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ANovel Role ofAIM2 Inflammasome-Mediated Pyroptosis in RadiofrequencyAblation of Hepatocellular Carcinoma

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Keywords:

Absent in melanoma 2; Hepatocellular carcinoma; Inflammasome;Pyroptosis;Radiofrequencyablation

Abbreviations:

AIM2: Absent in Melanoma 2; ELISA: Enzyme-Linked Immunosorbent Assay; FLICA: Fluorescent-Labeled Inhibitors of Caspase; GAPDH: Glyceraldehyde-3-PhosphateDehydrogenase;HBV:HepatitisBVirus;HCC:HepatocellularCarcinoma;HCV:HepatitisCVirus;H&E:Hematoxylin and Eosin; IL-1β: Interleukin-1β; IL-18: Interleukin-18; LDH: Lactate Dehydrogenase; NC: Nonspecific Control; qRT-PCR: Quantitative Reverse Transcription–Polymerase Chain Reaction; RFA: Radiofrequency Ablation; SD: Standard Deviation

1. Abstract

Background: Hepaticinflammationandinflammasome-me- diated mechanisms are involved in the pathogenesis of Hepato- cellular Carcinoma (HCC), and Absent in Melanoma 2 (AIM2) triggersactivationoftheinflammasomecascade.Untilnow,itremains unclear whether and how AIM2 plays a role in HCC and RadiofrequencyAblation (RFA). This study aimed to investigate whether RFA induces pyroptosis in HCC through AIM2-inflammasome signaling in vivo and in vitro.

Methods: BALB/c nude mice were used to generate HepG2 or SMMC-7721 cell-derived tumor xenografts. HCC cells with knockout or overexpression ofAIM2 were created for functional and mechanistic studies.

Results: We found that RFAsignificantly suppressed the tumorgrowthinmicebearingHCCxenografts.Flowcytometryanal- ysis revealed that RFA induced pyroptosis. Furthermore, AIM2, NLRP3, caspase-1, γ-H2AX, and DNA-PKc had significantly greaterexpressionlevelsinthelivertissuesfrommicetreatedwith RFAversusthoseofthecontrols.Inparallel,theexpressionlevels of Interleukin (IL)-1β and IL-18 were significantly greater in the HepG2andSMMC-7721cell-derivedxenograftmicetreatedwith RFAcomparedtothosewithoutreceivingRFA.Ofnote,agreater effectwasachievedintheRFAcompleteablationgroupversusthe partialablationgroup.Moreover,studiesincelllineswithknockoutoroverexpressionofAIM2demonstratedthatAIM2exerteda role in RFA-induced pyroptosis.

Conclusion: The findings of this study indicate that RFA suppresses tumor growth through inducing pyroptosis and that AIM2-mediatedpyroptosisisanimportantcelldeathmechanism. Therefore, intervention ofAIM2-mediated inflammasome signal- ing may assist in improving RFA treatment for HCC.

2. Introduction

Hepatocellular Carcinoma (HCC) is among the most common malignancies and a leading cause of cancer-related deaths globally [1]. It has been noted that the majority of HCC cases occurin Asian countries [2]. For instance, in China, the incidence of HCC is considerably high, accounting for approximately 50% of all newly diagnosed HCC cases across the world, and can be attributed to the particularly high prevalence of chronic Hepatitis B Virus (HBV) infection [2-4]. In fact, HBV and Hepatitis C Virus (HCV) are common causes of chronic hepatitis and progressive-

lycauseinflammationoftheliver.Althoughtheexactpathogenic

mechanisms underlying HCC remain to be elucidated, hepatic inflammation and inflammasome-mediated molecular mechanisms havebeenproposedtoplayaroleinthepathogenesisofHBV-and HCV-related HCC; in addition, HCC is considered an inflammation-relatedmalignancy[5-8].OfthecurativetreatmentsforHCC, RadiofrequencyAblation (RFA) has emerged as an effective and safe treatment for patients with a small-sized tumor, usually 3cm or less in diameter. In comparison with traditional hepatic resection and liver transplantation, RFAhas been associated with less invasiveness and a shorter hospital stay. Despite the advantages and increasing application of RFA in the treatment of HCC, the molecular mechanisms underlying the action of RFAare not well understood.

Absent in Melanoma 2 (AIM2) is a cytosolic receptor in the pyrin and HIN domain-containing protein family [9, 10].AIM2 has beenshowntosenseandbindtodouble-strandedDNAandtoapoptosis-associatedspeck-likeprotein,thustriggeringactivationof the inflammasome signaling cascade and orchestrating assembly of the AIM2 inflammasome [11-13]. Activation of the AIM2 inflammasome, which consists of multiple proteins, represents one key aspect of the inflammation pathways. TheAIM2 inflammasome can activate caspase-1, leading to induction, maturation, and release of key proinflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18) [14]. Therefore, theAIM2 inflammasome possesses both proinflammatory and propyroptotic propertiestomediatepyroptosis,whichhasbeenimplicatedinthe host defense, thus combating microbial invasion, carcinogenesis, andcancerprogression.AnumberofpreviousstudieshavereportedthatAIM2expressionisdecreasedinHCCtissuesversushistologicallynormaltissuesandthatlowerlevelsofAIM2aresignificantlycorrelatedwithmoreadvancedHCC.Untilnow,itremains largely unknown whether and howAIM2 plays a role in HCC. In addition,whetherandhowAIM2exertsaroleinthemechanismof action of RFA for HCC has not yet been explored.

