ESR1 regulates the obesity and metabolism-differential gene MMAA to inhibit the occurrence and development of hepatocellular carcinoma

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Abstract

Background Obesity is often regarded as one of the factors that promote tumorigenesis, but the role of obesity in promoting hepatocellular carcinoma (HCC) is still controversial.

Methods We compared the trend change of 14 obesity-related genes in formation and development of HCC in normal, adjacent and HCC tissues. Mendelian randomization (MR) analysis was used to verify the relationship between obesity and HCC occurrence. MMAA was discovered as an obesity and metabolism-differential gene, and its function in HCC was testified *in vivo* and *in vitro*. Finally, we explored how obese female with originally high expression of female estrogen receptor (ESR1), directly upregulated MMAA to interfere with the progression of HCC.

Results Fourteen obesity-related genes were downregulated in adjacent and tumoral tissues compared with normal liver tissues, which indicated that obesity may be inversely related to the occurrence of HCC and was consistent with the result in MR analysis. We also discovered MMAA is a metabolic gene closely related to the occurrence and development of HCC via mining TCGA database, and it functioned as an anti-tumor-promoting role in HCC by damaging the mitochondrial function and preserving the redox balance. We further verified obese female with originally high expression of ESR1 can regulate MMAA to protect HCC from progression.

Conclusions This study elucidates obesity might be a protective factor for female HCC patients, as they originally highly expressed ESR1 which could upregulate MMAA to suppress tumor growth and participate in metabolic reprogram.

Introduction

Hepatocellular carcinoma (HCC) constitutes the majority of liver cancer, which is ranked the 7th among new emergence of malignant tumors, causing a fatality rate of 8.3% [1]. There is a gender preference in the incidence and mortality of HCC, HCC favors male compared to female, with an incidence ratio of 7:1 ~ 10:1 [2]. According to the latest global cancer statistics, the incidence rate of HCC is 6.3% in male in comparison with 3.0% in female, with the mortality rate of 10.5% and 5.7% respectively [1]. Consistently, the hepatocarcinogenesis in animals also support the fact that males are more susceptible to HCC regardless of the etiology (chronic viral infection of hepatitis B or C, smoking, alcohol abuse and so on) [3]. This suggests that the occurrence of HCC may be related to the genetic information carried by different genders, but the underlying mechanism remains unknown.

Obesity is defined by the World Health Organization as the body-mass index (BMI) higher than 30.0 kg/m² and BMI between 18.5 to 24.9 kg/m² is defined as overweight. But for Asians and Westerners of the same weight, Asians have higher body fat content than Westerners, but less muscle content. It is therefore recommended that Asians whose BMI exceeds 25.0 kg/m² can be considered obese, which is a relatively safe reference value [4]. Obesity has become the second factor that promotes tumorigenesis only to smoking [5]. Also, obesity was commonly believed to potentially enhance the risk for tumor

progression and recurrence, including breast cancer, ovarian cancer, pancreatic cancer, gastric cancer, meningioma, etc [6]. However, obesity has a counterintuitive association with prognosis, especially in patients treated with immunotherapy. Among 330 patients with advanced chromocytoma treated with PD-1 inhibitors, the average life expectancy of obese patients was 13 months longer than patients of normal weight [7]. It was found that the expression level of PD-L1 increases with body weight regardless of species. The serotonin increases with the gradual growth of adipose tissue, until it is similar to cancer cells, which participates in the regulating the function of T cells and positively related with the expression of PD-1 checkpoint protein [8]. Moreover, adipose tissue produces estrogen, causing estrogen levels to rise [9]; female estrogen receptor (ESR1) which encodes estrogen receptor, is originally highly expressed in female compared with male [10]. Therefore, it is unclear that whether adipose tissue gene could be activated by ESR1 to cause the prognostic difference in female HCC patients.

When the adaptive lipid-storing function of adipose tissue fails, obesity becomes a metabolic disease, which affects the inner-membrane functions, submitochondrial localization and ultrastructural morphology of the mitochondria [11]. Obesity could lead to mitochondrial malfunction, manifesting as decreased synthesis and activity of mitochondrial substances, excessive production of reactive oxygen species, and increased autophagy, which can in turn affect the function of adipose tissue and result in the whitening of brown fat [12]. It was reported that CB-839 exerts anti-cancer effect by reducing the mitochondrial membrane potential and reducing the number of mitochondria, which could inhibit the tumor metabolism significantly [13]. Recent studies demonstrate that gender difference also occur in efficacy of cancer treatment [14]. Previous studies have confirmed that compared with males' mitochondria, females' have stronger biosynthesis and stronger anti-oxidant defense ability, which reduces production of cellular reactive oxygen species (ROS) thus postponing tumor progression [9]. This reminds us not to neglect the association between gender specificity, mitochondria and cancer. Moreover, Ke et al. discovered that the upregulation of 14 genes leads to obesity, thus the relationship between the 14 genes and the potential anti/oncogenic genes in HCC should be furthered explored [15]. Metabolism of cobalamin associated A (MMAA) is the key gene related to mitochondrial function, the lack of which would develop methyl acryluria (MMA) and eventually progress into hepatoblastic carcinoma due to the impaired tricarboxylic acid cycles and accumulation of oxidative stress as well as toxic metabolites [16]. There is currently no research on the correlation between MMAA and HCC, and considering that mitochondrial function changes caused by the defect of MMAA suggest that it may have a potential correlation with the occurrence and progression of HCC.

