TMB Signature-Related RCAN2 Promotes Apoptosis by Upregulating EHF/DR5 Pathway in Hepatocellular Carcinoma

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Abstract

Background: The tumour mutation burden (TMB) is a valuable indicator of the accumulation of somatic mutations, and is thought to be associated with the biological behaviour and prognosis of tumours. However, the related genetic mechanism for these association is still unclear. The aim of the present study was to identify the key gene(s) associated with TMB in hepatocellular carcinoma (HCC) and to investigate its biological functions, downstream transcription factors, and mechanism of action. Methods: Patients in The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) database were classified according to TMB signature-related genes. Key genes related to the TMB signature and tumour prognosis were identified. Immunohistochemistry and Quantitative Real-Time Polymerase Chain Reaction (qPCR) were then used to assess gene expression in clinical HCC tissues and HCC cells. Cells with altered gene expression were evaluated for the effect on cell proliferation and apoptosis, both in vitro and in vivo. Three independent databases and cell sequencing data were used to identify the mechanisms involved and the downstream transcription factors. The mechanism was also studied by altering the expression of downstream transcription factors in vitro. Result: The integrated cluster (IC) 2 group, characterized by 99 TMB signature-related genes, showed a significant different TMB score compared to the IC1 group (p < 0.001), as well as more favourable tumour prognosis (p = 0.031). We identified five key prognostic genes that were differentially expressed between IC2 and IC1 and were associated with overall survival. The expression of one of these key prognostic genes, RCAN2, was negatively correlated with TMB in 18 out of 33 tumour types examined. A high level of RCAN2 was correlated with better overall survival in HCC (p = 0.0009). Overexpression of RCAN2 enhanced apoptosis in vitro and in vivo, whereas knockdown of RCAN2 attenuated apoptosis. The mechanism by which RCAN2 promotes apoptosis may involve upregulation of the expression of ETS homologous factor (EHF) and of death receptor 5 (DR5). Conclusions: Downregulation of RCAN2 expression was found to correlate with elevated TMB in multiple cancer types. RCAN2 was also found to be a biomarker of HCC prognosis, and to promote the apoptosis of HCC cells through the EHF/DR5 pathway. These findings provide a new perspective on systemic treatment for advanced HCC with a high TMB.

Keywords: tumour mutational burden; RCAN2; EHF; hepatocellular carcinoma; apoptosis

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer type worldwide and has the third highest mortality rate [1,2]. Due to its insidious onset, a large number of patients are diagnosed with advanced HCC and systemic treatment is the main option for these patients [3]. However, a subset of patients still experiences tumour progression following the systemic treatment. Therefore, enhancing therapeutic efficacy remains the primary focus of clinical investigations.

Currently, some scholars have classified HCC into different subtypes based on gene expression, such as the Hoshida classification and the Epithelial Cell Adhesion Molecule (EpCAM) subtype [4,5]. In addition, some researchers try to explore the changes of HCC biofunction caused by these mutations by detecting peripheral circulating tumour cells and so on [6]. However, there is currently no widely recognized classification method based on gene expression for HCC. Somatic mutation is an important factor in the efficacy of systemic treatment, and the accumulation of mutations can increase the heterogeneity and malignancy of tumours [7]. In HCC, tumour suppressor genes such as Tumour protein p53 (TP53) and Catenin beta 1 (CTNNB1) are commonly mutated genes [8–10]. The tumour mutation burden (TMB) represents the cumulative number of somatic gene mutations within a tumour, and always be served as one of the crucial metrics for assessing somatic mutational accumulation [11]. In malignancies such as lung cancer, its level has been substantiated to be intricately associated with the prognosis of immunotherapy [12]. However, the mechanism between TMB and prognosis remains uncertain, and exploring the underlying mechanism is the research direction that interests us.
Apoptosis is a prevalent form of programmed cell death, primarily triggered by oxidative damage, calcium homeostasis imbalance, and mitochondrial impairment [18, 19]. It is characterized by the activation of specific caspase and mitochondrial control pathways. Under normal circumstances, tumour cells disrupt the apoptosis signalling during their occurrence and progression, resulting in reduced apoptotic response in tumour cells and enhanced growth and proliferation [20]. Previous studies have demonstrated that immune cells can secrete tumour necrosis factor-α (TNF-α) or other apoptotic inducers to activate the extrinsic apoptotic pathway in tumour cells [21,22]. The apoptotic inducers bind to the death receptors (DRs) located on the surface of tumour cells to initiate the activation of downstream apoptotic pathways and finally lead to the programmed cell death in tumour cells [23]. DRs belong to the TNF receptor superfamily, and the known DRs mainly include TNFR1, TNFR2, DR4, death receptor 5 (DR5) and so on [24]. The key to initiating the extrinsic apoptotic pathway lies in the recognition of exogenous apoptotic signals by DRs on tumour cells. Therefore, identifying transcription factors that regulate DRs and targeting this process may hold crucial significance for enhancing systemic therapy.

