M2 macrophage-derived exosomes inhibit ferroptosis via regulating circ_TNFRSF21/ miR-451a/SLC7A11 axis in cutaneous squamous cell carcinoma

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Research Article

Keywords: M2 macrophages, exosomes, ferroptosis, circ_TNFRSF21, miR-451a, SLC7A11, cutaneous squamous cell carcinoma

Posted Date: July 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1856562/v1

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Abstract

Background: Cutaneous squamous cell carcinoma (cSCC) is a malignant proliferation of cutaneous epithelium. Ferroptosis is a new type of cell death involved in cancer progression. M2 macrophage derived exosomes promote cancer proliferation and progression. We intended to investigate the role of M2 macrophages derived exosomes on ferroptosis in cSCC and explore the underlying mechanism.

Methods: cSCC samples and adjacent normal tissues were obtained from cSCC patients. Exosomes were isolated from M2 macrophages differentiated from human mononuclear macrophage (THP-1) cells by flow cytometry. Cell viability, iron level, Malondialdehyde (MDA), Lipid ROS, mitochondrial superoxide, mitochondrial membrane potential (MMP) were detected for the validation of ferroptosis. RNAs and proteins were measured using RT-qPCR and western blot. Binding relationships between miR-451a and circ_TNFRSF21 or SLC7A11 were revealed by dual luciferase assay.

Results: M2 macrophages were increased in cSCC tissues. M2 macrophages inhibited erastin-induced ferroptosis, which was reversed by exosome inhibitor GW4869. SLC7A11 was up-regulated in cSCC tissues. Erastin treatment reduced cell viability, increased the level of iron and Fe^{2+}, upregulated MDA, lipid-ROS, mitochondrial superoxide, and declined MMP, which were reversed by M2 exosomes. Interestingly, circ_TNFRSF21 knockdown antagonized these effects of M2 exosomes. Circ_TNFRSF21 targeted miR-451a to regulate SLC7A11. Erastin-induced ferroptosis was promoted by miR-451a mimics, which was reversed after SLC7A11 overexpression. M2 exosomes inhibited erastin-induced ferroptosis in cSCC cells, which was antagonized by SLC7A11 downregulation or miR-451a upregulation.

Conclusion: M2 macrophage exosomes inhibited ferroptosis in cSCC through circ_TNFRSF21/miR-451a/SLC7A11 axis. It is contributed to cSCC progression and may providing a novel target for therapy.

Introduction

Cutaneous squamous cell carcinoma (cSCC), representing 20–50% of skin cancers, is featured by malignant proliferation of cutaneous epithelium [1]. Given that the frequency of lymph node metastases is approximately 4% and the mortality is about 2% [2]. Surgery is the cornerstone on cSCC management, while radiotherapy is also employed occasionally [3]. Although most cases of cSCC are capable to be cured surgically, the extra work-up and therapies for high-risk cSCC are still limited [4]. The biomarker identification as well as deeper understanding of cSCC molecular mechanisms are needed urgently for developing effective therapeutics for cSCC.

Ferroptosis, a novel type of cell death, has been discovered in recent years [5]. As a non-apoptotic form of regulated cell death, ferroptosis is induced by the iron-dependent accumulation of lipid reactive oxygen species and the exhaustion of plasma membrane polyunsaturated fatty acids [6]. Currently, it has been presented that ferroptosis participates in the initiation and progression of various cancers [7]. It has been identified that the induction of ferroptosis can inhibit cancer development in cSCC [8, 9]. Nevertheless, the underlying mechanism of the ferroptosis process in cSCC progression remains elucidated.