Although many studies have shown that obesity promotes the onset of tumors, they are all based on traditional observational epidemiology methods, which often ignores the confounding bias. Especially in HCC, rare studies have verified the correlation between obesity and oncogenesis. Mendelian randomization (MR) is a tool for variable analysis that uses genetic variation (such as single nucleotide polymorphisms, SNPs) as the target to be studied. The variables of the exposure factors can effectively overcome these limitations and clarify the causality correlation between exposure factors and the cause of the tumor [17]. In this study, we found that the obesity is a protective factor in female HCC through MR analysis and the obesity differential gene—MMAA is selected via bioinformatic analysis. We investigated

the function of MMAA in HCC. Upregulation of MMAA in tumor tissues predicts longer survival for HCC patients. MMAA suppresses the proliferation and migration of HCC cells and regulates the redox balance, thereby highlighting MMAA as a critical target for treatment of HCC.

Results

Obesity is a protective factor for HCC occurrence in female

In order to analyze the trend of obesity-associated genes in patients, we calculated the expression of 14 genes/ GAPDH in GTEx normal, TCGA LIHC N and TCGA LIHC patients to see if obesity changed with HCC occurrence. The expression of 14 genes/ GAPDH decreased as the disease progressed (Figure 1A, 1B and 1C), including *ADAMTS9*, *DHX33*, *EIF3A*, *KAT8*, *NOTCH4*, *PSMC3* and *PUM2*. The gender of GTEx normal was stratified according to our manual (Supplementary figure 1) [18]. Moreover, the changes of 14 genes/ GAPDH expression in female were presented in the Supplementary figure 2. To study the effect of obesity genotype on the occurrence of HCC, we use genome wide association study (GWAS) database (<http://atlas.ctglab.nl/>) to search obesity-related (BMI > 25.0 kg/m², body mass index II id: 974) and HCC-related (liver/hepatocellular cancer II id: UKB-b 15443) SNPs, and to analyze the correlation between obesity and HCC by MR. It is suggested that obesity is not a risk factor affecting the occurrence of HCC in the general population. A further stratified analysis based on gender found that obesity is related to the incidence of HCC in female (Figure 1D and E). MR egger test showed a significant negative correlation between obese female and HCC incidence (P < 0.001, Figure 1F and G). It indicated from a genetic perspective, obesity may be a protective factor for HCC in female.

MMAA is a differential obesity gene and predicts better prognosis in HCC

To verify whether obesity has a positive impact on the prognosis of patients with HCC, we illustrated a flowchart to specify our screening criteria. First, we included normal liver samples from GTEx database (n=77) as well as 371 LIHC patients from the TCGA database. Secondly, we discovered that obese female benefited from HCC prevalence via MR analysis. Thirdly, we designed the following criteria to select the metabolism-related genes: 1) Built obesity-ssGSEA scores and classified TCGA LIHC patients into high & low expression groups; 2) Selected genes highly expressed in GTEx and low expressed in TCGA LIHC in female; 3) Intersected differential genes with genes highly expressed in GTEx in female; 4) Selected protective genes (HR<1) in TCGA LIHC female patients; 5) Correlated the at least 12 of the 14 obesity-related genes with genes filtrated from the previous 4 steps. Finally, considering that the dysfunction in mitochondria is closely related to obesity, therefore, we combined the requirements of genes associated with HCC oncogenesis and prognosis as well as mitochondrial function (Figure 2A). *MFN2*, *ODGH* and *MMAA* was eventually picked out for survival analysis, but only *MMAA* predicted better outcome in 5-year survival in female HCC (Figure 2B and Supplementary figure 3; generalized Wilcoxon analysis, P = 0.0468). To ensure the clinical characteristics would not interfere with the conclusion, we analyzed the clinical data baseline of the HCC patients in TCGA (Supplementary table 1). In order to verify the role that MMAA might play in HCC, we collected 55 tissue sections of HCC patients in our center to measure the

expression of MMAA. MMAA was mainly located in the cytoplasm, and was enriched higher in adjuvant tissues than in the tumoral tissues (Figure 2C and 2D). Consistently, the protein level of MMAA detected in four patients by western blotting also supported the IHC result (Figure 2E). This indicated that MMAA might play as an anti-oncogenic role in HCC, as high expression of which predicted superior outcome but still needed further validation.

MMAA inhibits cell proliferation and migration in HCC

Based on the high expression of MMAA in the non-tumoral tissues and its representativeness in obesity differential genes, we hypothesized that MMAA might influence cancer cell proliferation, colony formation ability, migration and apoptosis. We firstly established the stable transfected JHH-7 and LM3 cells with a pLVX-FLAG-puro-MMAA plasmid, and the overexpression efficiency was confirmed (Figure 3A and 3B). The cell proliferation rate was dramatically inhibited significantly in the OE-MMAA cells (Figure 3C). The colony formation ability was observed decreased in the in the OE-MMAA group (Figure 3D), suggesting that MMAA processed a vital function in liver cancer cell proliferation and tumorigenesis. To identify that whether MMAA has the potential of affecting liver cancer cell mobility, we next performed transwell assays and wound healing in vector-NC and OE-MMAA cells. OE-MMAA JHH-7 and LM3 cells both exhibited reduced numbers of migrating cells in the transwell assays (147 VS 14 and 97 VS 33, both $P < 0.001$, Figure 3E). Coincided with the result of transwell assays, the extent of wound closure was greatly inhibited by overexpressed MMAA (78% VS 32%, 93% VS 60%, both $P < 0.001$, Figure 3F).