In the present study, we aimed to investigate the roles of AIM2in HCC and the action of RFAfor the treatment of HCC in mice with xenograft tumors as well as in hepatoma cells. The findings obtained through conducting this study may offer a better understandingofthebiologicalfunctionofAIM2,theAIM2inflammasome, and pyroptosis in HCC, thereby assisting in improving RFA treatment for HCC.

3. MaterialsandMethods

ExperimentalAnimals

BALB/cnudemice(4–6weeksold)werepurchasedfromJiangsu SynthgeneBiotechnologyCo.,Ltd.(Nanjing,Jiangsu,China).The mice were housed under controlled conditions and were allowed tap water ad libitum throughout the period of the animal experiments. To establish the HepG2 and SMMC-7721 cell-derived tumor xenograft animal models, BALB/c nude mice were subcutaneouslyinjectedwithHepG2cells(1×107cellssuspendedin100 μLof serum-free RPMI1640 medium) or SMMC-7721 cells (1 × 107cellssuspendedin100μLofserum-freeRPMI1640medium). HepG2 or SMMC-7721 cell-derived xenograft nude mice were randomlyassignedtoreceiveRFAcompleteablation,RFApartial ablation,ornoablationasacontrol(nonablation).Fourweeksfollowingthetreatment,themicewereanesthetizedandsacrificedby cervical dislocation, and the tumors were collected. The weights and volumes of the excised tumors were analyzed.

The study involving experimental mice was reviewed and approved by the Ethics Committee of Changzhou First People's Hospital (Approval No. 2018-025).All methods were carried out in accordance with the local institutional and national guidelines and regulations. In addition, they were performed in compliance withtheinternationalregulationsfortheuseoflaboratoryanimals.

RFA

HepG2 or SMMC-7721 cell-derived xenograft nude mice were treatedwith RFAusing a Cool-tipTMRFAElectrodekit (Covidien IIc, Mansfield, MA, USA), according to the manufacturer's protocol. For the in vitro study, RFAwas performed using a thermal needle to treat SMMC-7721 cells.

Histology

Tumor tissues of the HepG2 and SMMC-7721 cell-derived xenograftnudemiceweref ixed,paraffin-embedded,andcutinto2-μmthick sections.After staining with hematoxylin and eosin (H&E), the slides were examined by light microscopy.

Immunohistochemistry

Immunohistochemical analysis was carried out to assess the proteinexpressionofAIM2,NLRP3,andcaspase-1inthelivertissues from HepG2 and SMMC-7721 cell-derived xenograft nude mice treatedwithorwithoutRFA.Thelivertissueswerecollected,fixed in10%formalin,andembeddedinparaffin.Theparaffinsections ofthelivertissueswerehydrated,andtheslideswerethenincubatedwithprimaryantibodies,includingthosetargetingAIM2(Bioss Antibodies, Beijing, China), NLRP3 (Abcam, Cambridge, UK), and caspase-1 (Abcam, Cambridge, UK), at 4 °C overnight. The resulting slides were then incubated with 3,3′-diaminobenzidine (DAB), a substrate for horseradish peroxidase using a DAB PeroxidaseSubstratekit,accordingtothemanufacturer'sinstructions (VectorLaboratories,Burlingame,CA,USA).Imagesweretaken byanOlympusdigitalelectronmicroscope(Olympus,Tokyo,Japan).Theimmunoreactivitiesoftheimmunohistochemicalimages were evaluated for each slide.

Cell Culture

TwohumanhepatocellularcarcinomacelllLines,includingHepG2 and SMMC-7721, were obtained from Shanghai Binsui Biotechnology (Shanghai, China) and used for the in vitro studies. The HepG2andSMMC-7721cellswereculturedinRPMI1640medi- um (Hyclone, Marlborough, MA, USA) supplemented with 10% (v/v)fetalbovineserum,100μg/mLstreptomycin,and100units/

mLpenicillin.

OverexpressionandKnockoutofAIM2

ThepcDNA3.1vectorwasusedtoconstructtheexpressionvector (OS-AIM2)byinsertingthecDNAsequenceencodingAIM2.The successful construction of the expression vector OS-AIM2 was verified by sequencing. SMMC-7721 cells were transfected with OS-AIM2foroverexpressionofAIM2usingLipofectamine2000 Reagent (Invitrogen, Carlsbad, CA, USA). Silencing of AIM2 wasachievedbyCRISPR/Cas-9geneeditingofthe AIMgene in SMMC-7721 cells. SMMC-7721 cells were transfected with CRISPR/Cas-9-AIM2forknockoutofAIM2usingLipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA).

LactateDehydrogenaseReleaseAssay

Cellulardamagewasmeasuredbyalactatedehydrogenase(LDH) release assay in which the LDH levels in the cell culture supernatant of SMMC-7721 cells were determined using an LDH release assaykit(Abcam,Cambridge,UK),accordingtothemanufactur- er's instructions.

FlowCytometryAnalysisofPyroptotic Cells

Pyroptosis was measured on a flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Fluorescent-labeled inhibitors of caspase (FLICA) probe assays (AAT Bioquest, Sunnyvale,CA,USA)wereconductedtoexaminethepyroptosis, accordingtothemanufacturer'sinstructions.Pyroptoticcellswere specificallystainedbyFAM-FLICA-caspase-1andpropidiumiodide staining.