Macrophages not only play a vital role for warding off foreign pathogens and maintaining cellular homeostasis, but also exert various supportive functions in different tissues [10]. Infiltration of macrophages in solid tumors is related with poor prognosis in most cancers [11]. Tumor-associated macrophages (TAMs) typically accelerate angiogenesis, contributing to tumor growth and metastasis as well as cancer cell proliferation [10]. Proinflammatory M1 macrophages improve tumor cell phagocytosis, whereas anti-inflammatory M2 macrophages, for example TAMs, play a significant role in tumor growth and invasion [12]. Exosomes derived from TAMs can facilitate tumor angiogenesis, metastasis and proliferation [13]. Furthermore, exosomes derived from M2 macrophage promotes cell migration and invasion in colon cancer [14], facilitates growth and angiogenesis in pancreatic ductal adenocarcinoma [15], promotes metastasis in hepatocellular carcinoma [16]. Current studies have proposed that the altering the polarization of macrophages may induce ferroptosis of cancer cells [17]. Whether M2 macrophage-derived exosomes can regulate the process of ferroptosis is still indistinct in cSCC. Circular RNAs (circRNAs) are a kind of endogenous non-coding RNAs containing a structure of covalent closed loop [18]. Increasing researches have indicated that circRNAs are differentially expressed in cSCC which play a critical role in cancer formation [19]. Currently, circRNAs have been reported to be implicated in biological processes of ferroptosis, therefore influencing cancer growth [20, 21]. For instance, circRNA RHOT1 inhibits ferroptosis and promotes progression in breast cancer [22]. CircDTL modulates apoptosis and ferroptosis, acting as an oncogene in non-small cell lung cancer cells [23]. M2 macrophage-derived exosomal circRNA (Circ_0020256) has been validated to facilitate cell proliferation, migration and invasion in cholangiocarcinoma [24]. Previous study has reported that circ_TNFRSF21, a newly identified circRNA, promotes formation of endometrial carcinoma via
declining miR-1227 expression [25]. In addition, up-regulation of circ_TNFRSF21 seems to be co-regulated with the expression of its host gene in cSCC [26]. However, the function of circ_TNFRSF21 remains elusive in cSCC, further exploration is also required to understand the potential molecular mechanisms.

In present study, we aimed to identify whether M2 macrophages derived exosomes were involved in the regulation of ferroptosis in cSCC. Moreover, we also investigated the roles of circ_TNFRSF21 on ferroptosis in cSCC. Our study presented evidence for further understanding the pathogenesis and new therapeutic targets for cSCC treatment.

**Materials And Methods**

**Patient samples**

This research was approved by the Ethics Committee of Nanfang Hospital Southern Medical University. 12 pairs of cSCC samples and adjacent non-tumorous tissues were obtained from cSCC patients. All tissues were instantly frozen at -80°C with liquid nitrogen, after surgical resection. Prior to this surgery, no patients were administrated with radiotherapy or chemotherapy. All patients have written informed consent before this research. The biopsy results were confirmed (blinded) by a board-certified dermatopathologist before inclusion.

**Immunohistochemistry**

Before fixed in ice-cold acetone, frozen sections were rewarmed at room temperature. After antigen retrieval with 0.3% triton X-100, sections were blocked at room temperature with 5% BSA. Then they were incubated at 4°C overnight with diluted primary antibody CD68 (1: 200, Abcam, Cambridge, MA, USA). The next day, sections were administrated for 30 min at room temperature with HRP labeled goat anti-rabbit IgG antibody (1: 500, Abcam). Color reaction by diaminobenzidine chromogen (DAB) was developed for 3 min. Sections were experienced with dehydration, permeabilization and mounting, after hematoxylin counterstaining for nucleus. Positive target protein expression was presented in brown-yellow particles. Lastly, we selected five fields randomly from each section and they were observed under a microscope.

**Cell culture**

Human mononuclear macrophage cell line (THP-1) was obtained from American Type Culture Collection (ATCC, Manassas, VA). They were cultured in RPMI-1640 medium with 10% FBS at 37°C with 5% CO2 until 90% confluence. Cells were administrated with 100 ng/mL PMA (Sigma Aldrich, St. Louis, MO, USA) for differentiation into macrophages for 48 h, 100 ng/mL LPS (Sigma) and 100 ng/mL IFN-γ (Sigma) for differentiation into M1 macrophages for 24 h, and 20 ng/mL IL-4 (Sigma) for differentiation into M2 macrophages for 72 h. A431 cells were obtained from ATCC. SCL-1 cells were purchased from Tongpai Biotechnology Co. (shanghai, China). Cells were cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified incubator with 5% CO2 at 37°C.

**Cell transfection**

Sh-circ_TNFRSF21 and sh-SLC7A11 interference vectors and corresponding shNC, SLC7A11 overexpression plasmid (OE-SLC7A11) and its NC, miR-451a mimics or inhibitor were acquired from GenePharma (Shanghai, China). When the cells reached 80–90% confluence, they were transfected with sh-circ_TNFRSF21, sh-SLC7A11 interference vectors or corresponding shNC, OE-SLC7A11 or its NC, miR-451a mimics or inhibitor using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then Erastin (10 μM, Sigma) was used to treat cells for 48h.

**Flow cytometry**

When cell confluency reached 80 ~ 90%, cells (1 × 10⁶ cells) were gently collected into centrifuge tubes for centrifugation and washed (1 × PBS, 0.5% BSA, 2 mM EDTA) twice, counted, and then incubated with respective primary antibody in dark for 30 min at 4°C. Cells were washed with cold PBS for three times before stained with secondary antibody. Monoclonal antibodies for macrophage were anti-CD68 and anti-CD11b (BD Biosciences, San Jose, CA). Besides, monoclonal antibodies for M2 macrophage were anti-CD206 and anti-CD163 (BD Biosciences). The stained cells were analyzed with FACS Calibur (BD Biosciences). FlowJo software 8.7.1 (Treestar Inc., USA) was used to access the results.