Overexpression of MMAA negatively regulates mitochondrial respiration in HCC

It is generally perceived that HCC has a high mitochondrial load and dysfunctional mitochondria takes up more than 75% of cell volume [19]. Considering MMAA is involved in the translocation of cobalamin into the mitochondrion, we started to explore whether it participated in the mitochondrial function of energy metabolism reprogramming. We first examined the apoptosis changes, which is often mediated by ROS to cause the opening of the mitochondrial membrane permeability transition channel, and the mitochondrial membrane system undergoes ruptures and can cause cell death. Figure 4A and 4B showed that MMAA was an apoptosis promoting molecule, especially in early apoptosis. The overexpression of MMAA increased the percentage of nucleus density and the depth of staining and the expression of Caspase-9 and Parp1 were also upregulated (Figure 4I), which also represented the promoted status of apoptosis (Figure 4C). Correspondingly, the process of cell apoptosis is often accompanied by the destruction of mitochondrial transmembrane potential. By measuring the mitochondrial transmembrane potential via JC-1 dye, we found that overexpressed MMAA decreased the membrane potential of JHH-7 and LM3 cells (Figure 4D and 4E). We continued to explore the changes in the ultrastructure of mitochondria in vector-NC and OE-MMAA cells through transmission electron microscopy (TEM). Consistent with the above results, the OE-MMAA cells demonstrated more swollen and rounded mitochondria and less interconnected mitochondria compared with vector-NC cells (Figure 4F). The number of mitochondria decreased by the overexpression of MMAA, indicating that MMAA was a vital negative regulator of mitochondria function (Figure 4G and 4H). In the meantime, we found that the

overexpression of *MMAA* increased the expression of two mitochondria-associated proteins compensatorily, Sirt3 and COX IV (Figure 4I).

Overexpression of *MMAA* changes metabolic phenotype in HCC cells *in vitro*

The abnormalities of mitochondrial metabolism led to sorafenib resistance in targeted therapy for HCC [20]. Also, as the permeability pores of the inner mitochondrial membrane of the abnormal mitochondria and the respiratory electron transport chain released ROS, we observed that the overexpression of *MMAA* increased ROS release (Figure 5A and 5B). We next explored the possible mechanism underlying in the changes of metabolic phenotype through RNA sequencing. Figure 5C showed that genes related to “Glycolysis/Gluconeogenesis” and “FoxO signaling pathway” expressed distinctively in OE-*MMAA* and vector-NC groups.

As the energetic metabolism usually alters from mitochondria respiration to aerobic glycolysis, we measured the extracellular acidification rate (ECAR) and O₂ consumption rate (OCR) to determine the metabolic phenotype in HCC cells. We discovered that the overexpression of *MMAA* reduced the glycolysis rate of HCC cells. The basal glycolysis and compensatory respiration were reduced HCC cells with lentiviral transduction of OE-*MMAA* in comparison with vector-NC (Figure 5D, 5E, 5J and 5K). Moreover, the mitochondria respiration of HCC cells also showed a decreasing trend by the overexpression of *MMAA*. As we can see from Figure 5F and 5G, the basal OCR, ATP-linked respiration, the maximal OCR level as well as the spare respiratory capacity were all weakened in the OE-*MMAA* compared with vector-NC (Figure 5L and 5M). To some extent, the reduction in ATP consumption means that the need for energy of tumor growth is reduced, and the malignancy of tumor is decelerate (Figure 5L). Considering that cellular redox balance interferes with the mitochondrial respiratory function, we continued to detect the redox level in HCC cells to see if *MMAA* could act as an antioxidative molecule. As expected, the GSH/GSSG ratio was descended in the OE-*MMAA* group in the JHH-7 and LM3 cell lines (Figure 5H). Consistently, the NADP⁺/NADPH ratio was also increased in the OE-*MMAA* group in comparison with the vector-NC group (Figure 5I). These results confirmed our hypothesis that the *MMAA* overexpression could influence the redox balance, and the antioxidative genes, such as *TXNIP*, *GCLC*, *GCLM*, *HMOX1*, *NOQ1* and *NRF2* were downregulated in the OE-*MMAA* group (Figure 5N). To validate if the expression of *MMAA* could affect the expression of antioxidative genes in large population samples, we found the expression of *MMAA* was negatively correlated with the antioxidative genes (*GPX4*, *FOXO1*, *GPX7* and *TNFR*) in the TCGA (Figure 5O). This indicated that antioxidative genes disturbed the unfavorable effect of *MMAA* on the mitochondrial function in HCC.

***MMAA* overexpression inhibits hepatic xenograft tumors to proliferate**

To further explicit the inhibitory function of *MMAA* in HCC, xenograft tumors were established in both human HCC cell line and murine cell line. As presented in Figure 6A and 6B, the tumor sizes were significantly shrunken in the OE-*hMMAA* group compared to the control group. More importantly, the Ki-67 expression was reduced in the OE-*hMMAA* group accordingly (Figure 6C) in HepG2. Furthermore, we

found that the expression of *ESR1* was also positively correlated with the expression of *MMAA* (Figure 6C and 6D), suggesting that *MMAA* might be regulated by its upstream regulator— *ESR1*. The ability of anti-cancer progression was also confirmed in murine xenograft (Figure 6F, 6G and 6H) following the implantation procedure in Figure 6E. This indicated that *MMAA* could inhibit the proliferation of HCC from the genetic level regardless of the species.

MMAA* is a downstream target of *ESR1

As we found that obese women have favorable prognosis in the development of HCC, we wondered whether the female estrogen receptor and the transcriptional factor *ESR1* is involved in the activation of *MMAA*. As we discovered that *MMAA* was upregulated with the increase of *ESR1* expression (Supplementary figure 4), we firstly conducted a ChIP assay to find out whether *ESR1* could bind with the consensus *ESR1* binding element. From Figure 7A we can see, *ESR1* could combine with the consensus *ESR1* binding element in the promoter region starting from -2000 to 0 of *MMAA* (Figure 7B). The luciferase assays illustrated that manipulation of *ESR1* expression promoted the *MMAA* promoter activity in 293A cells (Figure 7C). To further validate that *MMAA* is a downstream target of *ESR1*, we mutated the promotor sequence CATGTCAACATTACCTT into TTCAGTCGTGCGTAATG, and the expression level of *MMAA* failed to be upregulated by *ESR1* (Figure 7D).