Enzyme-LinkedImmunosorbentAssay(ELISA)

ELISAwascarriedouttodeterminetheserumlevelsofIL-1βand IL-18 in HepG2 and SMMC-7721 cell-derived xenograft nude mice treated with or without RFA as well as in the cell culture supernatantofSMMC-7721cellsusingELISAkits(Abcam,Cambridge, UK), according to the manufacturer's instructions.

Real-TimeQuantitativeReverseTranscription–Polymer- ase Chain Reaction (qRT-PCR)

Total RNA was extracted from SMMC-7721 cells using TRIzol (Invitrogen, Waltham, MA, USA). The total RNA samples were transcribed into cDNA, according to the manufacturer's instructions.qRT-PCRwascarriedouttomeasurethemRNAexpression of target genes (pyroptosis-related genes).The mRNAexpression ofβ-actinwasusedasaninternalcontrol.TherelativemRNAlev- els of target genes were obtained by using the 2-ΔΔCt method, with all assays performed in triplicate. Fold-change values were calculatedbycomparativeCtanalysisafternormalizationtoβ-ac- tin. The primers used in the qRT-PCR analysis were as follows: AIM2, forward primer: 5′-ATCAGGAGGCTGATCCCAAA-3′; reverse primer: 5′-TCTGTrCAGGCTTAACATGAG-3′; β-actin, forwardprimer:5′-GGCACCACACCTTCTACAATG-3′,reverse primer: 5′-TAGCACAGCCTGGATAGCAAC-3′.

WesternBlotAnalysis

Western blot analysis was performed to examine the hepatic protein levels of AIM2 as well as the key inflammasome- and pyroptosis-related proteins, such as NLRP3 (Abcam, Cambridge, UK), caspase-1 (Abcam, Cambridge, UK), γ-H2AX (Abcam, Cambridge, UK), and DNA-PKc (Sangon Biotech, Shanghai, China). The protein expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. In brief, 30–50 μg of total protein was separated on 4–15% sodium dodecyl sulfate–polyacrylamide gels. After electrophoretic transfer onto ImmunBlot polyvinylidene difluoride membranes, the resulting membranes were blocked with phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20, followed by incubation with primary antibody overnight at 4 ºC. The membranes were then incubated with secondary antibodies (dilution, 1:10,000) at room temperature for 1 h. An imaging system was usedtodeterminetherelativeopticaldensityofeachspecificband in the western blot analysis.

StatisticalAnalysis

StatisticalanalysiswasconductedwithSPSSsoftwareversion 16.0 for Windows (SPSS, Chicago, IL, USA). All experiments included at least triplicate samples for each treatment group, and data were expressed as the mean ± Standard Deviation (SD). For theimmunohistochemistryandwesternblotresults,representative imagesarepresented.AnalysisofVariance(ANOVA)wasapplied tocomparemeansofmultiplegroups.$P<0.05$indicatedasignifi- cant difference between groups.

4. Results

RFA Suppressed Tumor Growth in Subcutaneous Xenograft Nude Mice

We initially assessed the effects of RFAon in-vivo tumor growth insubcutaneousxenograftnudemice.AsshowninFigure1.A–B, HepG2andSMMC-7721cell-derivedxenografttumorsgrewprogressively in the control nude mice without the RFAtreatment as a control, whereas RFAtreatment markedly suppressed the tumor growthasdemonstratedbythesignificantlysmallertumorsizeand the lower tumor weight compared with those of the controls after four weeks of treatment. It was also noted that the mean tumor sizes were significantly smaller and the tumor weights were significantlylowerintheHepG2andSMMC-7721cell-derivedxenograftnudemicetreatedwithcompleteRFAablationthaninthose treatedwithpartialRFAablationafterfourweeksoftreatment($P<0.05$)(Figure1A–B).Theresultsalsoindicatedthattheinhibitory effectsonthein-vivotumorgrowthweregreaterintheHepG2and SMMC-7721 cell-derived xenograft nude mice treated with complete RFAthan in those treated with partial RFAablation (Figure 1A–B).ConsistentwiththechangesinthetumorsizeandweightH&Estainingoftumorsectionsrevealedasignificantreductionin thenumberoftumorcellsintheHepG2andSMMC-7721cell-derivedxenograftnudemicetreatedwithcompleteorpartialRFA

ablation,comparedwiththecontrolmice(Figure1C).Inaddition, flow cytometry analysis showed that the percentages of pyroptot- ic cells were 12.62%, 10.75%, and 5.49% (HepG2) and 26.42%, 9.84%,and3.53%(SMMC-7721)inthecomplete,partial,andno RFAablationgroups,respectively(Figure1D).Statisticalanalysis indicated that the proportion of pyroptotic cells was significantly increased in the tumor cells of the subcutaneous xenograft nude micetreatedwithRFApartialorcompleteablation,comparedwith no RFA ablation as a control ($P < 0.01$) (Figure 1D). Moreover, therewasasignificantlygreatereffectintheRFAcompeteablation group compared with the partial ablation group ($P < 0.01$).

