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Basic Study

Rifaximin on epigenetics and autophagy in animal model of hepatocellular carcinoma secondary to metabolic-dysfunction associated steatotic liver disease

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Abstract

BACKGROUND

Prevalence of hepatocellular carcinoma (HCC) is increasing, especially in patients with metabolic dysfunction-associated steatotic liver disease (MASLD).

AIM

To investigate rifaximin (RIF) effects on epigenetic/autophagy markers in animals.

METHODS

Adult Sprague-Dawley rats were randomly assigned ($n = 8$, each) and treated from 5-16 wk: Control [standard diet, water plus gavage with vehicle (Veh)], HCC [high-fat choline deficient diet (HFCD), diethylnitrosamine (DEN) in drinking water and Veh gavage], and RIF [HFCD, DEN and RIF (50 mg/kg/d) gavage]. Gene expression of epigenetic/autophagy markers and circulating miRNAs were obtained.

RESULTS

All HCC and RIF animals developed metabolic-dysfunction associated steatohepatitis fibrosis, and cirrhosis, but three RIF-group did not develop HCC. Comparing animals who developed HCC with those who did not, miR-122, miR-34a, tubulin alpha-1c (*Tuba-1c*), metalloproteinases-2 (*Mmp2*), and metalloproteinases-9 (*Mmp9*) were significantly higher in the HCC-group. The opposite occurred with *Becn1*, coactivator associated arginine methyltransferase-1 (*Carm1*), enhancer of zeste homolog-2 (*Ezh2*), autophagy-related factor LC3A/B (*Map1 Lc3b*), and p62/sequestosome-1 (p62/SQSTM1)-protein. Comparing with controls, *Map1 Lc3b*, *Becn1* and *Ezh2* were lower in HCC and RIF-groups ($P < 0.05$). *Carm1* was lower in HCC compared to RIF ($P < 0.05$). Hepatic expression of *Mmp9* was higher in HCC in relation to the control; the opposite was observed for p62/Sqstm1 ($P < 0.05$). Expression of p62/SQSTM1 protein was lower in the RIF-group compared to the control ($P = 0.024$). There was no difference among groups for *Tuba-1c*, Aldolase-B, alpha-fetoprotein, and *Mmp2* ($P > 0.05$). miR-122 was higher in HCC, and miR-34a in RIF compared to controls ($P < 0.05$). miR-26b was lower in HCC compared to RIF, and the inverse was observed for miR-224 ($P < 0.05$). There was no difference among groups regarding miR-33a, miR-143, miR-155, miR-375 and miR-21 ($P > 0.05$).

CONCLUSION

RIF might have a possible beneficial effect on preventing/delaying liver carcinogenesis through epigenetic modulation in a rat model of MASLD-HCC.

Key Words: Animal model; Autophagy; Epigenetic; Hepatocellular carcinoma; Metabolic dysfunction-associated steatotic liver disease; Rifaximin

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Core Tip: Managing metabolic dysfunction-associated steatotic liver disease (MASLD)-hepatocellular carcinoma (HCC) is a clinical challenge, with many unanswered questions, as autophagy and epigenetics appear to contribute to drug resistance. Additionally, the broad-spectrum oral antibiotic Rifaximin influences inflammation, energy metabolism, and fat storage. Utilizing animal models for MASLD-HCC is crucial in understanding pathophysiological mechanisms and potential therapeutic targets. Furthermore, Rifaximin may have a beneficial effect in rats by possibly preventing or delaying hepatic carcinogenesis through epigenetic modulation.

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INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) comprises a spectrum of histological abnormalities, ranging from isolated steatosis to steatohepatitis, characterized by inflammation, necrosis, and hepatocellular ballooning, and progression to fibrosis, cirrhosis, liver failure and/or hepatocellular carcinoma (HCC)[1,2]. Along with diet and sedentary lifestyle, many other factors often determine the progression of MASLD and the development of MASLD-associated carcinogenesis[3]. Although only 2.4% to 12.4% of cirrhotic MASLD patients develop HCC, it is expected that it will become the leading HCC cause by 2030[4,5].

Several MASLD-associated oncogenesis mechanisms, such as structural genomic defects, epigenetic alterations and autophagy promote significant changes in regulatory and signaling pathways, creating a favorable hepatic microenvironment for lesion progression and HCC development, which can occur with or without cirrhosis[6-8]. Among the epigenetic mechanisms, microRNAs act on the expression or suppression of genes responsible for the worsening of liver damage, that is, they have the potential to be used as new biomarkers for early diagnosis and/or therapeutic targets to HCC[8-10]. Autophagy is recognized for playing a beneficial role in the initial liver injury by contributing to the removal of protein aggregates, damaged organelles, and lipid droplets, preventing the formation of pre-tumor cells[6,7]. However, during tumor promotion, autophagy predominantly acts on the adaptive mechanism, contributing to tumor maintenance and growth through the supply of energy substrates and metabolic adaptation, which improves their survival ability in hypoxic and low-nutrient environments, promoting tumor progression[6,11]. Autophagy tends to play a complicit role in HCC treatment resistance, the interface between these two processes being multifactorial and therefore crosstalk can occur in different target proteins. In this sense, therapies for the epigenetic control of autophagy are promising targets for treatment of liver injury[6,11].

Currently, there is no approved pharmacological therapy for steatohepatitis, and several studies are being conducted with different targets. Dietary management, such as flavonoids, has shown beneficial effects on the epigenetic mechanisms of MASLD-HCC[12]. On the other hand, although microbiota plays an important role in MASLD pathogenesis, there is no definite evidence of a positive effect of its modulation in human steatohepatitis[13,14]. Rifaximin (RIF) is a minimally absorbed, broad-spectrum oral antibiotic that have a positive modulation on components of gut microbiota, improving endotoxemia, exerting an influence on inflammation, energy metabolism and fat storage[14-16]. Therefore, this study was designed to assess the effect of RIF treatment on the underlying pathophysiological mechanisms associated with epigenetic changes and autophagy in MASLD associated hepatocarcinogenesis.

MATERIALS AND METHODS

Animals and ethical procedures

Twenty-four adult (60-d-old) male Sprague Dawley rats weighing 250–400 g was included for this study. The animals were group-housed in polypropylene cages with sawdust-covered floors. Rats were maintained on a standard 12-h light/dark cycle, in a temperature-controlled environment (22 ± 2 °C). The Institutional Ethics Committee approved all experiments and procedures for the Use of Animals, No. 2021-0105. The procedures for scientific animal's use were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed, 2011) and law No. 11.794 (Brazil, 2008).