Hence, in this study, we regrouped patients into distinct cohorts based on the expression levels of TMB-related genes. By screening differentially expressed genes in various groups, we identified prognostic genes that are related to TMB. And then, RCAN2 was screened out for further mechanistic study. We validated the biofunction of RCAN2 in vitro and in vivo, and explored the underlying mechanisms through cross-validation in multiple independent databases. Finally, we elucidated the possible molecular pathways underlying the impact of RCAN2 on HCC apoptosis. It may provide a new perspective and target for systemic treatment in advanced HCC.

2. Materials and Methods

2.1 Availability of Data and Materials

Externally validated datasets were generated from The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) database (https://portal.gdc.cancer.gov) and the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo, ID: GSE76427 and GSE64041) [25]. SHI-sequencing data and MHCC-LM3 cell sequencing data used in this study can be accessed from Supplementary Material and used after the consent of the corresponding author upon reasonable request.

All clinical samples of HCC tissues and matched adjacent normal liver tissues were obtained from patients at Sun Yat-sen University Cancer Center between 2015 and 2018. These were used for high-throughput sequencing, quantitative real-time polymerase chain reaction (qPCR), western blot, and immunohistochemistry (IHC) analyses. All clinical samples and data were accessed after obtaining approval of the Ethics Committee of Sun Yat-sen university Cancer Center (No. 202011). Written informed consents from patients were waived due to the retrospective nature of our study.

2.2 Cell Culture

Hep-3B cells were purchased from the National Collection of Authenticated Cell Cultures (https://www.cellenbank.org.cn/). MHCC-LM3 cells were obtained from the Liver Cancer Institute, Fudan University (Shanghai, China). All cells were grown in Dulbeco’s modified Eagle’s medium (DMEM, Gibco, New York, NY, USA) with high glucose and supplemented with 10% foetal bovine serum (FBS, Gibco) in a 37 °C incubator with 5% CO2 atmosphere. Recombinant human TNF-α (Cat No: PRP1013) and TRAIL (Cat No: PRP1028) proteins were purchased from Abbkine (Wuhan, China).

Cell lines were authenticated by the Genomics Unit at TSINGKE, using Short Tandem Repeat profiling with a commercial kit from NUHIGHbio (Cat No: NH9347, NUHIGHbio, Suzhou, China). Mycoplasma test was performed in all cell lines every other week using the MycoAlert Mycoplasma Detection Kit (Vazyme, Nanjing, China). The mycoplasma test is negative.

2.3 Construction and Transduction of Plasmid Vectors

The full-length sequence of RCAN2 was cloned into the lentiviral expression vector pReceicer-Lv105 (GeneCopoeia, Guangzhou, China) to construct the pReceicer-Lv105-RCAN2 recombinant plasmid. Target sequences were synthesized and inserted into the shRNA expression vector psi-LVRU6GP (GeneCopoeia) in order to construct shRNA of RCAN2. The shRNA of ETS homologous factor (EHF) was constructed by the vector pLenti-U6 (OBIO, Shanghai, China) and the shRNA with a non-targeting sequence was used as a negative control.

Virus packaging in HEK 293T cells was performed by co-transfection of recombinant plasmid with Opti-MEM (Gibco) and Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia). Viral particles were harvested and used to transduce HCC cells after 48 h of transfection. HCC cells (1 × 10^5), including Hep-3B and MHCC-LM3 cells, were
infected with 2 mL of viral particles and 5 µg/mL polybrene (YEASEN, Shanghai, China). Transfected cells were cultured in medium supplemented with puromycin at least 2 weeks to select stably expressing cells for the later experiment. The expression of RCAN2 was validated by qPCR and western blot.