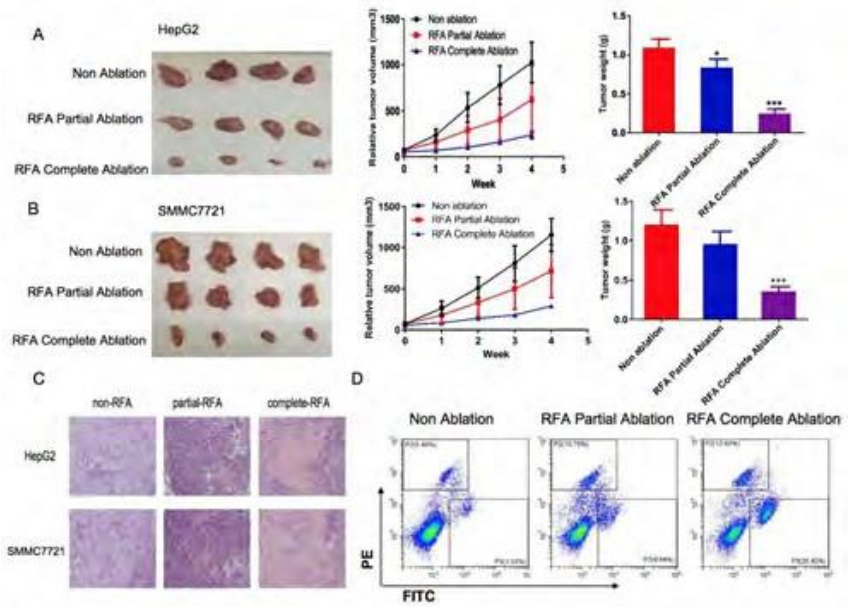

Figure 1: Inhibitory effects of radiofrequency ablation (RFA) on xenotransplant tumor growth in subcutaneous xenograft nude mice. HepG2 and SMMC-7721 cell-derived xenograft nude mice were used to examine the effects of RFA on tumor growth, and the mean tumor sizes and weights were analyzed in the following three groups: No ablation as a control (Nonablation), RFApartial ablation, and RFAcomplete ablation. (A) HepG2 cell-derived xenograft nude mice. Four weeks after RFApartial or complete ablation, the tumor growth was significantly suppressed as indicated by thesmallertumorsizeandlowertumorweightcomparedwiththecontrolmicewithoutRFAtreatment.(B)SMMC-7721cell-derivedxenograftnude mice.FourweeksafterRFAcompleteablation,thetumorgrowthwassignificantlysuppressedasindicatedbythesmallertumorsizeandlowertumor weightcomparedwiththecontrolmicewithoutRFAtreatment.(C)HistologicalfindingsoftumorsectionsfromHepG2andSMMC-7721cell-derived xenograftnudemice.HematoxylinandeosinstainingofthetumorsectionsrevealedasignificantreductioninthenumberoftumorcellsintheHepG2 andSMMC-7721cell-derivedxenograftnudemicetreatedwithcompleteorpartialRFAablationcomparedwiththecontrolmice.(D)Flowcytometry analysis of pyroptotic cells in subcutaneous xenograft nude mice treated with complete or partial RFAablation in comparison with the control mice. Data are presented as the mean ± SD, and $P< 0.05$ indicated a significant difference between groups. *$P<0.05$; ***$P<0.001$.

RFAInducedPyroptosisinSubcutaneousXenograftNude Mice

Giventheinhibitoryeffectsof RFAontumorgrowth,wenextas- sessed pyroptosis in subcutaneous xenograft nude mice at differ- ent time points after RFA treatment. Immunohistochemistry and westernblotanalysiswereperformedtoexaminethekeyproteins involvedintheinflammasomeandinpyroptosisinthelivertissues from the HepG2 and SMMC-7721 cell-derived xenograft nude mice. The immunohistochemistry results showed that the protein levels of AIM2, NLRP3, and caspase-1 were significantly greater in the liver tissue sections from the HepG2 and SMMC-7721 cell-derivedxenograftnudemicetreatedwithRFApartialorcom- pleteablation,comparedwiththoseinthecontrolgroup($P<0.05$) (Figure2A–B).Inaddition,agreatereffectofRFAontheprotein expression ofAIM2, NLRP3, and caspase-1 was observed in the HepG2andSMMC-7721cell-derivedxenograftnudemicetreated with RFA complete ablation in comparison with partial ablation (Figure2A–B).Westernblotanalysisrevealedsimilarfindingsto the immunohistochemistry results. Additionally, the mRNA and proteinlevelsofγ-H2AXandDNA-PKcweresignificantlygreater in the HepG2 and SMMC-7721 cell-derived xenograft nude mice treated with RFApartial or complete ablation versus no RFAab- lation ($P< 0.05$) (Figure 2C–F), and a greater effect was found in theRFAcompleteablationgroupversusthepartialablationgroup (Figure 2C–F).

The mean levels of serum IL-1β and IL-18 were significantly greater in the HepG2 and SMMC-7721 cell-derived xenograft nudemicetreatedwithRFApartialorcompleteablationafterfour weeks of treatment ($P< 0.05$) (Figs. 3A–B). Notably, there was a greatereffectofRFAontheserumlevelsofIL-1βandIL-18inthe HepG2andSMMC-7721cell-derivedxenograftnudemicetreated withRFAcompleteablationincomparisonwiththosetreatedwith partial ablation (Figure 3).