Study design

After acclimatization to the environment, the animals were randomized by weight into a control group ($n = 8$) that received a standard diet, water free of diethylnitrosamine (DEN, catalog number N0756, Sigma-Aldrich, United States) and gavage with vehicle (Veh) solution from the 5th week of the experiment until the 16th week; HCC-group ($n = 8$) that received a high-fat and choline-deficient diet (HFCD, catalog number RH19576, Rhooster, Brazil), 135 mg/L DEN in drinking water and gavage with Veh solution, during the same period previously described; and the RIF-group ($n = 8$) that received HFCD diet plus DEN and RIF (catalog number R9904, Sigma-Aldrich, United States) by gavage from the 5th week of the experiment until the 16th week of the experiment. The DEN dose and the experimental period for the development of this protocol were based on a previous study, which combine a dietary model of fatty liver based on high trans-fat with exposure to a known hepatic carcinogen as a means of provoking and accelerating more severe injury. The model replicated many features of MASLD including steatohepatitis with ballooning, fibrosis, cirrhosis, and HCC[17]. The experimental study design is presented in Figure 1. The animals were weighed twice a week during the experimental period. After 16 wk of the experiment, all the animals were euthanized by cardiac exsanguination. Serum samples and liver fragments were collected aseptically, frozen in liquid nitrogen, and stored in an ultra-freezer at -80 °C until the experimental procedures were carried out. A portion of each liver sample was fixed in 10% formalin for histological analysis.

Experimental diets

Animals in the control group received a standard rodent diet (Nuvilab CR-1; Quimtia SA, Brazil) with an energy value of 2.93 kcal/g. The diet consisted of 55.0% carbohydrates, 22.0% protein, 4.5% fat, and 18.5% of other nutrients such as fibers and vitamins. Animals in the intervention groups received an HFCD diet with an energy value of 4.3 kcal/g. This product consisted of 54.5% carbohydrates, 14.0% protein, and 31.5% fat (enriched with 54.0% trans fatty acids). The diet of the intervention group was chosen as such because it mirrors many of the phenotypes observed in humans with MASLD, as previously demonstrated by our research group[18]. The diet offered to the animals in the control and intervention

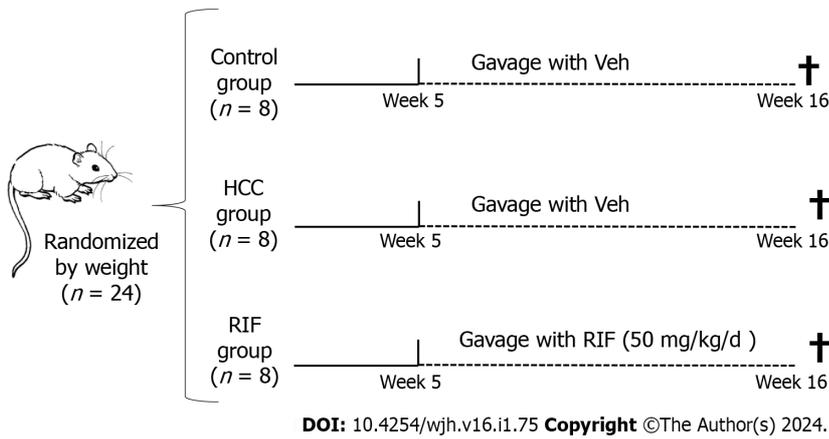


Figure 1 Experimental design. The control group ($n = 8$) received a standard diet, water without diethylnitrosamine (DEN) and gavage with vehicle (Veh) solution; hepatocellular carcinoma-group ($n = 7$) received a high-fat and choline-deficient (HFCD) diet, water with DEN and gavage with Veh solution; rifaximin (RIF)-group ($n = 7$) received a HFCD diet, water with DEN and gavage with RIF. After all the animals were euthanized. DEN: Diethylnitrosamine; HCC: Hepatocellular carcinoma; HFCD: High-fat and choline-deficient; RIF: Rifaximin; Veh: Vehicle.

groups was replaced every two days. Both the groups received water and food *ad libitum* during the experimental period.

Rifaximin treatment

The therapeutic intervention through the administration of daily gavage with RIF or the offer of Veh solution, also by gavage, in the respective experimental groups occurred daily from the 5th week of the experiment until the date of euthanasia. The animals in the control group and HCC-group received daily gavage with a Veh solution (0.5 mL/kg distilled water) in order to undergo the same stress conditions as those in the RIF-group. The RIF-group received a daily dose of 50 mg/kg/d of RIF by daily gavage from the 5th week of the experiment until the 16th week of the experiment. The dose of RIF used was in accordance with a previous study published in the literature[19].

Assessment gene expression of hepatocarcinogenesis and autophagy markers

The total RNA was extracted from fragments of the liver tissue using TRIzol (catalog number 15596026, Invitrogen, United States). The cDNA conversion, from 2 µg of RNA, was performed using the High-capacity cDNA Reverse Transcription kit (catalog number 4368814, Applied Biosystems, United States). Quantitative (q) real-time polymerase chain reaction (RT-PCR) with TaqMan assay (Applied Biosystems, United States) was used to assess the gene expression of aldolase-B (*Aldob*), tubulin alpha-1c (*Tuba1c*), alpha-fetoprotein (*Afp*), metalloproteinases-2 (*Mmp2*), *Mmp9*, autophagy-related factor LC3A/B (*Map1 Lc3b*), *p62/sequestosome-1 (p62/Sqstm1)*, beclin-1 (*Becn1*), enhancer of zeste homolog-2 (*Ezh2*) and coactivator associated arginine methyltransferase-1 (*Carm1*) in liver tissue. The *Actb* gene was used as a normalizer in both tissues. The probes used are described in [Supplementary Table 1](#). Values were calculated with the formula $2^{-\Delta\Delta Ct}$.

Analysis of the circulating microRNAs

To analyze the circulating microRNAs from serum, total RNA was extracted using the miRNeasy serum/plasma kit (catalog number 217184, Qiagen, United States). Then, cel-miR-39 (1.6×10^8 copies) spike in control (catalog number 21961, Qiagen, United States) was added to provide an internal reference. cDNA conversion was performed from 10 ng of total RNA using the TaqMan microRNA Reverse Transcription kit (catalog number 4366597, Applied Biosystems, United States). Analysis of the gene expression of miR-122, miR-34a, miR-26b, miR-224, miR-33a, miR-143, miR-155, miR-375 and miR-21, together with the cell-miR-39 normalizer, was performed by RT-qPCR using TaqMan assay (Applied Biosystems, United States). The sequences and codes of the assessed microRNAs are described in [Supplementary Table 1](#). Values were calculated by formula $2^{-\Delta\Delta Ct}$.

Analysis of protein expression of hepatic autophagy markers

For western blotting analysis, protein extraction was performed on samples of liver tissue from rats. The samples were homogenized in a solution containing Triton X-100, β-mercaptoethanol, tris-buffered saline (TTBS), ethylenediaminetetraacetic acid and proteases inhibitor cocktail (catalog number 29131, Santa Cruz Biotechnology, United States). The samples were then normalized to 40 µg of protein. Subsequently, proteins were separated by electrophoresis using a 12% w/v polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was washed with TTBS, and then incubated for one hour in a blocking solution containing 3% bovine serum albumin in TTBS. Following the blocking step, the membrane was washed three times with TTBS and incubated overnight at 4 °C in a blocking solution containing the following primary antibodies: Anti-actin (catalog number A5060, Sigma-Aldrich, United States), anti-LC3B (catalog number ab128025, Abcam, United Kingdom), and anti-SQSTM1 (catalog number ab56416, Abcam, United Kingdom). The primary antibodies were used at a dilution of 1:1000. After the overnight incubation, the membrane was washed three times with TTBS and then incubated for two hours in a solution containing a horseradish peroxidase-conjugated anti-IgG

secondary antibody in TBBS, at a concentration of 1:2000. For band detection, Clarity Western ECL Substrate (catalog number 1705062, BioRad, United States) was used, and the resulting image was captured using an ImageQuant LAS 500 (GE Healthcare Lifesciences) imaging system. Band intensities were quantified using the ImageJ software, and actin was used as a constitutive protein reference.