In conclusion, our findings demonstrated that obese females are benefit from the incidence of HCC, and the obesity-differentiated gene—*MMAA* is a novel predictive marker for the better outcome in HCC. *In vitro* cell lines and *in vivo* experiment showed that the overexpression of *MMAA* negatively regulate cell proliferation, migration and anaerobic glycolysis. Moreover, *MMAA* correlates with the antioxidative genes to perturb the balance of redox balance and reprogram the metabolic pattern of HCC via altering the mitochondria function. Finally, the current study uncovers the *ESR1*/*MMAA* axis as a potential therapeutic target for HCC. Further studies are needed to excavate this axis to improve the overall survival of HCC patients (Figure 7E).

Discussion

Primary HCC is more than two times as prevalent in men than women, which is also observed in male mice developing liver tumorigenesis via diethylnitrosamine compared to the incidence of female [21, 22]. Clinical findings have demonstrated that the incidence of HCC and the relating mortality rates are higher in men than that in women [23]. In the past few decades, great efforts have been made to identify the potential molecular target that could elucidate the sex dimorphism in HCC but failed. Herein, we identify that obesity might protect obese female from HCC, and discover *MMAA* as an obesity-differential gene as well as a prognostic index for HCC. The overexpression of *MMAA* in HCC cells inhibits cancer cell proliferation, migration and clone formation as well as promotes the apoptosis rate and maintains the redox balance as a result of mitochondria dysfunction. Moreover, we demonstrate that *MMAA* is a downstream target of *ESR1*, which further explains that *MMAA* benefits female HCC patients' outcome in the long term.

It is commonly believed that obesity led to the onset of cancer, however, recent studies discovered that obesity might not influence all cancer types in the same way referring to carcinogenesis. The increase in BMI promotes the proportion of lipocytes, which produces great amount of estrogen. It was reported in some studies that whether it is eating high-fat food or a normal diet, the probability of mice getting HCC has not changed in two groups but the probability rose when added cancer-promoting substances, which indicates that the obesity is not the direct cause of cancer. Men are more likely to suffer from HCC than women, for ovariectomy increases the incidence of HCC in women, while castration decreases the incidence in men [24, 25]. Therefore, ovarian hormones can prevent HCC, and androgens can promote tumor growth. Moreover, studies have shown that estrogen plays a role in the promotion phase of carcinogenesis rather than the initial phase. Also, ESR1 has been proved to suppress proliferation, migration, and invasion in HCC cells; the underexpression of which in male predicts a worse prognosis in HCC [26]. Therefore, regardless of many studies have reported that the incidence of HCC might be related to the obesity in a large population, some confounding factors might be neglected, such as serum lipids, sex hormones, blood glucose and hereditary factors. In order to reveal the real relationship between exposure and ending, genes are randomly assigned to offspring during meiosis, which is equivalent to randomly assigning gene effects to "subjects", thereby "controlling" the influence of other factors on gene effects. Researchers use genetic variation as an instrumental variable to study the relationship between instrumental variables and outcomes, and from the relationship between the two to deduce the effect of exposure (gene effect) on the outcome [27]. Thereby, to explicit whether obesity is the onco-carcinogenesis factor of HCC and whether it affects both genders equally, applying MR to excavate the GWAS database (<http://atlas.ctglab.nl/>) and correlate the SNPs of obesity (BMI > 25.0 Kg/m²) and the representative SNPs of HCC is a good way to explore the possible underlying mechanisms. Surprisingly, we found that obesity in the female may be a protective factor for HCC. Considering that ESR1 is a ligand-activated transcription factor, composed of several domains important for hormone binding, DNA binding and transcription activation and encodes ER, which is a substance that promotes the development of secondary sexual characteristics and the maturation of sexual organs in female animals. As adipose tissue produces estrogen, which might indicate an underlying relationship between ESR1 and obesity-related gene [10]. Clarifying the obesity differential genes in HCC patients and specifying their relationship with ESR1 is the key factor to illuminate how obese females benefit from HCC oncogenesis. Our results also showed that *ESR1* directly regulate obesity differential gene—*MMAA* to improve the prognosis of HCC patients.

As is known that the tumor environment was greatly affected by BMI in the tumoral and peritumoral adipose tissues, which thereby influences the efficacy of immunotherapy clinically and supplies sufficient energy for cancer cells to flexibly up-regulate the free fatty acid pathway, takes up more lipids, and indirectly inhibits the tumor infiltration and anti-tumor function of CD8 + T cells [28, 29]. The obesity-related genes might be involved in the metabolic reprogramming which is essential for the malignant stemness and cancer cell proliferation. Metabolic abnormality is one of the crucial characteristics of cancer cells, for they will undergo a series of metabolic reprogramming to meet their own rapid proliferation needs. The Warburg Effect proposed in the 1920s pointed out that even under aerobic