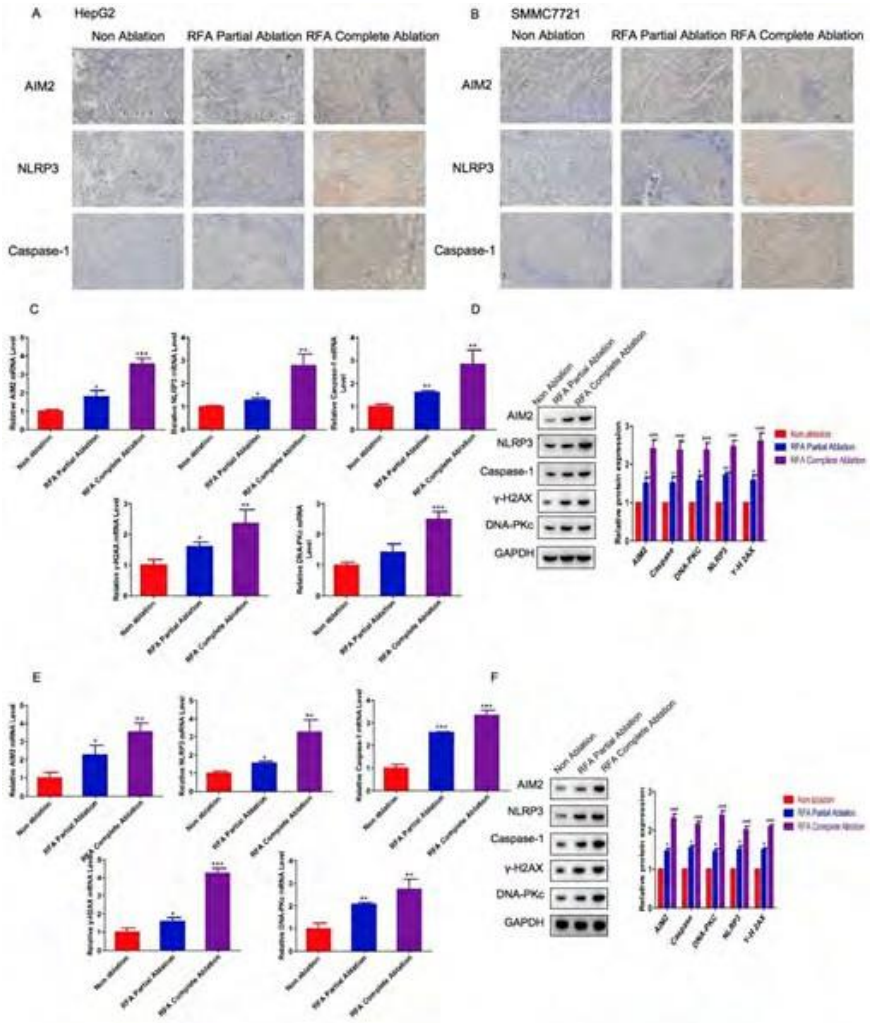

Figure 2: The levels of inflammasome- and pyroptosis-related molecules before and after radiofrequency ablation (RFA) in subcutaneous xenograft nude mice. HepG2 and SMMC-7721 cell-derivedxenograft nude micewere treatedwith RFApartialablation,FRAcompleteablation,or no ablation as a control (Nonablation).After 4 weeks, the liver tissues were collected for subsequent analysis.Immunohistochemical analysis of inflammasome proteinsbeforeandafterRFAin(A)HepG2cell-derivedxenograftnudemiceand(B)SMMC-7721cell-derivedxenograftnudemice.HepaticAIM2, NLRP3,caspase-1,γ-H2AX,andDNA-PKc(C)mRNAand(D)proteinexpressionlevelsinHepG2cell-derivedxenograftnudemice.HepaticAIM2, NLRP3,caspase-1,γ-H2AX,andDNA-PKc(E)mRNAand(F)proteinexpressionlevelsinSMMC-7721cell-derivedxenograftnudemice.Thehepat-icAIM2,NLRP3,caspase-1,γ-H2AX,andDNA-PKcmRNAandproteinexpressionlevelsweresignificantlygreaterintheHepG2andSMMC-7721 cell-derivedxenograftnudemicetreatedwithRFApartialorcompleteablationversusnoRFAablation,andthereweregreatereffectsontheseprotein levelsintheRFAcompleteablationgroupversusthepartialablationgroup.Dataareexpressedasthemean±SD.P<0.05indicatedasignificantdif- ference between groups. *P<0.05; **P<0.01; ***P<0.001.