Correlations between the analyzed markers

For this analysis, in relation to markers related to hepatocarcinogenesis we selected *Mmp2*, *Mmp9*, *Afp*, *Tuba1c* and *Aldob* and associated with the autophagy and epigenetic process we selected *Becn1*, *p62/Sqstm1*, *Map1 Lc3b*, *Ezh2*, *Carm1*, p62 (protein ratio), and LC3B (protein ratio). Regarding microRNAs, we selected miR-122, miR-26b, miR-224 and miR-34a, which are markers related to liver disease and which showed a significant difference between the experimental groups.

Histopathological analysis

Formalin-fixed liver tissue samples were embedded in paraffin and stained with hematoxylin and eosin (H&E) and picrosirius red. Histopathological lesions of the different evolutionary stages of nonalcoholic fatty liver disease were assessed according to the score by Liang *et al*[20], which is a highly reproducible scoring system applicable to experimental rodent models[20]. This scoring system was developed through the assessment of various experimental models, aiming to establish generic criteria for analysis. To validate the proposed scoring, biological material from rodents was evaluated by blinded pathologists in two separate assessments, with an interval of over 3 mo between them. In this validation, observers estimated the percentage of macrovesicular steatosis, microvesicular steatosis, hypertrophy, and the number of inflammatory foci per field[20]. The degree of fibrosis was evaluated using the slides stained with picrosirius red, and cancerous lesions were graded according to the Edmondson & Steiner classification[21]. The analysis was performed by an experienced pathologist, who was blinded to the experimental groups.

Sample size calculation and statistical analysis

The sample size estimation was performed using the WINPEPI 11.20 software (Brixton Health, Israel), based on a previously published study by the research group demonstrating HCC development in an experimental model[17]. Considering a power of 80% and a significance level of 5%, it was determined that 8 animals per experimental group would be required for conducting this study. The outcome used for the calculation was the prevention of HCC.

Normality was verified for all variables using Shapiro-Wilk test and histograms. Nonparametric data were analyzed using the Kruskal-Wallis followed by Dunn test. Quantitative variables were expressed as median and interquartile ranges (25th-75th) and percentage. Spearman's correlation coefficient was performed, with a moderate ($0.3 < r < 0.6$), strong ($0.6 < r < 0.9$) or very strong ($0.9 < r < 1.0$) correlation were adopted. Statistical significance was set at $P < 0.05$. Data were analyzed using the Statistical Package for Social Sciences (version 28.0; SPSS Inc., United States).

RESULTS

During the 16 wk of the experiment, one animal from the HCC-group and another from the RIF-group died, totaling 7 animals per experimental group. There were no deaths in the control group ($n = 8$) during the study period.

Expression of genes involved in hepatic disease pathogenesis

The data of gene expression of hepatocarcinogenesis markers are shown in Table 1. There was no difference between the experimental groups for the gene expression of *Tuba1c* ($P = 0.839$), *Aldob* ($P = 0.595$), *Afp* ($P = 0.837$) and *Mmp2* ($P = 0.101$). There was a significant difference between the experimental groups in the gene expression of *Mmp9* ($P = 0.035$). For this marker, there was a significant increase in its expression in the HCC-group compared to the control group ($P = 0.013$). However, there was no significant difference in the expression of *Mmp9* between the HCC-group and RIF-group ($P = 0.071$).

Data obtained for the gene expression of markers of autophagy are shown in Figure 2. There was a significant decrease in *Map1 Lc3b* gene expression in the HCC and RIF-groups compared to the control group ($P = 0.002$ and $P = 0.001$, respectively). There was no significant difference in *p62/Sqstm1* expression in the RIF-group compared to the control ($P = 0.078$) and HCC-group ($P = 0.890$); however, the HCC-group showed a significant decrease in its expression compared to the control ($P = 0.010$). *Becn1* and *Ezh2* showed a significant decrease in their expression in the HCC-group ($P = 0.001$ and $P < 0.001$, respectively) and RIF-group ($P = 0.009$ and $P = 0.010$, respectively) compared to the control. There was a significant reduction in *Carm1* expression in the HCC-group compared to the RIF-group ($P = 0.004$).

Gene expression of the circulating microRNAs

The results obtained from the gene expression of the circulating microRNAs related to liver damage are demonstrated in Table 1. There was a significant increase in miR-122 gene expression in the HCC-group compared to the control group ($P < 0.001$), however the RIF-group did not differ significantly from the control ($P = 0.118$). There was a significant difference between the experimental groups in the gene expression of miR-34a ($P = 0.007$). The expression of miR-34a was significantly lower in the RIF-group compared to the control ($P = 0.005$). There was a significant difference between the experimental groups in the gene expression of miR-26b ($P = 0.026$). The HCC-group showed a significant decrease in miR-26b expression compared to the RIF-group ($P = 0.008$), which was like the control ($P = 0.469$). The gene expression of miR-224 was significantly higher in the HCC-group compared to the RIF-group ($P < 0.001$), which is like the control

Table 1 Gene expression of markers related to hepatocarcinogenesis

Variables ¹	Control (n = 8)	HCC (n = 7)	RIF (n = 7)	P value
<i>Tuba1c</i>	1.28 (0.18-2.76)	4.27 (0.00-9.69)	1.38 (0.29-19.8)	0.839
<i>Aldob</i>	2.46 (0.01-5.78)	4.24 (0.00-9.92)	0.12 (0.00-2.69)	0.595
<i>Afp</i>	1.28 (0.18-2.76)	2.33 (0.00-9.69)	1.38 (0.29-19.8)	0.837
<i>Mmp2</i>	1.81 (0.03-5.15)	31.4 (0.25-62.2)	0.92 (0.16-18.4)	0.101
<i>Mmp9</i>	2.36 (0.02-4.25) ^a	23.6 (2.69-76.8) ^b	14.6 (1.32-28.7) ^{a,b}	0.035
miR-122	1.14 (0.34-2.34) ^a	7.07 (1.41-11.8) ^b	2.62 (1.20-6.00) ^{a,b}	0.005
miR-34a	0.94 (0.63-1.70) ^b	0.39 (0.33-0.44) ^a	0.33 (0.13-0.89) ^a	0.007
miR-26b	1.04 (0.61-1.57) ^{a,b}	0.36 (0.21-1.85) ^a	2.22 (1.46-2.96) ^b	0.026
miR-224	1.05 (0.41-2.45) ^{a,b}	2.97 (1.96-5.94) ^b	0.58 (0.13-0.85) ^a	0.005
miR-33a	0.98 (0.53 - 2.16)	0.60 (0.54-1.17)	1.4 (0.70-1.60)	0.445
miR-143	1.18 (0.25-3.67)	1.40 (0.54-5.16)	0.62 (0.17-2.54)	0.471
miR-155	1.46 (0.32-1.96)	0.63 (0.01-1.18)	0.34 (0.04-1.46)	0.074
miR-375	0.79 (0.61-2.42)	0.85 (0.17-1.09)	0.38 (0.14-1.76)	0.216
miR-21	1.12 (0.34-2.13)	1.49 (0.91-6.08)	1.69 (0.84-3.68)	0.188

¹Variables expressed by median (25th-75th percentiles).