2.4 High-Throughput Sequencing
Total RNA from clinical tissues and HCC cells was isolated and purified with TRizol reagent (Invitrogen, Carlsbad, CA, USA) for later sequencing. High-throughput sequencing of samples was performed on an Illumina HiSeq 4000 platform (GENEWIZ, Suzhou, China). Sequencing reads were then cleaned and processed by hisat2, fastqc, samtools and subread.

2.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR) Assay
Total RNA was extracted from cells and tissues using TRizol reagent (Invitrogen). ReverTra Ace qPCR RT Master Mix (TOYOBO, Shanghai, China) was used to perform reverse transcription, and qPCR was then performed using the THUNDERBIRD SYBR qPCR Mix (TOYOBO). The specific reaction conditions were as follows: 95 °C for 15 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. β-actin was used as the internal reference gene to normalize other gene expression levels. The primers used are listed in Table 1.

2.6 Western Blot
Total protein from clinical tissues and HCC cells was extracted using the RIPA buffer Kit (KeyGEN BioTECH Corporation, Nanjing, China). The protein concentration was determined using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibodies overnight at 4 °C and then with secondary antibodies at room temperature for 1–2 hours. Antibodies against RCAN2, caspase-3, caspase-8, caspase-9 and DR5 were purchased from Proteintech (http://www.ptgen.com/). The antibody against EHF was purchased from Thermo Fisher Scientific (https://www.thermofisher.cn/), while all other antibodies were purchased from CST (https://www.cellsignal.cn/).

2.7 Cell Viability

In vitro, the proliferation rate of HCC cells was measured using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. Hep-3B and MHCC-LM3 cells were seeded into 96-well plates at 2 × 10^4 cells per well in a final volume of 100 µL. Proliferation was measured at days 0, 1, 3, and 5 using a 10% CCK-8 solution. The absorbance at 450 nm was measured after incubation for 2 h at 37 °C and used to calculate the number of viable cells.

2.8 Immunohistochemistry (IHC)
Tissue microarrays were constructed by Guangzhou Yuebin Biotech Laboratory (Guangzhou, China). Slides with tissue microarray sections were incubated overnight (8–12 h) at 4 °C with the antibody to RCAN2 (1:250). The reaction time with chromogenic reagent was 30 s. After IHC staining, all slides were scanned using a Vectra 2 scanning system (Perkin Elmer, Waltham, MA, USA) and analysed by IHC scores with the Inform analysis system (Perkin Elmer). The median IHC score was used as the cut-off value, with patients categorized into high- and low-expression groups.

2.9 Image-Flow Cytometry Assay
Annexin V-FITC/propidium iodide (PI) staining assay (4ABiotech Corporation, Beijing, China) and Annexin V-PE/7-AAD staining assay (YEASEN, Shanghai, China) were used to detect cells in early and late apoptotic stages with the image-flow cytometry assay. The transfected cells were harvested and washed in cold PBS and resuspended in 500 µL solution with 5 µL Annexin V-PE and 10 µL 7-AAD. For the detection of MHCC-LM3 apoptotic cells following recovery of EHF expression, cells were incubated in 500 µL solution with 5 µL Annexin V-FITC and 10 µL PI. After incubation for 10–15 min at 15–25 °C, the cells were evaluated in a CytoFLEX S machine (Beckman Coulter, Brea, CA, USA) and analysed with CytExpert software (v 2.4.0.28, Beckman Coulter) for automated image-flow cytometry. A total of 20,000 cells were analysed per sample. Data were later analysed using Flowjo software (v10.5.3, BD Biosciences, Sussex, NJ, USA).

2.10 Animal Experiments
Four- to six-week-old male BALB/c nude mice were used for xenograft experiments. For the subcutaneous HCC

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Species</th>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
</tr>
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<tbody>
<tr>
<td>RCAN2</td>
<td>Human</td>
<td>Forward</td>
<td>CAGCCCGAGCTAGGATAGAG</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTGGCGTGGCATCGTTGAT</td>
</tr>
<tr>
<td>EHF</td>
<td>Human</td>
<td>Forward</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>CTGTGCTACCATAGTTGGTC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Human</td>
<td>Forward</td>
<td>CATGACGTTGTGATACGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTCCCTAATGTCAAGCAGT</td>
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implantation model, $3 \times 10^6$ cells were suspended in 100 µL of serum-free DMEM. Each nude mouse was inoculated in the subcutaneous tissue above the right groin with a micro syringe. All mice were sacrificed 4 weeks later. Tumour diameter and weight were recorded, and the diameter was calculated as follows: tumour diameter = (longest diameter + shortest diameter)/2. The animal study was approved by the Animal Care Review Committee of the Sun Yat-sen University (L025501202107011).

