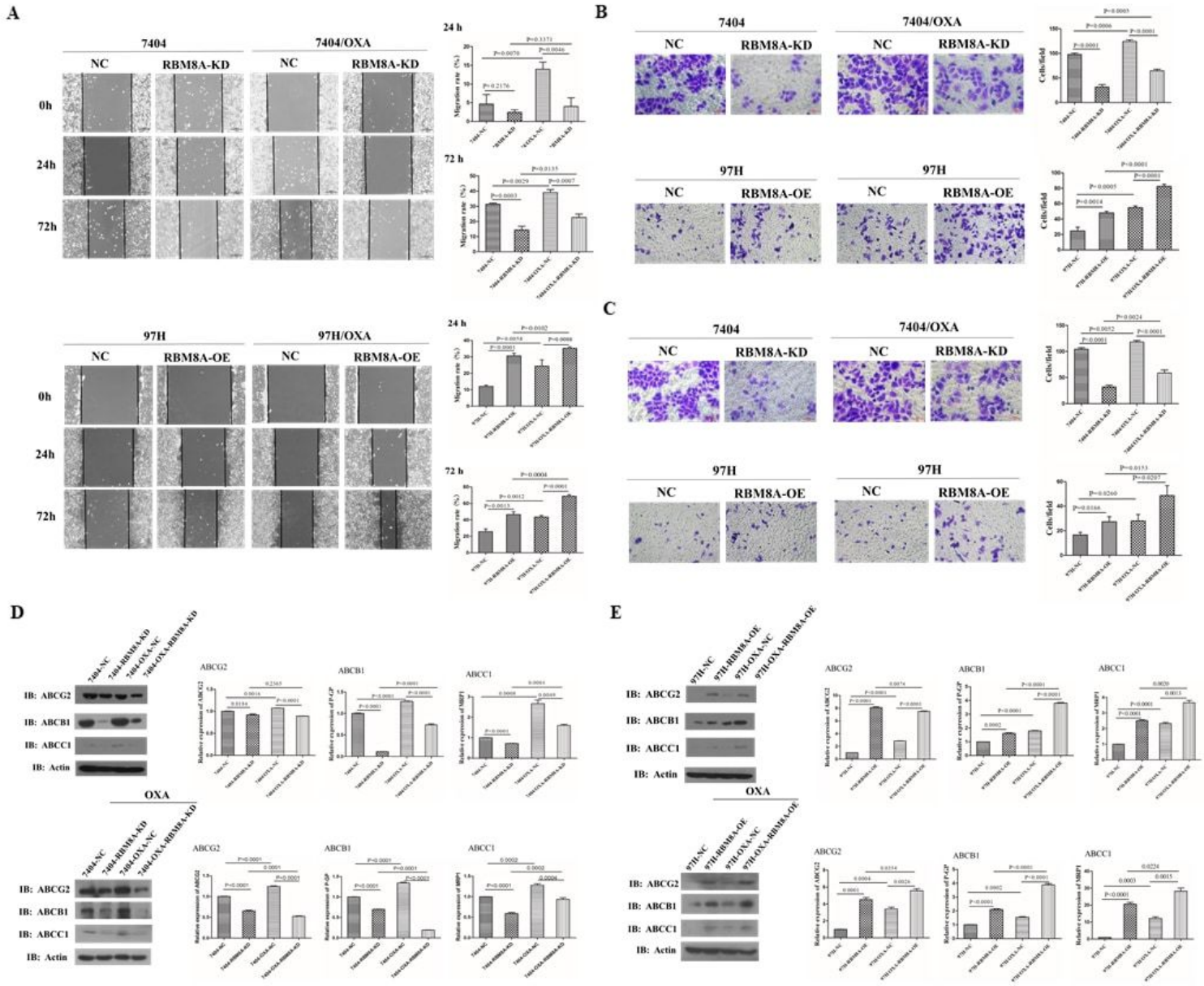

Figure 3

Modulation of RBM8A expression affects the migratory and invasive potential of PCLs and DR-HCC cells as well as the levels of drug resistance-related protein expression in these cells. (A). Wound-healing assay. The scraped areas were photographed at 0, 24 h, and 72 h postscraping (scale bar = 200 μm, 10× magnification). Quantification of migration efficiency at 24 h and 72 h after scraping (right panel). (B). Transwell Matrigel migration assay. Representative traces of each experimental group are shown (scale bar = 50 μm, 40× magnification). Quantification of migration efficiency at 24 h and 72 h (right panel). (C). Transwell Matrigel invasion assay. Representative photographs and quantification are shown (scale bar =