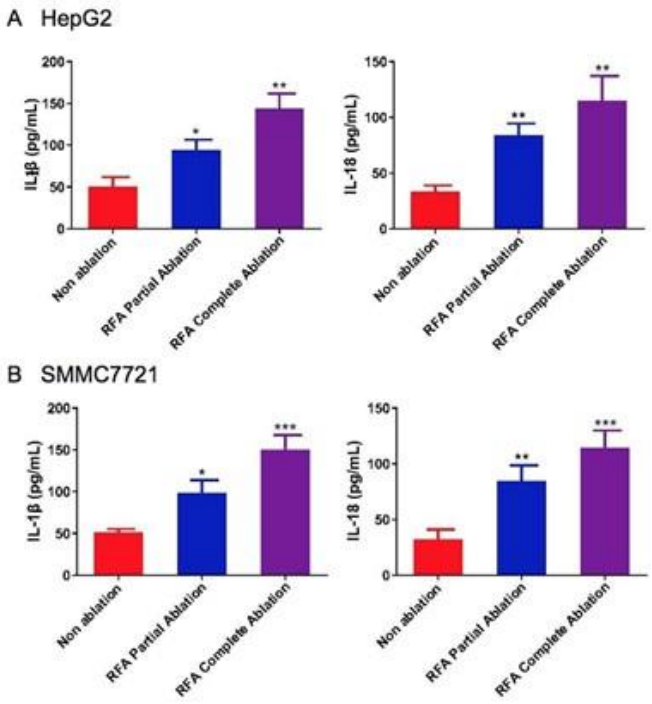

Figure3: SerumlevelsofIL-1βandIL-18beforeandafterradiofrequencyablation(RFA)insubcutaneousxenograftnudemice.Theserumlevelsof IL-1βandIL-18wereexaminedbyELISAin(A)HepG2cell-derivedxenograftnudemiceand(B)SMMC-7721cell-derivedxenograftnudemiceafter 4weeksofRFApartialablation,FRAcompleteablation,ornoablation.Dataareexpressedasthemean±SD.P<0.05indicatedasignificantdifference between groups. *P<0.05; **P<0.01; ***P<0.001.

AIM2 Exerted a Role in RFA-Induced Pyroptosis in Hepatoma Cells

Intriguedbytheanimalstudyfindings,wefurtherinvestigatedthe biological role ofAIM2 in RFA-induced pyroptosis in hepatoma SMMC-7721 cells.Acell proliferation assay revealed that down-regulation of AIM2 enhanced the proliferation of SMMC-7721 cells in a time-dependent manner (Figure 4A); by contrast, over-expression of AIM2 attenuated the proliferation of SMMC-7721 cells in a time-dependent manner (Figure 4B). In addition, exposuretoRFAresultedinamarkedincreaseinLDHreleaseincomparison with no RFA treatment. Furthermore, overexpression of AIM2withLv-AIMenhancedtheLDHreleasecomparedwiththe control SMMC-7721 cells (Figure 4C).

Pyroptosis is a form of programmed cell death that can affect tumorcellproliferation.Therefore,weexaminedtheeffectsofRFA onpyroptosisinhepatomaSMMC-7721cellswithoverexpression or silencing of AIM2. Flow cytometry assays showed that RFA induced pyroptosis compared with no RFA(Fig. 5A).Additionally, overexpression ofAIM2 with Lv-AIM2 enhanced pyroptosis, whereassilencingofAIM2withshAIM2diminishedpyroptosisin SMMC-7721 cells (Figure 5A).

qRT-PCRrevealedthatthehepaticγ-H2AXandDNA-PKcmRNA levelsweresignificantlygreaterintheSMMC-7721cellstreated with RFAversus those not treated with RFA. Moreover, overexpression of AIM2 increased hepatic γ-H2AX and DNA-PKc mRNA expression, while silencing of AIM2 led to a decrease in the hepatic γ-H2AX and DNA-PKc mRNAlevels in the SMMC-7721cells(Figure5B).Similarly,thewesternblotresultsshowed that the hepatic γ-H2AX, DNA-PKc, and caspase-1 levels were significantly greater in the SMMC-7721 cells treated with RFA versusthosenottreatedwithRFAandthatoverexpressionofAIM2 increased hepatic γ-H2AX and DNA-PKc protein expression; by contrast, silencing of AIM2 suppressed the hepatic γ-H2AX and DNA-PKc protein levels in SMMC-7721 cells (Figure 5C).

Furthermore, RFA treatment caused increases in hepatic IL-1β andIL-18mRNAexpressioninSMMC-7721cellsaswellasproteinexpressioninthecellculturesupernatant(Figure6).Notably, SMMC-7721 cells with overexpression of AIM2 exhibited dramaticincreasesinbothhepaticIL-1βandIL-18mRNAexpression as well as protein expression in the cell culture supernatant. In contrast, silencing of AIM2 in SMMC-7721 cells inhibited both hepatic IL-1β and IL-18 mRNAexpression as well as protein expressioninthecellculturesupernatant(Figure6).Consistentwith thechangesinhepaticIL-1βandIL-18mRNAexpression,thepro- tein levels in the cell culture supernatant were also altered. These resultswereinagreementwithAIM2exertingabiologicalrolein RFA-induced pyroptosis in HCC.

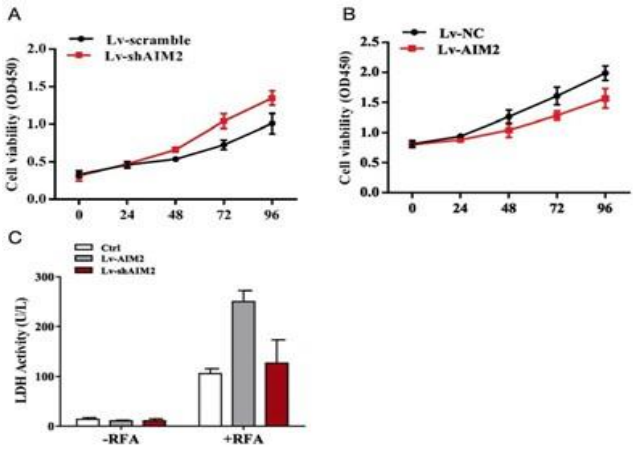

Figure 4: Effects of silencing or overexpression ofAIM2 on the proliferation of SMMC-7721 cells and LDH release.Aproliferation assay showed the effects of (A) silencing or (B) overexpression ofAIM2 on the cell viability of SMMC-7721 cells. Silencing ofAIM2 withAIM2-specific shRNA (shAIM)enhancedtheproliferationofSMMC-7721cellscomparedwiththescrambledshRNAcontrol.Bycontrast,overexpressionofAIM2withLv-AIMattenuatedtheproliferationofSMMC-7721cellsincomparisonwiththenonspecificcontrol(NC).Alactatedehydrogenase(LDH)releaseassay revealed(C)theeffectsofsilencingoroverexpressionofAIM2onthesupernatantLDHactivitywithorwithoutexposuretoRFA.Dataareexpressed as the mean ± SD. $P < 0.05$ indicated a significant difference between groups.