conditions, tumor cells will maintain anaerobic glycolysis to produce energy and raw materials for cell proliferation and sustain its malignant property [30]. Besides ATP, Sanchez-Perez et al noted that the oxidative stress interferes with the DEN metabolism by increasing the ROS and oxidized thiol proteins to accelerate the HCC carcinogenesis process, which indicated that it might be possible to focus on the development of targeted therapies aiming at disturbing the redox balance [31]. As all roads lead to NADPH in cancer, NADP is frequently converted to NADPH by glucose 6-phosphate dehydrogenase and malic enzymes, accompanied by the overexpression of mutant p53 in tumor cells [32]. Notably, p53 could partially activate detoxification cytochrome P450 to control ROS and limit HCC progression [33]. The main mechanism used by cancer cells to reduce ROS levels relates to the production of glutathione and its application in the reaction $\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$, where GSH stands for reduced glutathione. Also, blocking glutamine metabolism helps inhibiting the growth and proliferation of HCC cells by sealing the production and utilization of glutamine metabolites. Therefore, p53 expression interacts with the glutamine metabolism in order to preserve the HCC distinct redox state [34]. NADH + is an indispensable molecule for mitochondrial function, serving roles in oxidative phosphorylation as well as redox balance [35]. Thereby, we boldly hypothesized that HCC cells were trained to develop to protect themselves from respiratory injury to maintain the redox balance.

Once the energy factory—mitochondria are destructed, the cancer cells would grab mitochondria from surrounding healthy cells to restore their functions. We observed that the apoptosis rate is elevated in the OE-*MMAA* group in HCC, which is often mediated by the ROS to cause the structural changes, ruptures, and fragmentation of the mitochondrial membrane and result in the alteration of permeability as well as cell death. The *MMAA* gene is essential for the coding of Cbl reductase and the mitochondrial transporter of vitamin B12 [36]. The defect of *MMAA* could lead to the reduction in AdoCbl synthesis located in mitochondria, which also leads to methylmalonic acidemia in clinical practice [37]. Watanabe et al discovered an NADPH-dependent aquacobalamin reductase in rat liver from one CblA cell line, which further confirmed that *MMAA* protein is mitochondria-targeted [38]. Thus, we hypothesized that *MMAA* could participate in the oxidative injury and the biological behavior of mitochondria. Our finding shows that the overexpression of *MMAA* could increase the ROS level and restrict the mitochondrial function in HCC cells. It is well-recognized that when the cancer suppressor gene is overexpressed, the mitochondria function is recovered and that the glycolysis level is downregulated. However, *MMAA* might be an exception, because we identified that the *MMAA* balanced the redox status to resist the potential oxidative stress. Therefore, we assumed that *MMAA* was ineluctable for the mitochondrial respiration and metabolic reprogram in HCC.

Due to the heterogeneity of tumor metabolism, different hypoxia conditions and pH, etc., different types of cancer have distinct sensitivity to oxidative damage. Molecule that disturb the active oxygen scavenging system can also perturb the glutamine glycolysis, which inhibit the growth and proliferation of HCC cells [39]. It was reported that NRF2 acts as an important transcription factor to maintain redox homeostasis, which influences the mitochondrial membrane potential. Also, the RNA sequence in our study indicated that overexpression of *MMAA* altered the genes associated with

Glycolysis/Gluconeogenesis” and “FoxO signaling pathway, which closely influenced the metabolic reprogram. We reckoned that the overexpressed-MMAA might affect the expression of antioxidant response element (ARE). The activation of the NRF2-KEAP1 pathway is one of the most important mechanisms for anti-tumorigenesis, which can cause the conformational change of KEAP1-CUL3-E3 ubiquitin ligase and subsequently translocate *NRF2* to the nucleus, and binds to the ARE [40]. This would induce the expression of a series of cytoprotective genes, such as *NQO1*, *GST*, *HMOX1*, *GCLC* and so on. We further confirmed it in HCC cell lines that the expression of *TXNIP*, *GCLC*, *GCLM* and *ME1* were downregulated in the OE-MMAA group compared with the control. Moreover, we explored the TCGA database and identified *GPX4*, *FOXO1*, *GPX7* and *TXN* were all negatively correlated with the expression of MMAA ($P < 0.05$). Thus, we concluded that MMAA might take part in interacting with ARE genes to impair the ROS detoxification and disturb the mitochondrial function.

Taken together, our study illustrated that the possible mechanism that the obese females have a better prognosis in HCC. As ESR1 promotes the development of secondary sexual characteristics in female animals, we demonstrate a novel function for it to directly regulate the obesity differential gene-MMAA and affects the cancer biological behavior of HCC. Moreover, MMAA mediates the mitochondrial function and alters the metabolic reprogramming through influencing the oxidative stress and ARE genes. Thus, the obesity differential gene-MMAA is oxidative and plays a specific role as a cancer suppressor, which provide certain therapeutic value for HCC patients, especially for female patients.

Materials And Methods

Comparison of the expression levels of 14 obesity-related genes among GTEx liver, TCGA normal liver and tumor tissues

The 14 obesity-related genes included *ADAMTS9*, *NOTCH4*, *EYS*, *DHX33*, *EIF6*, *GNL3*, *KAT8*, *POLR1D*, *PSMC3*, *PUM2*, *RRP12*, *TCF12*, *TCF7L2* and *ZFHX3*. We compared the expression of the 14 genes/ GAPDH among GTEx liver, TCGA normal liver and LIHC tissues. A student *t* test was used to compare the ratio of 14 genes/ GAPDH between groups, respectively. We performed a cluster analysis via Python (Version 3.7) to distinguish male and female patients in GTEx based on the expression of two genes (XIST and RPS4Y1) [18]. GTEx is a commonly used normal tissue database, but for the first time, we developed a manual to apply unique male and female genes as gender identification markers and the related information was attached in the supplementary materials. A single sample gene-set enrichment analysis (ssGSEA) was conducted based on the expression level of 14 obesity-associated signatures using the R package “GSEABase” and “GSVA”. The differential genes generated from high and low obesity score groups based on ssGSEA were extracted ($\log_{2}FC < -0.3$), then intersected with genes highly expressed in female GTEx compared with those in male ($\log_{2}FC > 0.3$). After the first intersection, genes highly expressed in female GTEx in comparison with TCGA LIHC tumor were screened out ($\log_{2}FC > 0$) and were calculated via Univariate Cox analyses to select the protective genes in HCC (Hazard ratio < 1). After the third selection, the remaining 291 genes were correlated with the 14 obesity genes ($R > 0.5$; correlated with 12 genes or more as the boundary and $P < 0.05$) and those relevant to metabolic pathways were