2.11 Statistical Analysis

Differences between variables were assessed by Wilcoxon analysis, Mann-Whitney test or two-tailed Student’s $t$ test. Kaplan–Meier analysis and log-rank tests were
Fig. 2. Differences between IC1 and IC2 and the screening of key prognostic genes related to TMB. (A,B) Differences in overall survival and TMB between IC1 and IC2 (****p < 0.0001; Mann-Whitney test of two independent samples). (C) KEGG analysis of differentially expressed genes between IC1 and IC2. (D) The results of multivariate Cox analysis for differentially expressed genes and overall survival. (E) Pan-cancer analysis of the correlation between RCAN2 expression and TMB. TMB, tumour mutation burden; IC, integrated cluster; KEGG, Kyoto Encyclopedia of Genes and Genomes. *p < 0.05, **p < 0.01, ***p < 0.001; Spearman analysis between RCAN2 expression and TMB.
Fig. 3. The RCAN2 expression in clinical HCC tissues and its correlation with overall survival. (A,B) Comparison of RCAN2 mRNA and protein expression between HCC and normal tissues (scale bar = 200 µm). (C,D) IHC staining of RCAN2 expression in clinical HCC tissues, and the Kaplan-Meier survival analysis of patients grouped according to their IHC score. HCC, hepatocellular carcinoma; IHC, immunohistochemistry (scale bar = 100 µm).

used to compare survival between groups. Multivariate Cox analysis was performed to identify independent factors of prognosis. Data are presented as the mean ± significant difference (SD), with the significance threshold considered as $p < 0.05$.

3. Results

3.1 Identification of RCAN2 as a Key Prognostic Gene Related to TMB

The workflow for this study is shown in Fig. 1. The TCGA-LIHC database was used to examine the correla-
Fig. 4. Overexpression of *RCAN2* inhibits the growth of HCC *in vitro* and *in vivo*, whereas HCC cells with *RCAN2* knockdown show enhanced proliferation. (A) *RCAN2* mRNA and protein expression in Hep-3B and MHCC-LM3 cells with altered *RCAN2* expression. (B) Overexpression of *RCAN2* inhibits the *in vitro* proliferation of Hep-3B and MHCC-LM3 cells, whereas HCC cells with *RCAN2* knockdown show enhanced proliferation. HCC cell proliferation was assessed by CCK-8 assay. (C,D) Overexpression of *RCAN2* inhibits the *in vivo* proliferation of MHCC-LM3 cells, whereas HCC cells with *RCAN2* knockdown show enhanced proliferation. Cell proliferation was assessed by measurement of the tumour diameter and weight (*p* < 0.05, **p** < 0.01, ***p*** < 0.001, ****p*** < 0.0001; *t*-test of two independent samples). CCK-8, Cell Counting Kit-8.
tion between gene expression and TMB. This resulted in the identification of 99 TMB signature-related genes when using the thresholds of \( p < 0.05 \) and \( |R| > 0.2 \). By using the expression of TMB signature-related genes as the clustering standard, 353 patients were divided into three subgroups: integrated cluster (IC) 1 (\( n = 197 \)), IC2 (\( n = 155 \)) and IC3 (\( n = 1 \)) (Supplementary Fig. 1A,B). Due to its small sample size, IC3 was eliminated from the study, leaving only IC1 and IC2. The overall survival of IC1 patients was worse than that of IC2 patients \( (p = 0.031, \text{Fig. 2A}) \), while the TMB score of IC1 patients was also significantly higher \( (p < 0.001, \text{Fig. 2B}) \). Using a \( p \)-adjusted value of <0.05 as the standard for significant difference, we identified 5443 differentially expressed genes between IC1 and IC2.