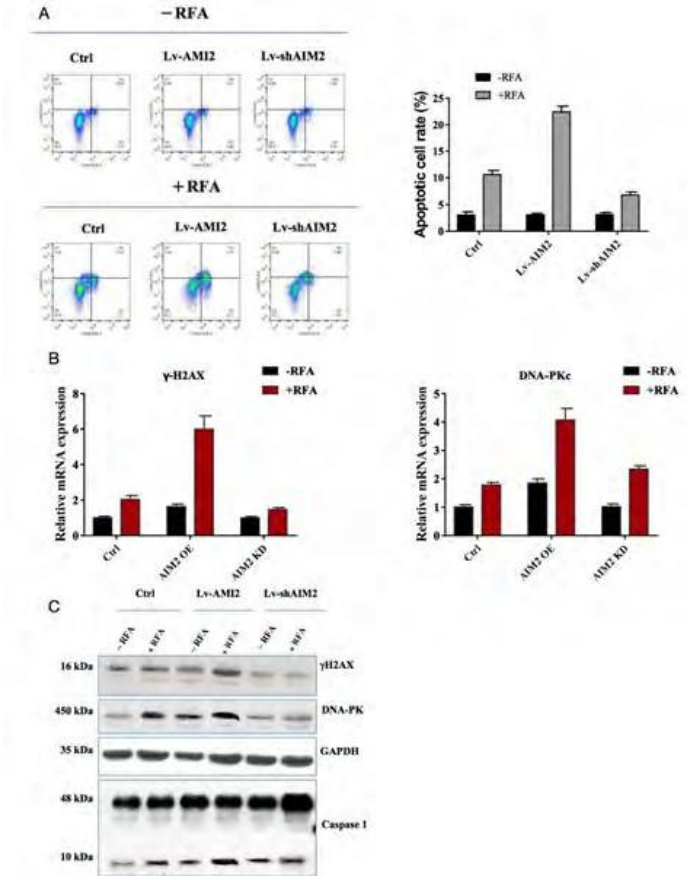

Figure 5: Effects of overexpression and silencing of AIM2 on radiofrequency ablation (RFA)-induced pyroptosis and pyroptosis-related moleculesinSMMC-7721cells.(A)EffectsofoverexpressionandsilencingofAIM2onradiofrequencyablation(RFA)-inducedpyroptosis.Pyroptosiswasde-termined by flow cytometry. Overexpression ofAIM2 with Lv-AIM induced the pyroptosis of SMMC-7721 cells in comparison with the nonspecific control (NC). By contrast, silencing ofAIM2 withAIM2-specific shRNA(shAIM) inhibited the pyroptosis of SMMC-7721 cells compared with the scrambled shRNA control. (B) RT-PCR analysis of the effects of overexpression and knockout of AIM2 on the mRNA levels of pyroptosis-related moleculesbeforeandafterRFAinSMMC-7721cells.(C)Westernblotanalysisoftheeffectsofoverexpressionandknockoutof AIM2ontheprotein levels of pyroptosis-related molecules before and after RFA in SMMC-7721 cells.

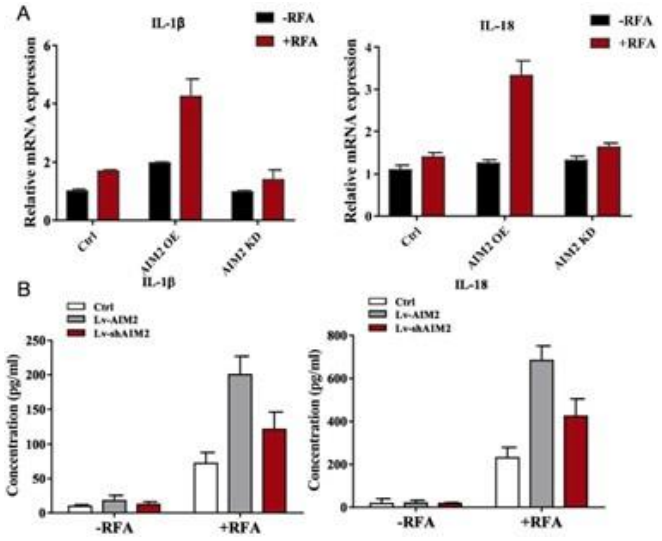

Figure 6: Effects of overexpression and silencing ofAIM2 on the cytokine levels in SMMC-7721 cells and in the cell culture supernatant. qRT-PCR analysis was conducted to measure the mRNAexpression levels of IL-1β and IL-18, and ELISAwas performed to determine the serum levels of IL-1βandIL-18inthecellculturesupernatantofSMMC-7721cells.(A)TheeffectsofoverexpressionofAIM2withLv-AIM2onthecytokinelevelsin SMMC-7721 cells and in the cell culture supernatant. (B) The effects of silencing ofAIM2 with Lv-shAIM2 on the cytokine levels in SMMC-7721 cells and in the cell culture supernatant.