^aP < 0.05. Different letters indicate a significant difference between groups.

^bP < 0.05. Different letters indicate a significant difference between groups.

HCC: Hepatocellular carcinoma; RIF: Rifaximin; *Afp*: Alpha-fetoprotein; *Aldo-B*: Aldolase-B; *Mmp*: Metalloproteinases; *Tuba1c*: Tubulin alpha-1c.

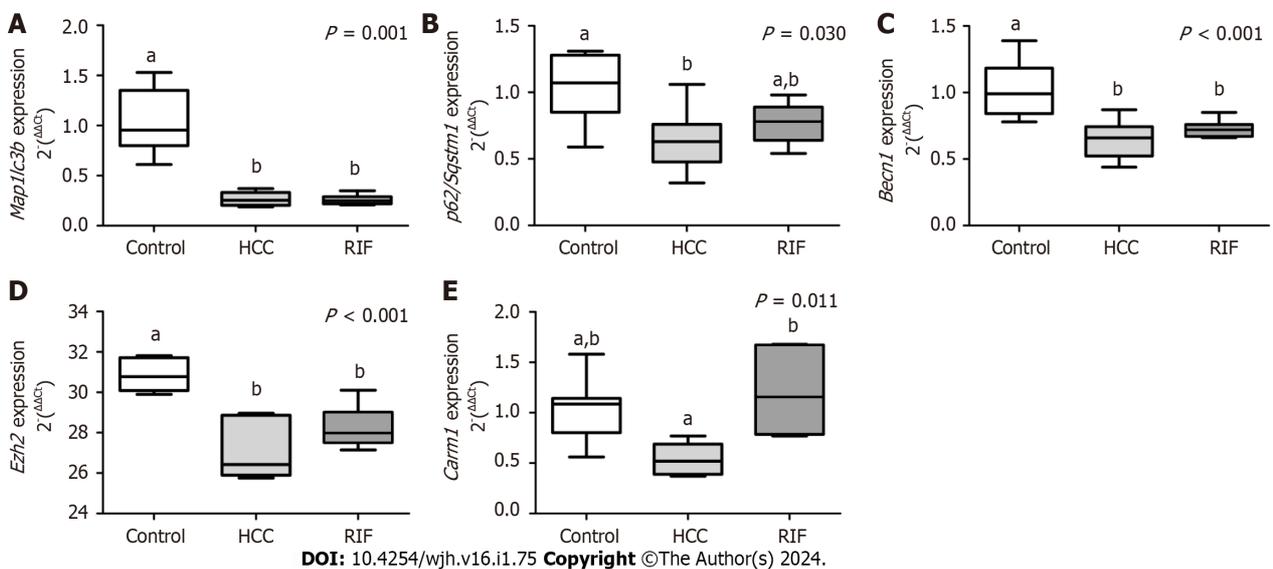


Figure 2 Hepatic gene expression of autophagy markers. A: Autophagy-related factor LC3A/B; B: P62/sequestosome-1; C: Beclin-1; D: Enhancer of zeste homolog-2; E: Coactivator associated arginine methyltransferase-1. Data expressed as median (25th-75th percentile), Kruskal-Wallis test. Different letters indicate a significant difference between groups ($P < 0.05$). ^a $P < 0.05$, ^b $P < 0.05$. Different letters indicate a significant difference between groups. *Becn1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh2*: Enhancer of zeste homolog-2; HCC: Hepatocellular carcinoma; *Map1 Lc3b*: Autophagy-related factor LC3A/B; *p62/Sqstm1*: Sequestosome-1; RIF: Rifaximin.

group ($P = 0.099$). There was no significant difference between groups in the gene expression of miR-33a ($P = 0.445$), miR-143 ($P = 0.471$), miR-155 ($P = 0.074$), miR-375 ($P = 0.216$) and miR-21 ($P = 0.188$).

Protein expression of autophagy markers

The data obtained from the protein expression analysis of p62/SQSTM1 and MAP1LC3B, conducted through the Western blot technique, are represented in **Figure 3**. A difference in the expression of the MAP1LC3B protein between the experimental groups was observed ($P = 0.024$). A significant decrease in its expression was noted in the RIF-group animals

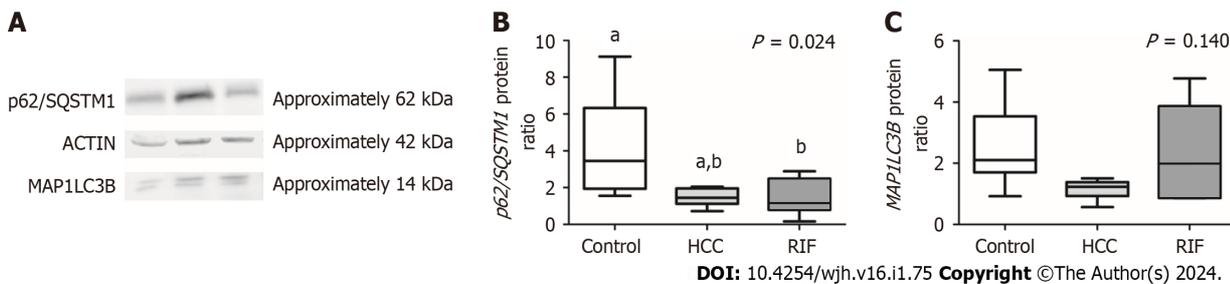


Figure 3 Protein concentration of autophagy markers. A: Protein expression bands obtained through the Western blot technique; B: p62/SQSTM1 expression (densitometry analysis); C: MAP1LC3B expression (densitometry analysis). Data expressed as median (25th-75th percentile), Kruskal-Wallis test. Different letters indicate a significant difference between groups ($P < 0.05$). ^a $P < 0.05$, ^b $P < 0.05$. Different letters indicate a significant difference between groups. HCC: Hepatocellular carcinoma; MAP1LC3B: Autophagy-related factor LC3A/B, p62/SQSTM1: Sequestosome-1; RIF: Rifaximin.

compared to the control group ($P = 0.039$). No significant differences in MAP1LC3B expression were found between the animals receiving RIF treatment and the animals in the HCC-group ($P > 0.05$). Regarding the protein expression of p62/SQSTM1, no significant differences were observed between the experimental groups ($P = 0.140$).

Correlations between hepatocarcinogenesis, autophagy and epigenetic markers

The values obtained from the correlations between markers of hepatocarcinogenesis, autophagy and epigenetic are described in Table 2. There was a negative correlation between the expression of miR-122 and miR-34a, both related to the severity of liver injury compared to the autophagy markers *Map1 Lc3b*, *p62/Sqstm1* and *Becn1* that act in the development of the autophagosome. Additionally, this negative correlation was also demonstrated between these microRNAs and *Ezh2*, an enzyme that modulates the expression of autophagy genes. There was a positive correlation between the three autophagy indicators (*Map1 Lc3b*, *p62/Sqstm1* and *Becn1*), however there was no correlation between *Ezh2* and *Carm1*, demonstrating that these enzymes that perform epigenetic control of autophagy act in different ways. *Ezh2* positively correlated with *Map1 Lc3b*, *p62/Sqstm1* and *Becn1*. While the correlations were strong between the autophagy markers *Map1 Lc3b*, *p62/Sqstm1* and *Ezh2*, it was only moderate with *Becn1*. As *Ezh2* can catalyze the methylation of lysine to histone (H3K27), which can decrease the expression of target genes, an attempt was made to assess whether *Becn1* would be on the target of *Ezh2*-H3K27 axis. The result was described in Supplementary Figure 1. Metalloproteinases were positively correlated with the expression of *Afp* and *Tuba1c*, markers related to hepatocarcinogenesis. *Aldob* expression was positively correlated with *Mmp2*, *Afp* and *Tuba1c*. The protein expression of p62 correlated positively with the gene expression of the markers *Becn1*, *p62/Sqstm1*, and *Map1 Lc3b*.