Next, the differentially expressed genes \( (n = 5443) \) selected above were studied by Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of biological function. This revealed that PI3K-Akt signalling was the top enrichment pathway (Fig. 2C), and suggests that phosphorylation-related pathways may underlie the phenotypic differences between IC1 and IC2. Cox proportional hazard analysis was used to identify 5 key genes related to prognosis: Galectin 3 \( (LGALS3) \), C-X-C motif chemokine ligand 1 \( (CXCL1) \), Solute carrier family 22 member 1 \( (SLC22A1) \), C-type lectin domain family 3 member B \( (CLEC3B) \) and RCAN2 (Fig. 2D). RCAN2 is a member of the regulator of calcineurin family that controls various cellular functions by affecting calcineurin activity. It was therefore selected for subsequent in vitro and in vivo studies.

The correlation coefficient between RCAN2 and TMB was calculated for multiple cancer types in the TCGA database. RCAN2 expression was found to correlate with TMB in 18 of 33 cancer types examined (Fig. 2E, Supplementary Table 1). In the large majority of these cancer types \( (17/18) \), a significant inverse correlation was observed between RCAN2 expression and TMB. This result suggests that accumulation of mutations reduces RCAN2 expression in tumours, thereby affecting prognosis.

3.2 HCC Patients with High RCAN2 Expression have Better Survival Than Patients with Low Expression

RCAN2 mRNA was extracted from 10 clinical samples, including HCC and adjacent normal tissues, and the level of RCAN2 was measured by qPCR. Corresponding tissues were used to validate RCAN2 protein expression by IHC staining. The results of these assays showed that the RCAN2 mRNA and protein expression were lower in HCC than in adjacent normal tissues (Fig. 3A,B).

To investigate the relationship between RCAN2 protein expression and the prognosis of HCC, surgical specimens were collected from 144 HCC patients for IHC staining. RCAN2 protein expression in each specimen was analysed and recorded as the IHC score using the Inform analysis system (Perkin Elmer, USA). Patients were subsequently divided into high-RCAN2 and low-RCAN2 groups according to the median IHC score of 120. The overall survival of the high-RCAN2 group was significantly better than the low-RCAN2 group \( (\text{log-rank } p = 0.0009, \text{Fig. 3C,D}) \).

3.3 Overexpression of RCAN2 Inhibits the Growth of HCC In Vitro and In Vivo, Possibly by Inducing Apoptosis

The basic levels of mRNA and protein expression of RCAN2 in tumour cells and normal hepatocytes HL-7702 are shown in Supplementary Fig. 2. Based on the above results, we selected Hep-3B with a high mRNA expression level and LM3 with a low of mRNA expression level for our study.

To explore the mechanism by which RCAN2 expression might impact the prognosis of HCC, the RCAN2-OE and RCAN2-SH plasmids were transfected into HCC cells (Hep-3B and MHCC-LM3). The expression of RCAN2 in these cells was then evaluated using qPCR and western blot analysis (Fig. 4A).

The proliferation rate of HCC cells was assessed using the CCK-8 assay to determine cell viability at different time points. HCC cells that overexpressed RCAN2 showed a reduced proliferation rate, whereas HCC cells with RCAN2 knockdown showed enhanced proliferation (Fig. 4B). These findings suggest that upregulation of RCAN2 can effectively inhibit the proliferation of HCC cells in vitro.

The biological function of RCAN2 in BALB/c nude mice was also investigated using MHCC-LM3 cells with altered RCAN2 expression. Following subcutaneous injection of cells, the tumour diameter and weight were measured to evaluate tumour growth (Fig. 4C,D). Mice injected with cells overexpressing RCAN2 (RCAN2-OE) showed significantly smaller diameter \( (p = 0.021) \) and weight \( (p = 0.0003) \) compared to mice injected with cells overexpressing vector only (vector-OE). In addition, mice injected with cells that were knocked down for RCAN2 (RCAN2-SH) showed significantly greater tumour diameter \( (p = 0.008) \) and weight \( (p = 0.0063) \) than the negative control (NC) group. These findings suggest that RCAN2 has a significant biological role in HCC, and that its overexpression can effectively suppress both the in vitro and in vivo growth of HCC.