50 μm, 40× magnification). Data are either representative of three similar observations or are shown as the mean±S.D. of three experiments. Western blot analysis of the expression of ABCG2, ABCB1 and ABCC1 in PCL-Bel7404-NC, PCL-Bel7404-RBM8A-KD, DR-Bel7404-KD and DR-Bel7404-KD (D) or in PCL-MHCC97H-NC, PCL-MHCC97H-RBM8A-OE, DR-MHCC97H-KD and DR-97J-KD cells (E). The protein expression of ABCG2, ABCB1 and ABCC1 in each experimental group without or with OXA treatment (second and third lines, respectively). Data are either representative of three similar observations or are shown as the mean±S.D. of three experiments.

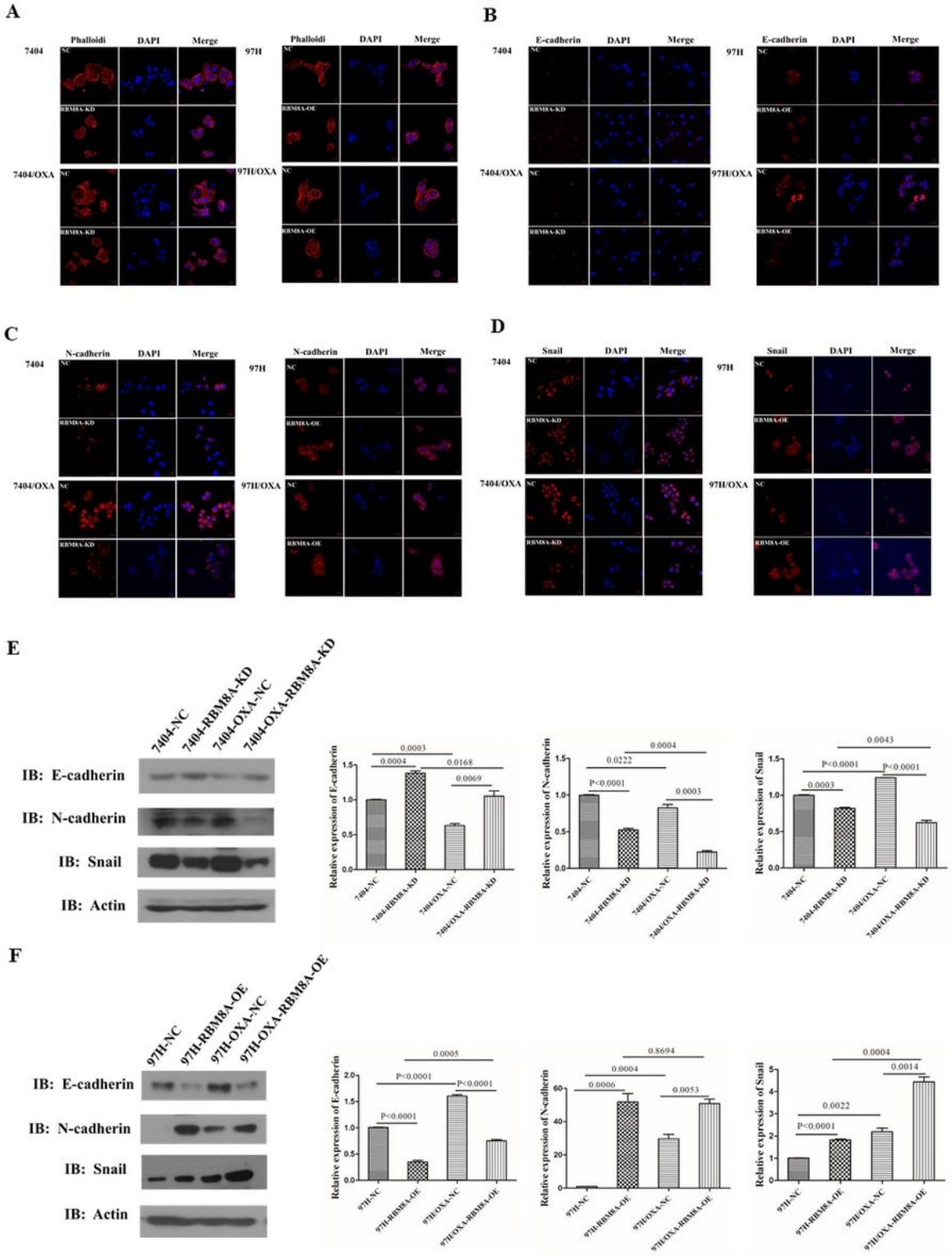

Figure 4

Modulation of RBM8A expression affects EMT transition in PCL and DR-HCC cells. (A). Immunofluorescence staining of the cytoskeleton. Confocal microscopy imaging of Phalloidin (red) merged with DAPI (blue) in PCLs and DR-HCC cells upon RBM8A knockdown or overexpression. (B). Immunofluorescence staining of E-cadherin. Confocal microscopy imaging of E-cadherin (red) merged with DAPI (blue) in PCLs and DR-HCC cells upon RBM8A knockdown or overexpression. (C). Immunofluorescence staining of N-cadherin. Confocal microscopy imaging of N-cadherin (red) merged with DAPI (blue) in PCLs and DR-HCC cells upon RBM8A knockdown or overexpression. (D). Immunofluorescence staining of Snail. Confocal microscopy imaging of Snail (red) merged with DAPI (blue) in PCLs and DR-HCC cells upon RBM8A knockdown or overexpression. (E). Western blot analysis of E-cadherin, N-cadherin and Snail protein expression in PCL-Bel7404 and DR-Bel7404 cells with or without RBM8A knockdown. (F). Western blot analysis of E-cadherin, N-cadherin and Snail protein expression in PCL-MHCC97H and DR-MHCC97H cells with or without RBM8A overexpression. Data are expressed as the mean±S.D. of three independent experiments or are representative of three independent observations. (Scale bar = 20 μm).