picked. A total of 33 genes were later searched whether they were related to oncogenesis of HCC. *MFN2*, *OGDH* and *MMAA* were chosen, and *MMAA* was the only gene associated with better prognosis in female HCC.

Human tissues and cell culture

The clinical tissue samples were obtained from patients who were diagnosed at Sir Run-Run Shaw Hospital (SRRSH) between 2007 and 2012. The current study conformed to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the SRRSH. All patients gave written informed consent for the use of their tissues for scientific research. Pathological diagnosis was based on the morphological and immunohistochemical criteria provided by the 8th edition of American Joint Committee on Cancer (AJCC) [41]. The JHH-7, LM3, HepG2 and Hepa1-6 were purchased from the American Type Culture Collection and verified by DNA fingerprinting. The culture environment was maintained at 37 °C with 5% CO₂.

Plasmids

The pLVX-FLAG cloning vector (Addgene) was used to generate OE-*MMAA* constructs and the 41 base pairs (bp) targeted against *MMAA* were CTGTTCCAGGGGCCCCACCGGTCCCATGCTGCTACCACATCC and CTCAGCGAATTGGCGAATTCTTAGTCTCTGCTTTTAAAAGCTTTTAACA. Overexpression lentivirus particles were produced by co-transfecting lentivirus constructs with pMD2.G and psPAX2 in a 3:2:1 ratio and then added to the HEK-293T cells. The pLVX-FLAG vector was designed as a control plasmid.

Quantitative real-time PCR (qRT-PCR) detection and quantitative analysis

Total RNA was isolated using RNA-Quick Purification Kit (ES Science, China), and was assessed for quality and quantity using absorption measurements. RNA was then reversely transcribed to cDNA by using an Evo M-MLV RT Premix kit (Accurate Biology, China). The cDNA was added with Hifair™ qPCR SYBR Green Master Mix (Yeasen, China) to form a mixture, then the expression levels of the candidate genes were determined using an ABI 7900HT Real-Time PCR system (Applied Biosystems, USA). Comparative data analysis was performed via the $2^{-\Delta\Delta C_t}$ method using the PCR Array Data analysis web portal (<http://gncpro.sabiosciences.com/gncpro/gncpro.php>) to determine relative expression differences between the comparison groups. All reactions were run in triplicate, and primer sequences are listed in Supplementary Table 2.

Animal model

A xenograft mouse model was used to verify the anti-oncogenic function of *MMAA* *in vivo* in both human and murine HCC cell lines. BALB/c-nu female mice, four weeks of age, were housed in sterile and filter-capped cages for one week before subcutaneous tumor implantation. Mice were randomly divided into two groups (n = 4/group) and injected with 100 µL PBS containing 3×10^6 HepG2 (human) / Hepa1-6 (murine) OE-*MMAA* cells or vector-NC type. After 1 week of tumor formation, the sizes of the xenograft

were measured twice a week and the volume was determined using the formula volume: length $\times$ width² / 2. All the tumor specimens were harvested after 3-4 weeks of tumor implantation and fixated with 4% paraformaldehyde before being sectioned into tissue slices for immunohistochemical staining (IHC). All animal experiments were conducted with consent from the Committee of the Use of Live Animals in Teaching and Research at SRRSH.

Western blot

Western blot was performed as previously described [42]. The antibodies used in the current study were anti-MMAA antibodies (Novus biologicals, USA; Abcepta, China), anti-Anti-ESR1 α antibody (Abcam, USA), anti-Caspase-9 antibody (Abclonal, China), anti-PARP1 antibody (Abclonal), anti- β actin antibody (Proteintech, USA) and anti- α Tubulin antibody (Proteintech).

Cell proliferation and colony formation assay

Cell proliferation was detected by the MTS assay. A total number of 2×10^3 cells were seeded into 96-well plates. MTS Reagent (Abcam) was added into the plates every day and the cells were cultured for an additional two hours. The absorbance values were measured at a wavelength of 490 nm by SpectraMax M2e Microplate reader (Molecular Devices, USA). In the colony formation assay, 1×10^3 cells were seeded into the 6-well culture plates. The colonies were fixed in 4% paraformaldehyde, and were stained with crystal violet solution (Beyotime Biotechnology, China) and counted under a microscope after two weeks.

Chromatin immunoprecipitation (ChIP) assay

ChIP was carried out using SimpleChIP[®] Enzymatic Chromatin IP Kit (Cell signaling technology, USA) on the basis of the manufacturer's protocol. In brief, Hep3B cells were cross-linked with formaldehyde and then lysed via SDS lysis buffer. Total nuclear DNA were sheared by enzymatic digestion with micrococcal nuclease. Protein-DNA complexes were precipitated by ESR1 α antibody or normal rabbit IgG as negative control respectively. Following by eluting the complex from the antibody, the PCR analysis was carried out with primers specific for *MMAA* promoter. The primers of P1, P2 and P3 fragments were listed in Supplementary Table 3.