Liver histopathological analysis

No hepatic histopathological changes were observed in the liver tissue of the control group (Figure 4A and B). The animals in the HCC-group had predominantly macrovesicular steatosis along with microvesicular steatosis of moderate intensity, with inflammatory activity and mild hypertrophy (Figure 4C and D). The RIF-group presented mild macrovesicular and microvesicular steatosis, no inflammation was observed and in only two animals the presence of hypertrophy was evidenced. In the staging of the histopathological lesion, seven animals in the HCC-group and RIF-group developed steatohepatitis (Table 3). In the evaluation of hepatic fibrosis, through H&E and picrosirius red staining respectively, five animals from the HCC-group and RIF-group developed fibrosis with multiple septa without the presence of cirrhosis and two animals from both experimental groups developed liver cirrhosis (Figure 4E and F). The breakdown by experimental group observed for microvesicular steatosis, macrovesicular steatosis, hypertrophy, inflammation, fibrosis, and HCC as a percentage is shown in Figure 4G.

In the evaluation of the tumor classification by the Edmondson & Steiner score, we reported a lesion grade three and four, corresponding to the presence of poorly differentiated and undifferentiated cancer, respectively, in the HCC-group. This finding was replicated for some animals in the RIF-group; however, three animals did not develop cancer.

Comparison between animals that developed or not hepatocellular carcinoma

Additionally, to better detail the results obtained between the autophagy and epigenetics markers with the hepatic histopathological results, we performed the subdivision of the experimental groups. Initially, we stratified the RIF-group, and performed a comparison analysis between the animals that developed HCC ($n = 4$) and those that did not develop HCC ($n = 3$). No significant differences ($P > 0.05$) were observed in gene expression of microRNAs, markers of hepatocarcinogenesis and autophagy between animals that developed or did not develop HCC in the RIF-group, as detailed in Table 4.

Subsequently, we carried out a new stratification of the experimental groups to compare the results obtained between the animals that developed HCC and those that did not develop HCC. The comparison of results is detailed in Table 5. In total, eleven animals developed HCC and eleven did not develop this clinical condition at the end of the study. Regarding epigenetic markers, we reported a significant increase in the gene expression of miR-122 ($P = 0.029$) and miR-34a ($P = 0.012$) in animals with HCC compared to animals without HCC. There was a significant increase in the gene expression of *Mm2* ($P = 0.017$), *Mm9* ($P = 0.013$) and *Tuba1c* ($P = 0.017$), markers related to hepatic hepatocarcinogenesis, in animals with HCC compared to those that did not develop HCC. Regarding autophagy markers, there was a significant reduction in

Table 3 Distribution of liver histopathological findings according to general non-alcoholic fatty liver disease scoring system for rodent models, *n* (%)

Variable ¹	Control (<i>n</i> = 8)	HCC (<i>n</i> = 7)	RIF (<i>n</i> = 7)
No MASLD	8 (100)	0 (0.0)	0 (0.0)
MASLD	0 (0.0)	0 (0.0)	0 (0.0)
MASH	0 (0.0)	7 (100)	7 (100)

¹Variables described as frequency (%).

HCC: Hepatocellular carcinoma; MASLD: Metabolic-dysfunction associated steatotic liver disease; MASH: Metabolic-dysfunction associated steatohepatitis; RIF: Rifaximin.

Table 4 Comparison between animals in the rifaximin-group that developed or not hepatocellular carcinoma

Variables ¹	Developed HCC (<i>n</i> = 4), median (min–max)	Did not developed HCC (<i>n</i> = 3), median (min–max)	<i>P</i> value
miR-122	2.27 (1.20–4.57)	2.97 (1.51–5.98)	0.571
miR-375	0.42 (0.14–1.76)	0.33 (0.30–0.67)	1.000
miR-26b	1.97 (1.10–3.38)	2.41 (1.97–3.99)	0.571
miR-224	0.46 (0.13–0.85)	0.58 (0.46–0.82)	0.857
miR-34	11.0 (7.17–18.9)	10.0 (8.64–10.5)	0.786
miR-21	2.36 (0.84–3.68)	1.68 (1.00–1.69)	0.700
miR-143	0.76 (0.17–2.53)	0.48 (0.25–1.96)	1.000
miR-155	0.69 (0.06–1.46)	0.13 (0.03–0.36)	0.250
miR-33a	0.88 (0.52–1.86)	1.55 (1.40–1.58)	0.400
<i>Mm2</i>	7.95 (0.58–18.4)	0.71 (0.16–0.92)	0.229
<i>Mm9</i>	24.0 (1.32–28.7)	8.48 (1.73–14.6)	0.400
<i>Afp</i>	6.99 (1.38–19.8)	0.46 (0.29–0.48)	0.057
<i>Tuba1c</i>	4.63 (0.30–7.12)	0.26 (0.25–0.30)	0.057
<i>Aldob</i>	2.32 (0.11–2.69)	0.07 (0.00–0.12)	0.114
<i>Becn1</i>	0.70 (0.67–0.85)	0.74 (0.66–0.76)	1.000
<i>p62/Sqstm1</i>	0.84 (0.54–0.98)	0.72 (0.64–0.79)	0.629
<i>Map1lc3b</i>	0.24 (0.22–0.35)	0.27 (0.21–0.29)	1.000
<i>Ezh2</i>	29.0 (28.0–30.1)	27.5 (27.2–28.0)	0.114
<i>Carm1</i>	0.79 (0.77–1.67)	1.23 (1.08–1.68)	0.400
p62/SQSTM1 protein	1.07 (0.16–2.89)	1.78 (1.19–2.36)	0.355
MAP1LC3B protein	2.00 (0.87–4.78)	2.20 (0.86–3.57)	0.643

¹Variables expressed by median (25th–75th percentiles).

P < 0.05 is considered significant.

Aldob: Aldolase B; *Afp*: Alpha-fetoprotein; *Becn1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh2*: Enhancer of zeste homolog-2; HCC: Hepatocellular carcinoma; *Map1lc3b*: Autophagy-related factor LC3A/B; *Mmp*: Metalloproteinases; *p62/Sqstm1*: P62/sequestosome-1; RIF: Rifaximin; *Tuba1c*: Tubulin alpha 1c.