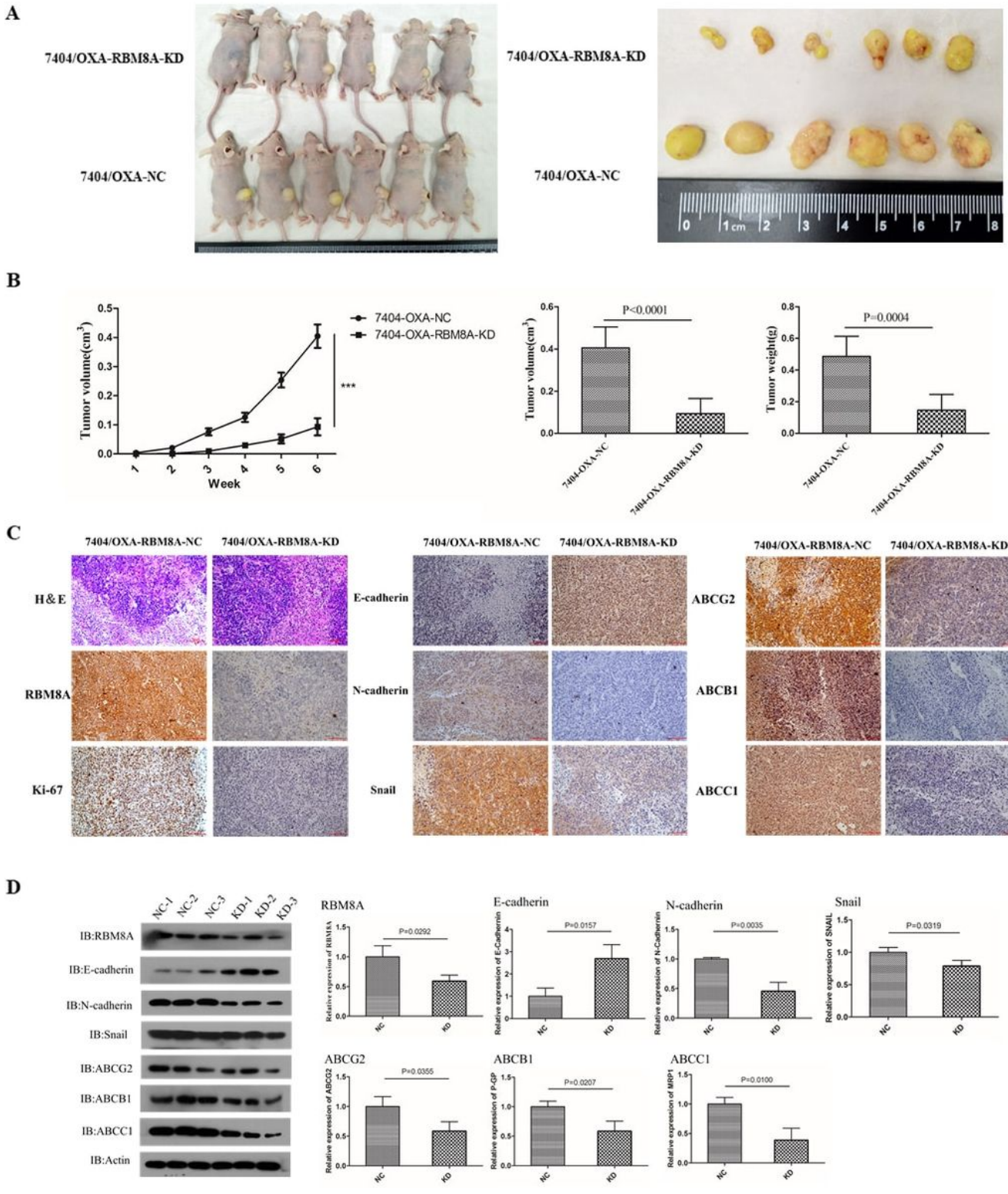

Figure 5

The biological effects of RBM8A on DR-HCC cell tumorigenesis in vivo. (A). The growth of xenografted Bel7404/OXA-RBM8A-KD and control cells orthotopically injected into mammary fat pads of nude mice and injected with OXA at 10 mg/kg around the tumor at 1 week, 2 weeks, 4 weeks and 6 weeks. The growth of tumors was followed up over the 6-week period. (Right) Photographs of primary tumors collected for each condition. (B). The mice injected with Bel7404/OXA-RBM8A-KD cells formed smaller (p

<0.0001) and lighter (p = 0.0004) tumors than did mice injected with control cells (NC). ***<0.001 (C). Immunohistochemical staining showing that tumors originating from Bel7404/OXA-RBM8A-KD cells had decreased RBM8A and Ki-67 levels compared to tumors originating from control cells. Immunohistochemical staining (C) and Western blot (D) showing that tumors originating from Bel7404/OXA-RBM8A-KD cells had increased E-cadherin and decreased N-cadherin, Snail, ABCG2, ABCB1 and ABCC1 levels compared to those originating from control cells. Data are expressed as the mean±S.D. of three independent experiments or are representative of three independent observations (scale bar = 100 μm, 20× magnification).