ROS evaluation

The cells were stained with H2DCF-DA provided in the ROS Assay Kit (Beyotime Biotechnology) according to the manufacturer's instructions. Cells were washed, resuspended in ice-cold PBS, and collected. The fluorescence intensities of DCF, formed by the reaction of DCF with ROS was detected by a FACScan flow cytometer (BD Biosciences, USA), an excitation wavelength at 488 nm and emission wavelength at 530 nm.

Evaluation of the intracellular reduced glutathione oxidized/glutathione ratio (GSH/GSSG) and NADP⁺/NADPH ratio

According to the manufacturer's protocol, GSH/GSSG Ratio Detection Assay Kit (Elabscience, China) was used to measure the intracellular GSH/GSSG ratio, with an absorbance at 412 nm. The NADP⁺/NADPH Assay Kit (Elabscience) was used to determine the NADP⁺/NADPH ratio. Both analyses were performed to measure the oxidation state of HCC cells.

IHC

IHC was used to evaluate the expression of MMAA in tumoral and normal hepatic tissues. After being processed for paraffin embedding, 5 µm sections of tissue samples were prepared. First, these sections were deparaffinized and rehydrated. For antigen retrieval, the sections were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 10 min in a microwave oven. Then, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Non-specific binding sites were blocked with 5% normal goat serum for 30 min. The sections were incubated with an antibody against MMAA (1:1000, Novus) overnight at 4 °C. The sections were then incubated with the secondary antibody, and the expression of MMAA in the tissues was observed via microscopy after DAB staining and haematoxylin staining.

Dual-luciferase reporter assay

The *MMAA* promoter region was obtained from the UCSC Genome Database (<https://genome.ucsc.edu/>), spanning from -2000 to 0 at the TSS (transcription start sites) was amplified from genomic DNA using the primers (forward: ATTTCTCTATCGATAGGTACCTGCTAGCAGTGAGCAAGGCTC, reverse: ACTTAGATCGCAGATCTCGAGAAATCCCAATGTGACAGTGTTG). Analysis of putative transcription factor binding sites on *MMAA* promoter was performed using JASPAR (<https://jaspar.genereg.net/>). The wild type (CATGTCAACATTACCTT) or mutant (TTCAGTCGTGCGTAATG) seed binding site of *MMAA* promoter region was subcloned into pGL3 luciferase reporter vector and verified by sequencing. HEK-293T cells were transfected with the wild type or mutant reporter construct, pRL-TK plasmid, and the luciferase activities were detected using a dual-luciferase system (Promega, USA) based on the manufacturer's protocol 36 hours later.

Detection of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) in cardiomyocytes was assessed using 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Beyotime Biotechnology). Briefly, cells were incubated in 6-well plates after indicated treatments, and equal volumes of JC-1 staining solution (5 µg/ml) were added, incubated at 37 °C for 20 min, and washed twice with PBS. Cells were analyzed using a multi-label plate reader (PerkinElmer, USA) with 488 nm excitation; 530 nm emission for green and 590 nm emission for red, respectively. Alteration in the ionic equilibrium results in the mitochondria depolarization. Mitochondrial depolarization was indicated by an increase in the red/green fluorescence intensity ratio.

Evaluation of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR)

Cellular mitochondrial function and glycolytic capacity were measured using the Seahorse Bioscience XF96 Extracellular Flux Analyzer, according to the manufacturer's instructions of Seahorse XF Cell Mito Stress Test Kit or Glycolysis Stress Test Kit (Seahorse Bioscience, USA). JHH-7 and LM3 were plated in XF96 Cell Culture Microplates (Seahorse Bioscience) at an initial cellular density of 4×10^4 cells/well the day before determination. Then we washed the cells with Seahorse buffer, with 175 μ L of Seahorse buffer plus 25 μ L each of 1 mmol/L FCCP [carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone], 1 mmol/L oligomycin, and 1 mmol/L rotenone was automatically injected to measure the OCR. For ECAR measurement, 10 mM glucose, 1 μ M oligomycin, and 100 mM 2-deoxy-glucose were automatically added to measure ECAR value. The OCR and ECAR values were calculated after normalization to the cell numbers and were plotted as the mean $\pm$ SD.

Measurement of cell apoptosis

The FITC Annexin V Apoptosis Detection Kit (Multi Sciences, China) was used to measure the apoptotic rate. Cells were complied with the manufacturer's instructions and counted using a FACSCalibur flow cytometer.

Statistics

SPSS software (version 22.0, IBM Corp., USA) was used for the data analysis and Student *t* tests were applied for most occasions. Experiments were repeated at least three times and were presented as the mean $\pm$ SD. One-way ANOVA was used to analyze the differences among three or more groups. Statistical differences were regarded significant at *, **P* < 0.05; ***P* < 0.01, and ****P* < 0.001.

Declarations

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Authors' contributions

Conceptualization: YYZ, QJM, CZ; Methodology: YYZ, JXC, QJM; Investigation: YRL, QMX, PC, CZ; Visualization: YRL, XXF; Supervision: DFH, QJM, HL; Writing – original draft: YYZ, JXC; Writing – review & editing: YYZ, CZ, HL, DFH.

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Availability of data and materials

The datasets analyzed in our study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the SRRSH (ethical code: 20200210-126) and written informed consent was obtained from all patients. All of the animal experimental protocols were approved by the Committee of the Use of Live Animals in Teaching and Research at SRRSH (ethical code: SRRSH20210130).