To explore the mechanism by which RCAN2 regulates the proliferation of HCC, we screened for genes related to RCAN2 in the TCGA-LIHC database. All genes were ordered by their \( R \) value and analysed through GSEA analysis. The top three signalling pathways found to be associated with RCAN2 expression were TNF-\( \alpha \)-related extrinsic apoptotic pathway may play a pivotal role in HCC cells with differential expression of RCAN2.

Based on the results of GSEA analysis, flow cytometry was employed to evaluate apoptosis in cells with al-
Fig. 5. The pathway enrichment analysis of \textit{RCAN2} in TCGA-LIHC database and the different proportions of apoptotic cells in different HCC groups with altered \textit{RCAN2} expressions. (A) Pathway enrichment analysis of the differentially expressed gene between \textit{RCAN2}-high and \textit{RCAN2}-low group in TCGA-LIHC database. (B) After 12 h treatment of transfected cells with recombinant human TNF-\(\alpha\) and TRAIL protein, the proportion of apoptotic cells in HCC cells was assessed by image-flow cytometry assay (*\(p\) < 0.05, **\(p\) < 0.01, ****\(p\) < 0.0001; t-test of two independent samples). TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; TNF-\(\alpha\), tumour necrosis factor-\(\alpha\).
Fig. 6. The protein expression of caspase-8, caspase-9 and caspase-3 in HCC cells cultured with or without 10 ng/mL TNF-α and 100 ng/mL TRAIL.
Fig. 7. Expression of EHF was positively correlated with RCAN2 in three independent databases and validation in HCC cells with altered RCAN2 expression. (A) Correlation of five genes that collectively met the threshold in three independent databases (GSE64041, GSE76427 and SHI-sequencing data; *p < 0.05, **p < 0.01, ***p < 0.001; Spearman analysis in independent databases). (B) Volcano plot showing the expression of five key genes in the NC group vs. RCAN2-SH group for MHCC-LM3 cells. (C) The mRNA expression of EHF was validated in Hep-3B and MHCC-LM3 cells with altered RCAN2 expression. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; t-test of two independent samples. (D) The protein expression of EHF and DR5 was validated in Hep-3B and MHCC-LM3 cells with altered RCAN2 expression. NC, negative control; EHF, ETS homologous factor; DR5, death receptor 5. Under different incubation conditions with various apoptotic inducers, changes were also observed in the expression of apoptosis-related proteins, including caspase-8, cleaved caspase-8, caspase-9, caspace-3 and cleaved caspase-3. Meanwhile, apoptosis-related proteins were down-regulated in RCAN2-SH group. Of note, caspase-9 is implicated in the mitochondrial apoptotic pathway, suggesting that RCAN2 may modulate the apoptosis of HCC cells via the mitochondrial pathway of extrinsic apoptosis.

3.4 Expression of the Transcription Factor EHF was Positively Correlated with RCAN2 in Several Independent Databases

To explore the mechanism by which RCAN2 regulates apoptosis in HCC, we screened genes related to RCAN2 in three independent databases (GSE76427, GSE6404 and SHI-sequencing data) using a threshold of p < 0.05. Five key genes (RORB, GAS6, SEMA6A, RFTN1 and EHF) were selected after intersecting the related genes in the three databases (Fig. 7A).
Fig. 8. The proportion of apoptotic cells, cell proliferation, DR5 protein expression and caspase-8/9/3 protein expressions in HCC cells with altered RCAN2 expression, and with or without recovery of EHF expression. (A) After 12 h treatment of transfected HCC cells with recombinant human TRAIL protein, the proportion of apoptotic cells was assessed by image-flow cytometry assay (**p < 0.01; t-test of two independent samples). (B) Cell proliferation was assessed in HCC cells (MHCC-LM3) with altered RCAN2 expression, and with or without recovery of EHF expression (****p < 0.0001; t-test of two independent samples). (C) Protein expression of RCAN2, EHF, DR5, caspase-8, caspase-9 and caspase-3 in HCC cells (Hep-3B and MHCC-LM3) with altered RCAN2 expression, and with or without recovery of EHF expression.
The relationship between five key genes and RCAN2 was validated in the MHCC-LM3 cell sequencing dataset. Compared with the RCAN2-SH group, the expression of EHF in the NC group was significantly upregulated, while the expression of the other four genes was not significantly different (Fig. 7B). Because of the positive correlation between RCAN2 and EHF in multiple independent databases, EHF was selected for subsequent mechanism verification.