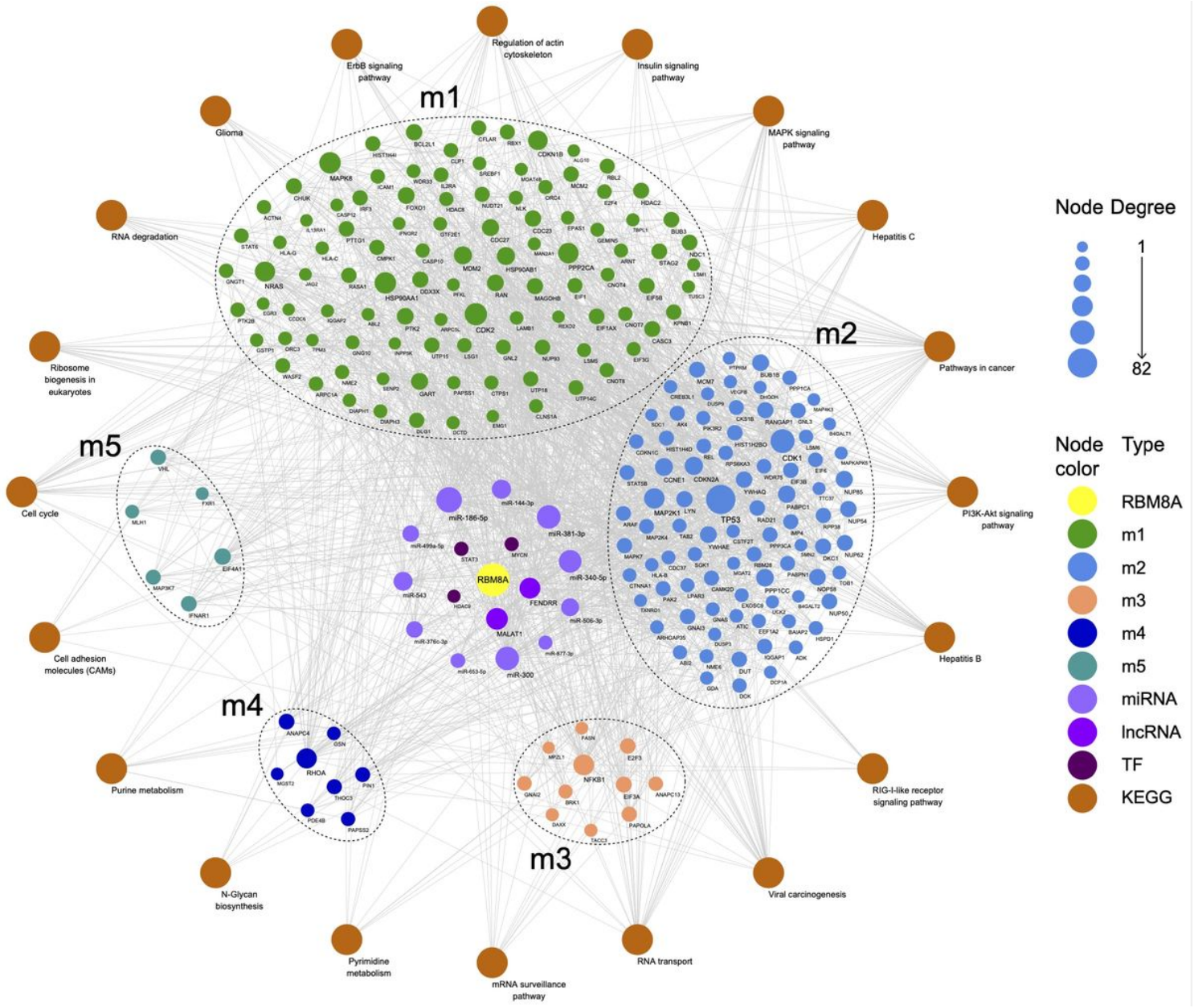

Figure 6

Comprehensive landscape of RBM8A regulating OXA resistance in HCC. Bioinformatics analysis was used to integrate the regulatory information of RBM8A on module genes and pivot factors and to construct a comprehensive overview of RBM8A-mediated OXA resistance in HCC. In the landscape,

lncRNAs, miRNAs and TFs serve as important mediators to assist RBM8A-associated regulatory module genes and then influence the signaling pathways involved in drug resistance of HCC.