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

Figure 1

The TCGA analysis and MR results reveal causal association between obesity and female HCC. (A) The expression of 14 obesity-related genes/ GAPDH in GTEx liver and TCGA Liver hepatocellular carcinoma (LIHC) normal samples. (B) The expression of 14 obesity-related genes/ GAPDH in GTEx liver and TCGA LIHC tumor samples. (C) The expression of 14 obesity-related genes/ GAPDH in TCGA LIHC normal and tumor samples. (D) A stratified analysis demonstrated that obesity was not related to the incidence of

HCC in the total population. (E) A stratified analysis further revealed that obesity was negatively related to the incidence of HCC in female. (F) and (G) MR egger test showed a significant negative correlation between obese female and HCC incidence.

Figure 2

The obesity differential gene-MMAA is a positive indicator in the prognosis of HCC. (A) A flowchart elucidating the screening criteria for obesity differential genes. (B) MMAA was associated with better outcome in female HCC patients but not in male. (C) The immunohistochemistry stainings of MMAA in HCC patients in terms of negative, low, moderate and high expression. (D) and (E) MMAA was highly expressed in adjacent tissues compared with tumoral tissues in HCC (scale bar, 200 μm and 40 μm).

Figure 3

The loss of MMAA is required for cell proliferation and migration in HCC *in vitro*. (A) Analysis of relative gene expression data for MMAA using qRT-PCR. (B) Analysis of MMAA protein expression using a Western blotting assay. (C) A MTS assay was used to detect the proliferation of HCC cells transfected with OE-MMAA. (D) A colony formation assay was conducted to confirm the effect of MMAA overexpression in two different cell lines and quantitation of the data was shown. (E) Cell migration analysis following MMAA overexpression and quantitation of the data was shown (scale bar, 100 μ m). (F) OE-MMAA JHH-7 and LM3 cells both exhibited significantly decreased cell motility in the wound healing assay and quantitation of the data was shown (scale bar, 100 μ m). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; three biological repeats per group.)

Figure 4

The loss of MMAA is required for cell proliferation and migration in HCC *in vitro*. (A) Apoptosis rates of the MMAA-overexpressed cell lines compared with vector-NC, quantification of the data was presented in (B). (C) Increased apoptosis in OE-MMAA HCC cell lines was accompanied by the shrinkage of the nucleus morphology (scale bar, 10 μ m). (D) Mitochondrial potential was measured by detecting the JC-1 decrease in JHH-7 and LM3 cells with the overexpression of MMAA. The decrease in mitochondrial

potential was quantified in (E) as mean $\pm$ SD. (F) TEM analysis of mitochondrial morphology after being stably infected with MMAA in JHH-7 and LM3 cells (scale bar, 2 μ m and 1 μ m). (G) Quantification of the percentages of swollen mitochondria and interconnected mitochondria through TEM data. (H) Quantification of the number of mitochondria per 20 mm² via TEM data. (I) Immunoblot analysis of mitochondria-related protein expressions in JHH-7 and LM3 cells infected with MMAA. (*P < 0.05, **P < 0.01, ***P < 0.001; three biological repeats per group.)

Figure 5

MMAA regulates redox balance to alter mitochondrial function in HCC. (A) ROS production was measured by flow cytometry in JHH-7 and LM3 cells with overexpression of MMAA; quantification of ROS production was shown in (B). (C) PCR array analysis of the effect of MMAA overexpression on changes in glycolysis and redox state related to mitochondrial function. (D) and (E) Overexpression of MMAA decreased the ECAR in JHH-7 and LM3 cells; quantification of the glycolysis and glycolytic capacity was shown in (J) and (K). (F) and (G) Overexpression of MMAA increased the OCR in JHH-7 and LM3 cells; quantification of the ATP production and maximal respiration was shown in (L) and (M). (H) Determination of the GSH/GSSG ratio in JHH-7 and LM3 cells with overexpression of MMAA. (I) Determination of the NADP⁺/NADPH ratio in JHH-7 and LM3 cells with overexpression of MMAA. (N) Quantitative PCR was performed to confirm that the mRNA expression of ARE-related genes was downregulated by overexpression of MMAA. (O) Pearson correlation analysis of different genes expressed in HCC patient tissues in the GEPIA2 dataset; *MMAA* expression was negatively correlated with *GPX4*, *FOXM1*, *GPX7*, and *TPM* (n = 369). (*P < 0.05, **P < 0.01, ***P < 0.001; three biological repeats per group.)

Figure 6

MMAA inhibits HCC progression in xenograft models and is positively correlated with ESR1 *in vivo*. (A) Stably infected OE-MMAA or vector-NC HCC cells were injected into nude mice and were measured every three days. (B) The tumor sizes were calculated and compared during the day of resection; (C) and (D) the tissue sections were stained for Ki-67, MMAA and ESR1 and the immunohistochemistry scores showed a positive correlation between ESR1 and MMAA (scale bar, 40 μm). (E) In order to verify whether MMAA can indeed inhibit the growth of HCC, we constructed a murine OE-MMAA HCC cell line and established xenograft models follow the procedure. (F) and (G) The tumor sizes were measured by electronic Vernier calipers in OE-MMAA and vector-NC groups. (H) Tumor growth curves were created based on the tumor volume. (*P < 0.05, **P < 0.01, ***P < 0.001; ≥ three biological repeats per group.)

Figure 7

ESR1 regulates MMAA/ARE activation HCC. (A) ESR1 occupied the binding site on the MMAA promoter region, as revealed by ChIP assays. (B) Position of the potential sequence in the MMAA promoter. (C) and (D) ESR1 increased the luciferase activity of the MMAA promoter in 293A and 293T cells. (E) A working model to illustrate the anti-oncogenic function of MMAA in HCC. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; three biological repeats per group.)

Supplementary Files

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