DISCUSSION

In this study, carried out in an experimental model of HCC secondary to MASLD, with the objective of evaluating the effect of treatment with RIF in relation to autophagy and epigenetic markers, we demonstrated: (1) A positive correlation between *Map1lc3b*, *p62/Sqstm1* and *Becn1* in HCC, however, there was no significant difference in the expression of these markers between the HCC-group and RIF-group; (2) a lack of correlation between *Ezh2* and *Carm1*, demonstrating that these enzymes regulate different signaling pathways, although RIF promoted a significant increase in *Carm1* expression;

Table 5 Comparison between the animals that developed and did not develop hepatocellular carcinoma

Variables ¹	HCC (n = 11), median (min–max)	No HCC (n = 11), median (min–max)	P value
miR-122	4.49 (1.20–11.8)	1.41 (0.34–5.98)	0.029 ^a
miR-375	0.64 (0.14–1.76)	0.72 (0.30–2.42)	0.512
miR-26b	1.10 (0.46–3.38)	1.23 (0.59–3.99)	0.656
miR-224	1.40 (0.13–5.94)	0.82 (0.41–2.45)	0.545
miR-34a	7.73 (2.29–18.9)	1.79 (0.14–10.5)	0.012 ^a
miR-21	1.92 (0.84–6.08)	1.46 (0.34–2.13)	0.109
miR-143	1.08 (0.17–5.16)	1.13 (0.25–3.67)	0.756
miR-155	0.66 (0.01–1.46)	0.60 (0.03–1.95)	0.349
miR-33a	0.66 (0.52–1.86)	1.40 (0.34–2.64)	0.200
<i>Mm2</i>	18.4 (0.25–62.2)	1.17 (0.03–5.15)	0.017 ^a
<i>Mm9</i>	23.6 (1.32–76.8)	2.76 (0.02–14.6)	0.013 ^a
<i>Afp</i>	3.23 (0.00–19.8)	0.62 (0.18–2.76)	0.190
<i>Tuba1c</i>	7.12 (0.12–27.8)	0.76 (0.12–3.71)	0.017 ^a
<i>Aldob</i>	2.32 (0.00–9.92)	1.35 (0.00–5.78)	0.549
<i>Becn1</i>	0.68 (0.44–0.87)	0.88 (0.66–1.39)	0.004 ^a
<i>p62/Sqstm1</i>	0.65 (0.32–1.06)	0.92 (0.59–1.31)	0.052
<i>Map1lc3b</i>	0.25 (0.19–0.37)	0.86 (0.21–1.53)	0.004 ^a
<i>Ezh2</i>	28.4 (25.8–30.1)	30.2 (27.2–31.8)	0.010 ^a
<i>Carm1</i>	0.69 (0.37–1.67)	1.10 (0.56–1.68)	0.026 ^a
p62/SQSTM1 protein	1.27 (0.16–2.39)	2.87 (1.19–9.12)	0.013 ^a
MAP1LC3B protein	1.29 (0.57–4.78)	2.10 (0.86–5.05)	0.183

¹Variables expressed by median (25th–75th percentiles).

^aP < 0.05 is considered significant.

Aldob: Aldolase B; *Afp*: Alpha-fetoprotein; *Becn1*: Beclin-1; *Carm1*: Coactivator associated arginine methyltransferase-1; *Ezh2*: Enhancer of zeste homolog-2; HCC: Hepatocellular carcinoma; *Map1lc3b*: Autophagy-related factor LC3A/B; *Mmp*: Metalloproteinases; *p62/Sqstm1*: P62/sequestosome-1; RIF: Rifaximin; *Tuba1c*: Tubulin alpha 1c.

(3) a significant increase in miR-26b expression in the RIF-group compared to the HCC-group; the inverse was observed for miR-224; (4) a negative correlation between the expression of miR-122 and miR-34a related to liver injury with *Map1lc3b*, *p62/Sqstm1* and *Becn1*; and (5) finally, steatohepatitis developed in all animals of the HCC-group and RIF-group, but three animals treated with RIF did not develop HCC.

RIF, a semisynthetic derivative of rifamycin, has an intestinal absorption of approximately 0.007%, which prevents it from exerting systemic or adverse effects[13,16]. It has broad-spectrum *in vitro* activity against aerobic and anaerobic enteric bacteria and has been used in the treatment of hepatic encephalopathy, traveler's diarrhea, and irritable bowel syndrome[13,22]. Recent studies report that RIF inhibited hepatic fibrosis in rodent models of alcoholic liver injury and steatohepatitis[23,24]. In clinical practice, its use seems to be effective and safe in MASLD, promoting the reduction of serum endotoxemia, improvement of insulin resistance, inflammatory process, and histopathological score[13,16]. To our knowledge, no studies evaluate the effect of RIF on the progression from MASLD to HCC in relation to autophagy and epigenetic markers. We emphasize that the treatment with RIF in the animals started from the fifth week of the experiment when the animals probably already had steatohepatitis, as demonstrated in previous studies[17]. In the histopathological evaluation, all animals in the HCC and RIF groups developed steatohepatitis; however, three animals treated with RIF did not develop HCC. This interesting finding continues to be investigated by our research group and we seek to understand the role of RIF in this process. A multicenter, double-blind, randomized, placebo-controlled study in patients with biopsy-proven steatohepatitis investigated the effects of daily administration of RIF for six months[13]. The results obtained demonstrated that patients treated with RIF showed a significant decline in cytokeratin-18 Levels, a biomarker capable of predicting the histopathological manifestations of steatohepatitis[13]. However, Cobbold *et al*[14] reported that RIF therapy in patients with steatohepatitis for six weeks caused no changes in liver triglyceride content, insulin sensitivity, or systemic inflammation[14]. Due to the lack of studies evaluating the effect of RIF on HCC secondary to steatohepatitis, further studies are needed in the search for new therapies for the prevention of disease progression and future clinical application.