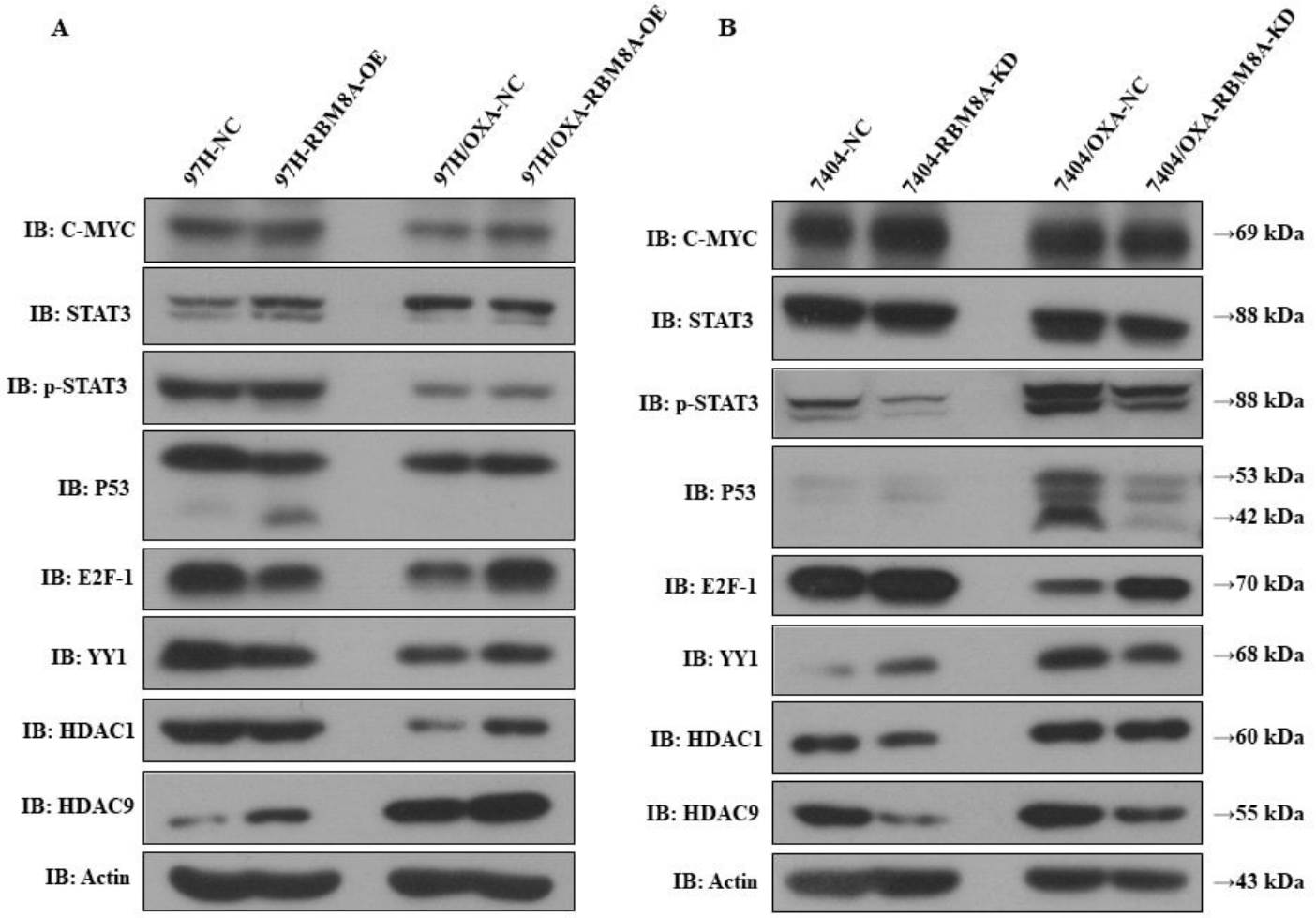

Figure 7

Western blot analysis of transcription factor expression in HCC cell lines. Western blot analysis of the expression of the transcription factors MYC, STAT3, P53, E2F1, YY1, HDAC1, and HDAC9 in HCC cell lines. After overexpression or knockdown of RBM8A in parental cell lines (PCLs) and DR-HCC cells, Western blotting revealed that HDAC9 expression regulated by RBM8A was associated with OXA resistance in HCC cells.