We also detected the correlation in mRNA and protein expression between RCAN2 and EHF in transfected HEP-3B and MHCC-LM3 cells, the changes in EHF mRNA and protein expression mirrored those of RCAN2, as determined by qPCR and western blot (Fig. 7C,D). This result suggests that overexpression or knockdown of RCAN2 can upregulate or inhibit the expression of EHF (Fig. 7D), and corroborates a previous study that found EHF acts as an upstream transcription factor which regulates the expression of DR5 [28].

3.5 RCAN2 Up-Regulates Extrinsic Apoptosis through the EHF/DR5 Pathway and Induces Apoptosis through the Caspase-8/9/3 Pathway

It is unclear RCAN2 promotes the apoptosis of HCC cells by regulating the expression of EHF. To address this, we transfected an EHF-SH plasmid into MHCC-LM3 cells with RCAN2-OE, as well as the corresponding control cells. Cell proliferation and apoptosis were measured by CCK-8 kit and image-flow cytometry assay, respectively. The proportion of apoptotic cells in the image-flow cytometry assay was reduced after the recovery of EHF expression (Fig. 8A). Moreover, the proliferation rate of HCC cells also increased after the recovery of EHF expression (Fig. 8B). At day 4, the cell proliferation rate of the RCAN2-OE/NC group was lower than that of the vector-OE/NC group (p = 0.004). When the expression of EHF was knocked down, the proliferation rate of cells improved significantly (RCAN2-OE/EHF-SH group vs. RCAN2-OE/NC group, p = 0.0003).

After 12 h treatment of HCC cells with recombinant TRAIL, the expression levels of caspase-8, cleaved caspase-8, caspase-9, caspase-3 and cleaved caspase-3 were evaluated to assess apoptosis. It showed that the protein expression of DR5, caspase-8, cleaved caspase-8, caspase-9, caspase-3 and cleaved caspase-3 were downregulated after the recovery of EHF expression (Fig. 8C). This result suggests that knockdown of EHF can reverse the effect of RCAN2 upregulation on the apoptosis of HCC cells, thereby restoring cell proliferation. Based on these preliminary observations, we propose that RCAN2 can promote extrinsic apoptosis of HCC cells by upregulating the expression of EHF and DR5, thus inducing apoptosis through the caspase-8/9/3 pathway.

4. Discussion

Abnormal proliferation in hepatocytes is always accompanied by somatic mutations of some important anti-tumour genes such as TP53, CTNNB1 and so on [8–10]. These processes of gene mutation and inactivation are considered important characteristics in the development of HCC [7,29]. The accumulation of mutations in tumours is associated with poor prognosis due to more malignant cellular behaviour [30,31]. The cellular behaviour is frequently characterized by an augmented rate of proliferation or an enhanced invasive capacity, and clinically manifested by accelerated rates of tumour progression or distant metastasis. It finally results in the limited response to systemic therapy [32,33]. TMB is one of the crucial metrics for assessing the mutational load in tumours and has been proven to be correlated with the efficacy of systemic therapy in many tumour types [11]. To improve the treatment of advanced HCC, it is therefore important to explore the molecular mechanism that link TMB with prognosis.

Previous studies have shown that different levels of effector protein phosphorylation can lead to different phenotypes, including chemoresistance and proliferation, in many cancer types [34–37]. Bioinformatics analyses conducted in the present study resulted in similar findings: Different tumour subgroups related to TMB show significant survival differences. Differences in the PI3K-Akt signalling pathway may be responsible for this effect on prognosis. Genes that regulate phosphorylation may therefore play an important role in determining the prognosis of different TMB subgroups in HCC.

Previous studies have shown that RCAN2 participates in phosphorylation by inhibiting the activation of calcineurin [13,14]. In our study, RCAN2 was one of the significantly different genes between IC1 and IC2 clustered by TMB signature-related genes. And it was also identified as the genes related to overall survival in the TCGA-LIHC database. In addition, RCAN2 showed a negative correlation with TMB in the pan-cancer analysis, suggesting that inhibition of RCAN2 expression may be a common phenomenon in many cancer types with accumulation of somatic mutations. Thus, RCAN2 may be a key biomarker in determining the overall survival of cancer patients, and it is important for enhancing the HCC treatment efficacy to explore the underlying mechanism of RCAN2 influencing the prognosis of HCC.