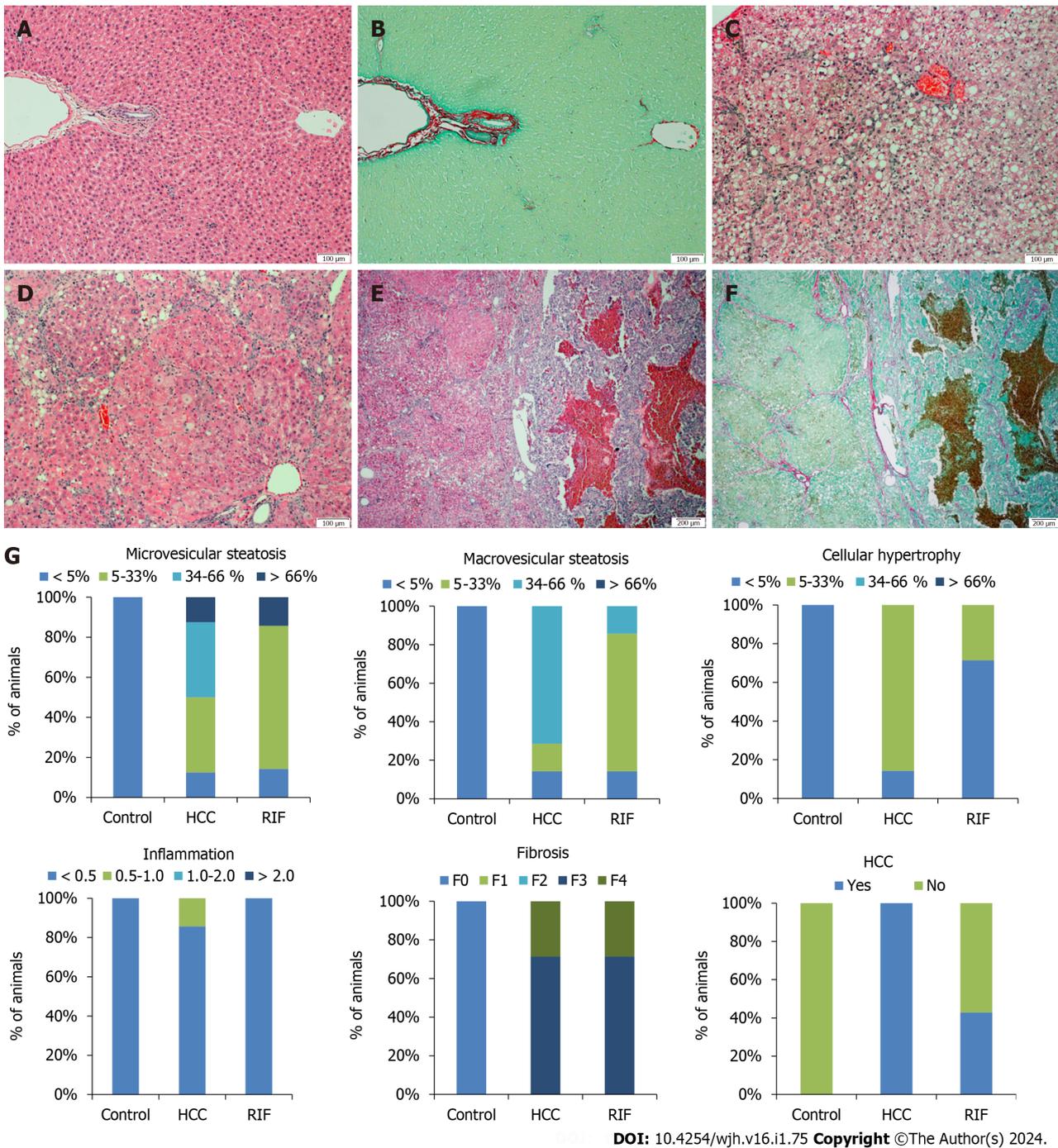


Figure 4 Histological evaluation of hepatic tissue in different experimental groups and percentage description for the presence of microvesicular steatosis, macrovesicular steatosis, hypertrophy, inflammation, fibrosis and hepatocellular carcinoma. A and B: The images correspond to hematoxylin & eosin (H&E) and picrosirius red staining's in the control group, showing a normal hepatic parenchyma with no significant changes; C and D: In the hepatocellular carcinoma (HCC)-group, animals exhibited macrovesicular steatosis and hypertrophy; E and F: Tumoral lesions with multiple fibrosis septa were observed in the HCC-group and rifaximin-group, as evidenced by H&E and picrosirius stained slides, respectively; G: Percentage description for the presence of microvesicular steatosis, macrovesicular steatosis, hypertrophy, inflammation, fibrosis, and HCC. Images A-D are magnified at 100 ×, and images E-F are magnified at 40 ×. HCC: Hepatocellular carcinoma; H&E: Hematoxylin & eosin; RIF: Rifaximin.

It is known that MASLD-HCC is a tumor of specific molecular characteristics compared to other etiologies of HCC. In the tumor microenvironment, the adaptive response caused by cellular hypoxia promotes the activation of pathways that intensify the process of angiogenesis, inflammation, fibrogenesis, and autophagy[9,25]. This response is dependent on hypoxia-inducible transcriptional factors, such as *Aldob*, *Tuba1c*, and metalloproteinases, among other markers. In this study animals of the HCC-group showed a significant increase in the expression of *Mmp9* in relation to the control group, and the treatment with RIF caused a reduction of this expression, obtaining values like the control. Additionally, we reported an increase in the gene expression of *Mm2*, *Mm9* and *Tuba1c* in animals that developed HCC compared to animals that did not develop this clinical condition. The increased expression of these markers is linked to the process of hepatic hepatocarcinogenesis, as it is known that metalloproteinases are master regulators in the process of cell prolifer-

eration and migration, and play a role in cell apoptosis, tissue regeneration, and immune response[26,27]. It is known that inflammation drives the progression of MASLD; however, the mechanisms associated with this process are not fully elucidated. In clinical practice, assessing the dietary inflammatory index and the systemic immunological inflammation index are tools that can be used to predict and evaluate the prognosis of liver disease, considering the associated genetic, environmental, and dietary factors[28,29].

Autophagy contributes with cellular homeostasis, thus avoiding cell transformation and tumor initiation. However, in the advanced stages of the tumor, autophagy acts mainly as a suppressor of cell death, allowing the adaptation of cancer cells to stressful conditions[30,31]. The autophagy process is regulated by different markers, including *Map1 Lc3b*, *p62/Sqstm1*, and *Becn1*[30]. In this study, we demonstrated a significant decrease in the expression of these genes in the HCC-group and RIF-group compared to healthy animals. It is known that there is no pattern in the expression of these markers in different cell types and tissues of origin[10,30]. An example of this process is the expression of *Becn1* which is reduced in glioblastoma, ovarian, lung, and esophageal cancer, but increased in colorectal and gastric cancer cells[32-34]. The results obtained in this study have a similar pattern of autophagy markers in HCC and hepatic fibrosis that was found in previous studies[10,35-38]. *Becn1* acts as an initiator of autophagy and its deregulation increase the susceptibility of cells to transformation, that is, its lower expression is predictive of inferior survival in HCC[10,33,37,38]. *Becn1* deficiency is also associated with increased angiogenesis, which indirectly corroborates the results obtained in this study[39]. Al-Shenawy[10] reports that autophagy and apoptosis in the liver are interrelated processes, in which elevated levels of *Becn1* in patients with chronic hepatitis may limit liver damage and interact with progression to cancer, where *Becn1* Later it is suppressed in aggressive cases of HCC[10]. The absence of microtubule-associated protein-1 Light chain-3 expression, an essential component in the formation of autophagosomes, is predictive of immediate mortality from HCC [35]. Duran *et al*[36] report that *p62/Sqstm1* deficiency increased the activation phenotype of hepatic stellate cells, inhibiting their anti-fibrotic and anti-inflammatory functions[36]. Although we reported a significant decrease in the expression of these autophagy markers in the HCC and RIF groups, we did not observe a significant difference with the treatment of RIF. It is known that the greater the difference in the levels of autophagy between cancer and normal tissue, the worse the prognosis of the lesion, a result observed in this study.

Epigenetics is involved in autophagy “turn on, turn off” along the carcinogenesis, through the activity of methyltransferases such as *Ezh2* and *Carm1*[40-42]. In this study, there was a significant decrease in *Ezh2* expression in the HCC-group and RIF-group compared to the control. Treatment with RIF promoted a significant increase in *Carm1* expression. No significant correlation was observed between the expression of *Ezh2* and *Carm1*, probably because they act in different ways of epigenetic control of autophagy. *Ezh2* represses the expression of genes related to the mammalian target of rapamycin pathway and *Carm1* acts on transcription factors such as p53, and factor nuclear-κB[40,41]. Inhibition of *Ezh2* activity induces autophagy, through the formation of LC3B and consequently the formation of the autophagosome, which corroborates with this study, given the lower expression of *Map1 Lc3b*[40,42]. The function of *Carm1*, like other epigenetic controllers, is dependent on the stage of lesion development, acting as a tumor repressor or promoter[41].