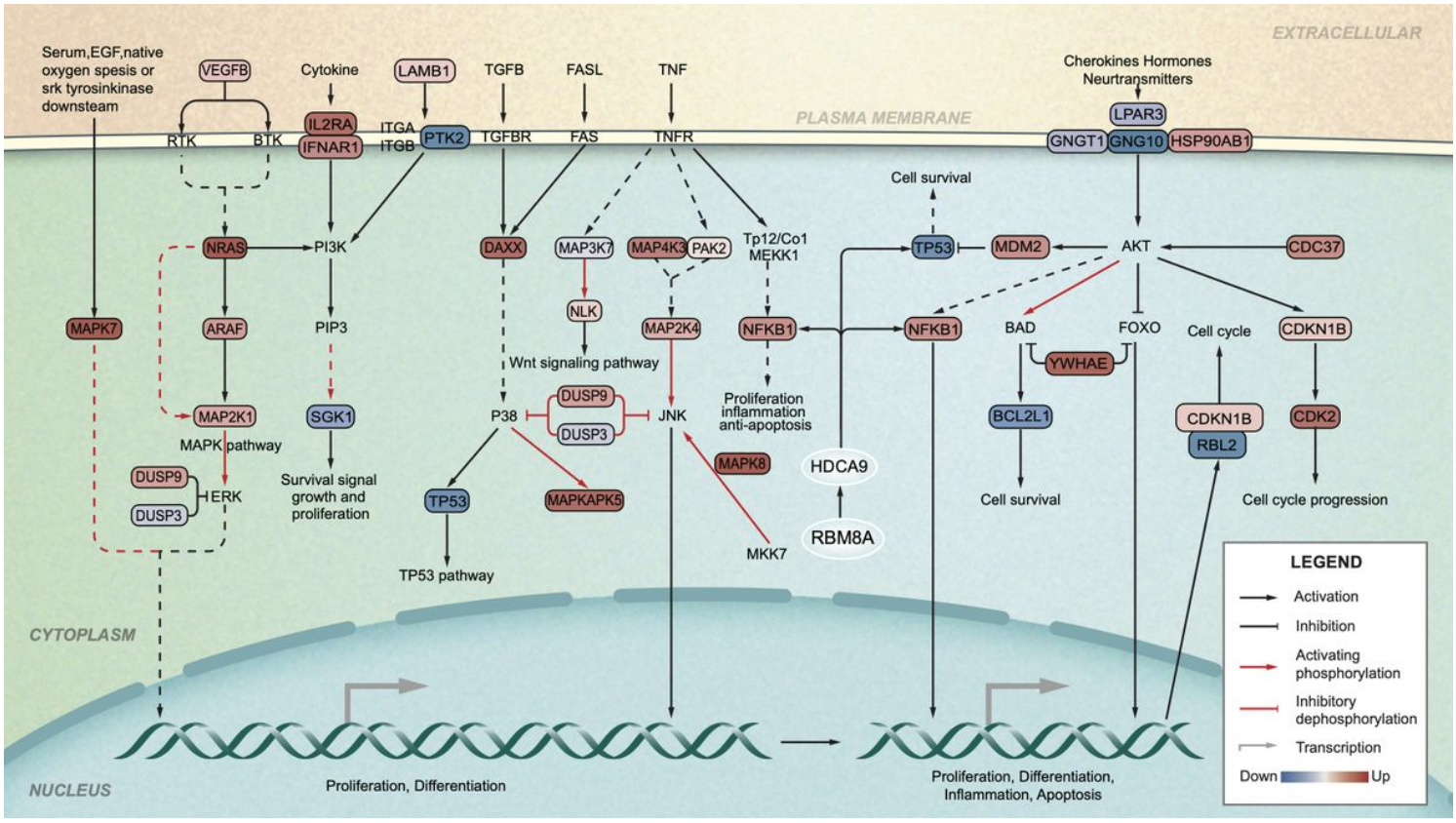

Figure 8

Molecular network by which the RBM8A-HDAC9 axis regulates OXA resistance in HCC. Bioinformatics analysis combined with qRT-PCR and Western blotting revealed that HDAC9 is the pivotal transcription factor that is most closely related to the RBM8A-mediated regulation of OXA resistance in HCC. The HDAC9-module gene-KEGG signaling pathway was extracted, and the potential mechanism by which the RBM8A-HDAC9 axis regulates drug resistance in HCC was identified.

Supplementary Files

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- SI.zip