However, the biological functions of RCAN2 appear to vary in different tumour types. In gastric carcinoma, RCAN2 stimulates cell growth and invasion by upregulating the phosphorylation of AKT and ERK [15]. Moreover, RCAN2 can suppress cell proliferation in KRAS-mutated colorectal cancer (CRC) by inhibiting the calcineurin-NFAT signalling pathway [16]. The present study found that high RCAN2 expression was associated with better overall survival in HCC. Furthermore, RCAN2 performed relevant biological functions by affecting apoptosis in vitro.

EHF is one of the ETS transcription factors that regulates expression of the downstream gene DR5 by binding to the ETS sequence in the promoter, thereby affecting tumour cell proliferation [28]. Through experiments involving the
recovery of EHF expression, we demonstrated that RCAN2 regulated extrinsic apoptosis through the EHF/DR5 pathway. Although the specific mechanism for this is still unclear, calcineurin and oxidative phosphorylation may play important roles in the process. When HCC cells are stimulated by external apoptotic inducers, the apoptotic signalling can mediate activation of apoptotic proteins such as caspase8/9/3 through up-regulation of DR5, eventually leading to cell apoptosis.

A shortcoming of this study was that the specific model of molecular interaction between RCAN2 and EHF remains unclear. In addition, the change of NFκB related pathway is not confirmed after overexpressing or knocking down of RCAN2 expression. The shortcomings mentioned above requires further exploration in future. Although there are some limitations, this study clarified the biological function of RCAN2 in HCC, as well as that of the RCAN2/EHF/DR5 pathway. This may provide a potential new target for the treatment of advanced HCC with high TMB.

5. Conclusions

RCAN2 expression is a biomarker of prognosis in HCC and is negatively correlated with TMB. It has been demonstrated that RCAN2 regulated extrinsic apoptosis through EHF/DR5 pathway in our study. When HCC cells are stimulated by external apoptotic inducers, the apoptotic signalling can mediate activation of apoptotic proteins such as caspase8/9/3 through up-regulation of DR5, ultimately leading to cells apoptosis. This study provides a potential new target for the treatment of advanced HCC with high TMB.

Abbreviations

HCC, Hepatocellular carcinoma; TP53, Tumour protein p53; CTNNB1, Catenin beta 1; TMB, Tumour mutational burden; TCGA-LIHC. The Cancer Genome Atlas-Liver hepatocellular carcinoma; qPCR, Quantitative real-time polymerase chain reaction; IHC, immunohistochemistry; EHF, ETS homologous factor; IC, Integrated cluster; KEGG, Kyoto Encyclopedia of Genes and Genomes; LGALS3, Galectin 3; CXCL1, C-X-C motif chemokine ligand 1; SLC22A1, Solute carrier family 22 member 1; CLEC3B, C-type lectin domain family 3 member B; OE, Overexpression of gene; vector-OE, plasmid with overexpression vector; SH, Knocking down of gene; NC, Negative control; CCK8, Cell counting kit-8; GSEA, Gene Set Enrichment Analysis; DR5, Death receptor 5, also called TNFRSF10B, TNF receptor superfamily member 10b; EHF, ETS homologous factor; RCAN2, Regulator of calcineurin 2.

Availability of Data and Materials

The externally validated datasets were generated from The Cancer Genome Atlas-Liver Hepatocellular Carci- noma (TCGA-LIHC) database (https://portal.gdc.cancer.gov) and Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo, ID: GSE76427 and GSE64041) [14]. The data generated and/or analyzed during the current study can be accessed and used after the consent of the corresponding author upon reasonable request by email.

Author Contributions

SZ, MKH, and YJX designed the research and reviewed and revised the manuscript; MS and WLG revised the design of the research and the manuscript. YJX, ZCL and AK performed the data analysis and experiments and drafted the manuscript; YJX, HL, YH, YYL and JFW and ZFW generated the figures. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Sun Yat-sen university Cancer Center (No. 202011) and written informed consents from patients were waived due to the retrospective nature of our study. The animal study was approved by the Animal Care Review Committee of the Sun Yat-sen University (L025501202107011).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2907243.

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