Epigenetic regulation is also carried out by microRNAs, which are short RNA sequences that function as modulators of mRNA expression, by either impairing translation or promoting its degradation[43,44]. Subtle dysregulation of anyone step in microRNAs biogenesis may lead to tumorigenesis. The miR-122 acts in the balance of proliferation and differentiation of hepatocytes, however its physiological role in carcinogenesis is variable and the mechanism by which it contributes to the progression of the lesion is undetermined[43]. In this study, the HCC-group showed a significant increase in miR-122 expression compared to healthy animals, a result that corroborates the literature[43,45]. We emphasize that this increase in gene expression was maintained in animals that developed HCC compared to animals that did not develop this clinical condition, in the stratified analysis of experimental groups. However, contradictory data are also reported, a possible explanation being the heterogeneity of the samples under different environmental conditions [44]. We did not observe a significant difference in the expression of miR-34a between the HCC-group and RIF-group, however there was a significant increase in its expression in the RIF-group compared to healthy animals. This increase in expression may represent a beneficial effect of RIF, as miR-34 inhibits the process of carcinogenesis through the regulation of p53, promoting apoptosis, cell cycle arrest, and senescence[46,47]. This data corroborates the increase in the expression of *Carm1* regulator of autophagy through the expression of p53. Additionally, we reported a significant increase in miR-26b expression in RIF treated animals. We infer that it is a beneficial effect of the treatment since the lower expression of this microRNA is associated with a worse prognosis of HCC[48,49]. The suppression of miR-26 may result in an increase in the expression of *Ezh2*, in opposite to our results. However, we know that the expression of these epigenetic markers is variable according to the tissue and stage of the lesion[50]. Another possible beneficial effect of RIF treatment was the decrease in the expression of miR-224, which acts as an oncomiR in HCC cells, and its upregulation promotes the proliferation and migration of malignant hepatocytes[51,52]. *Carm1* regulates the expression of transcription factors such as p53 and nuclear factor-κB which regulate the expression of miR-224. Possibly this signaling mechanism is activated and RIF acts in its modulation, however further studies are necessary to be carried out for the elucidation of the process[51,52]. Additionally, we compared the results of animals that developed or not HCC, and we found no difference within them in the RIF-group, probably due to the small number of animals. However, analyzing the entire group of animals, there was a difference between miR-122, miR-34a, *Tuba1c*, metalloproteinases and autophagy markers between the groups that developed cancer *vs* those who did not present HCC. These interesting findings also may demonstrate the influence of epigenetic and autophagy markers in the development of HCC in this scenario.

So far, there is no specific medication approved by the Food and Drug Administration in clinical practice for the treatment of MASLD; therefore, counseling focuses on lifestyle to prevent progression to HCC. Biological aging and the progression of liver disease occur through the interference of various factors, including environmental, genetic, and dietary issues[28]. The assessment of these mechanisms can contribute to the search for preventive strategies in the development of MASLD-HCC. In this study, animals were subjected to a HFCD with the aim of developing the liver

condition, allowing the study of the use of RIF as a preventive measure. Since dietary management tools are essential for controlling this clinical condition, one can speculate if including additional dietary intervention in the model, such as high fiber diet or flavonoids, for instance, could alleviate liver damage[12,53,54].

CONCLUSION

In summary, MASLD-HCC management is a clinical challenge, and many questions need to be addressed, including the response to the new immunotherapy agents. The process of autophagy and epigenetics tends to play a complicit role in drug resistance and the interface between the two is multifactorial and crosstalk occurs in different proteins of each process[6,55]. In this study, we observed, mainly in relation to the epigenetic markers evaluated, a possible beneficial effect of the treatment with RIF in rats with MASLD-HCC, suggesting it could be useful to prevent or delay carcinogenesis. On the other hand, the study has some weaknesses to be considered, like the small number of animals and the gene expression markers analysis. Thus, new preclinical studies are needed to evaluate the epigenetic and autophagy mechanisms in MASLD-HCC for a better understanding of the role of RIF, as there are factors that need to be better explored, such as the variability of the course of the disease, the complexity of the autophagy mechanism and the individualized treatment requirements.

ARTICLE HIGHLIGHTS

Research background

Metabolic-dysfunction associated steatotic liver disease (MASLD) incidence is increasing worldwide. Hepatocellular carcinoma (HCC) is a complex and heterogeneous neoplasm, and there's evidence showing MASLD-related HCC has some unique features, including gut microbiota (GM). However, current treatment does not take this heterogeneity into account, dealing with viral and non-viral HCC in the same way. This study is intended to characterize autophagy and epigenetics in experimental MASLD-HCC and its response to rifaximin (RIF), a minimally-absorbed broad-spectrum oral antibiotic, that may interfere in GM-derived inflammation.

Research motivation

Epigenetic changes, autophagy and GM are involved in hepatocarcinogenesis, but there is no definite evidence of a positive effect of its modulation in human steatohepatitis. RIF may influence in these complex mechanisms. Understanding GM influence on epigenetics and autophagy can help not only as a diagnostic tool but also as a target for new therapies.

Research objectives

The main objective was to investigate rifaximin (RIF) effects on epigenetic and autophagy markers in experimental HCC secondary to MASLD. Future research in humans with MASLD can open a new therapeutic pathway to decrease HCC burden in this setting.

Research methods

We conducted an innovative RIF experiment in a MASLD-HCC model with 24 adult Sprague-Dawley rats, randomly assigned in three groups ($n = 8$, each) and treated from 5-16 wk. We compared the results of control animals to RIF group and MASLD (animals in the last two groups received a high-fat choline deficient diet plus diethylnitrosamine in drinking water. Gene expression of epigenetic and autophagy markers was obtained at the end of experiment.

Research results

All animals in RIF and MASLD groups developed steatohepatitis, fibrosis, and cirrhosis. All MASLD animals also presented HCC, but in RIF group three rats did not develop tumor. Some microRNAs, metalloproteinases and aggressivity markers were higher in rats that developed HCC comparing with those that not developed, and the opposite occurred with the autophagy markers.

Research conclusions

The results suggest that autophagy and epigenetics could exert influence on MASLD-HCC *via* GM interference with RIF and support clinical studies in the area.

Research perspectives

RIF may have effect on autophagy and epigenetic markers as shown in this study. These initial results in animals shall be confirmed in other preclinical and clinical studies before recommending its use in high-risk patients with MASLD cirrhosis.

FOOTNOTES

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Author contributions: Michalczuk MT and Longo L performed the conceptualization, methodology, formal analysis, investigation, data curation, writing of the original draft, writing-review, and editing; Keingeski MB, Guerreiro GTS, Ferrari JT, Filippi-Chiela E, Uribe-Cruz C and Cerski CTS performed the methodology and formal analysis, writing review and editing; Basso BS performed the methodology; Vargas JE performed the analysis; Oliveira CP performed the methodology and writing review; Álvares-da-Silva MR performed the conceptualization, methodology, formal analysis, investigation, data curation, writing of the original draft, writing-review, editing and research fundraising.

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