5. Discussion

Themajornovelfindingsofthisstudyaresummarizedasfollows: [1]RFAtreatmentsignificantlyinhibitedtumorgrowthinBALB/c nudemicebearingHepG2orSMMC-7721cell-derivedxenografts comparedwiththecontrolsnotreceivingRFA(Figure1).[2]RFA induced pyroptotic cell death of HepG2 or SMMC-7721 cells in the xenograft nude mice, as evidenced by the fact that the levels ofAIM2,NLRP3,caspase-1,γ-H2AX,andDNA-PKcintheliver tissues were significantly elevated in the mice treated with RFA versusthoseofthecontrols(Figure2);theserumlevelsIL-1βand IL-18 were significantly greater in the HepG2 or SMMC-7721 cell-derived xenograft mice treated with RFA compared to those notreceivingRFA(Figure3);andgreatereffectswereobservedin theRFAcompleteablationgroupversusthepartialablationgroup ofxenograftnudemice(Figure2–3).(3)Functionalandmechanis-ticstudiesperformedinvitroindicatedthatAIM2exertedadirect roleinRFA-inducedpyroptosis;thesefindingsweresupportedby studies using knockout or overexpression of AIM2 (Figure 4–6). TheseresultssuggestthatRFAsuppressestheinhibitoryeffectsof RFAon the proliferation of hepatoma cells involved in the induc-tion of pyroptosis through AIM2-inflammasome signaling.

Ithasbeenwelldocumentedthathepaticinflammationrepresents a key event in the development of HCC [6]. The last decade has witnessed rapid progress in the understanding of activation of the inflammasome in the pathogenesis of HCC. For instance, it has been found that AIM2-like receptors are capable of inducing the activationofinflammasomesandtherebyfurtheractivatingcaspas- es. Once activated, caspases can mediate the generation, matura-tion,andsecretionofproinflammatorycytokines,mainlyIL-1β and IL-18.As a result, excessive secretion of these inflammatory cytokines (e.g., IL-1β and IL-18) ultimately causes a form of cell death,referredtoaspyroptosis[15-17].HBVandHCVinfections arewellknowncausesofHCC.Infact,nearly90%ofHCCcases areassociatedwithchronichepaticinflammation,forwhichHCC isagoodexampleofinflammation-relatedcancer[18,19].There- fore, inflammasome-associated molecular mechanisms have been a major focus in HCC research. In the present study, we found thatRFAtreatmentinducedcelldeathintheformofpyroptosisin nudemicebearingHepG2orSMMC-7721cell-derivedxenografts as well as in hepatoma cells. Our findings further supported that the AIM2-activated inflammasome activated caspase-1, through which it enhanced the formation and secretion of inflammatory cytokines (IL-1β and IL-18) and resulted in pyroptosis.

Numerous previous studies have shown that the hepatic AIM2 levels are significantly reduced in HCC samples compared with matched histologically normal tissues from the same patients and thatalowerAIM2expressionissignificantlycorrelatedwithmore advanced HCC [20-22]. In addition, it has been found that low-er AIM2 levels are significantly correlated with more advanced HCC, poorer tumor differentiation, and greater invasion and me-tastasis abilities, suggesting that the loss of AIM2 may contrib-ute to the progression of HCC [20, 21]. Moreover, Ma et al. have shown that the overexpression of AIM2 significantly suppressed the tumor growth in a xenograft mouse model [20]. Furthermore, thepotentialroleofAIM2inthedevelopmentofHCChasbeenre-centlyinvestigated,andtheresultsdemonstratethatgeneticsilenc-ingofAIM2orcaspase-1/11protectsmiceagainsttheoccurrence ofHCC[23,24].Ourfindingsareconsistentwiththeseprevious

studies. To date, the exact roles ofAIM2 andAIM2-activated inflammasomes in the action of RFA treatment for HCC have not been explored. Our results indicate that the inhibitory effects of RFA on the proliferation of hepatoma cells may involve the inductionofpyroptosisthroughAIM2-inflammasomesignaling.We postulated that exposure to RFAcan cause cellular damage, triggeringthereleaseofself-DNA,whichcanalsobesensedbyAIM2 andtherebyleadtotheassemblyandactivationoftheinflammas- ome complex in response to RFAbut in the absence of infection.

Our study may have some potential limitations. For instance, we found that AIM2 was markedly elevated in response to RFA in the mice bearing HepG2 or SMMC-7721 cell-derived xenografts as well as in cell cultures, but the molecular pathways through whichAIM2isupregulatedremainunknown.Weproposethat,in the absence of infection caused by pathogens, AIM2 may sense RFA-associatedDNA,assemble,activatetheinflammasomecomplex, and promote secretion of inflammatory cytokines, thus inducing pyroptosis. Given that compelling evidence of the actions of AIM2 beyond the inflammasome complex has been reported [25-30],itwouldbeinterestingtoexplorewhetherAIM2canplay aninflammasome-independentroleinHCCandRFAtreatmentfor HCC.Furtherin-depthinvestigationsontheunderlyingmolecular mechanisms are currently underway in our laboratory.

6. Conclusions

Takentogether,wefoundthatRFAsuppressedtumorgrowth,and, morenotably,RFAtreatmentinducedpyroptosisinHCC.Inaddi- tion, AIM2-mediated activation of inflammasome signaling was identified as an important cell death mechanism.Therefore, these findings advance our understanding of the biological function of AIM2,andinterventionofAIM2-meidatedinflammasomesignaling may assist in improving RFA treatment for HCC.

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