**Exosome isolation and identification**
Ultracentrifugation was used to isolate exosomes. To remove the precipitates, cell culture supernatant of M2 macrophages was centrifuged for 10 min at 500 g. To remove cell debris, supernatant was centrifuged at 4°C for 10 min at 2000 g. With a 0.22-µm filter, obtained supernatant was filtered followed by centrifuged in an ultracentrifuge tube at 100 000 g for 4 h. The precipitates were then resuspended in PBS before centrifuged for 70 min at 100 000 g. Acquired precipitates were exosomes. A transmission electron microscope was utilized for exosome morphology measurement. For Nanoparticle tracking analysis (NTA), exosomes (3 × 10^7 – 5 × 10^7 particles/mL) were measured by a ZetaView PMX 110 instrument and accessed by ZetaView 8.04.02 SP2. Western blot analysis was applied for exosome surface markers detection and BCA kit (Thermo Fisher, Shanghai, China) was utilized for exosome protein content measurement. SDS-PAGE was prepared before protein denaturation and electrophoresis. Exosomes were removed to membrane followed by examination of exosome-specific marker protein CD63, CD9, CD81, TSG101 (1:500, Abcam) expression. Tubulin (1:1000, Abcam) was detected as internal reference. Then, PKH67 green membrane dye (Sigma) was used to label exosomes fluorescently. Briefly, 5 µL of PKH67 with 1 mL of diluent C was added to 20 µg of protein equivalents of exosomes and incubated for 15 min at room temperature. To stop labeling, 1 mL of 1% BSA was added. After that, mixture was added to 22 mL of PBS before centrifuged for 80 min at 4°C to eliminate excess PKH67 dye. The supernatant was removed, and the pellets of PKH67-labeled exosomes were resuspended in PBS (100 µL).

**MTT assay**

Cells in logarithmic phase were removed into 96-well plates (2 × 10^3 cells/well). Each well was added with MTT solution (Sigma) and cells were incubated for 4 h, then DMSO (150 µL) addition and incubation for 15 min. Optical absorbance at 570 nm wavelength was detected.

**Iron assay**

Iron assay kit (Abcam) was utilized for intracellular ferrous iron (Fe^{2+}) level measurement following manufacturer’s guideline. In brief, samples were collected and washed in cold PBS. 5 × volumes of iron assay buffer were applied to homogenize samples on ice. The supernatant was collected, and each sample was added with iron reducer before mixing, and then they were incubated for 0.5 h. After addition with iron probe to each sample before mixing, they were incubated for 1 h. Colorimetric microplate reader (OD 593 nm) was applied to analyze the output.

**Malondialdehyde (MDA) assay**

Lipid Peroxidation Assay Kit (Abcam) was applied to analyze MDA concentration in cell lysates following instructions. In brief, the MDA in sample reacted with TBA was used to produce MDA-TBA adducts, which was measured colorimetrically (optical density (OD) = 532 nm).

**Lipid ROS analysis**

Lipid ROS was stained by C11-BODIPY 581/591 (Thermo Fisher). In brief, cells were trypsinized before incubated in HBSS with C11-BODIPY 581/591 (2 µM) in dark for 30 min at 37°C and then resuspended in fresh HBSS. Oxidation of the polyunsaturated butadienyl portion of the dye led to a shift of fluorescence emission peak (~ 590 nm to ~ 510 nm) measured by FACS Calibur™ flow cytometer.

**Mitochondrial superoxide**

Mitochondrial superoxide was detected with fluorescent probes MitoSOX™ (Thermo Fisher). Briefly, cells (1 × 10^5 cells/well) were plated in 12 well plates and allowed to attach overnight. Subsequently, cells were incubated with 2.5 µM MitoSOX for 20 min. Flow cytometry at 488nmEx/585nmEm was utilized for fluorescence detection.

**Determination of mitochondrial membrane potential (MMP)**

Mitochondria Staining Kit (JC-1, MultiSciences Biotech, China) was used to evaluate the MMP. Cells were collected and loaded with JC-1 (2 µM) for 20 min at 37°C, 5% CO2, then accessed with flow cytometry (BD Biosciences) with excitation at 488 nm and emission at 530 nm and 590 nm. MMP was exhibited at 590/530 nm emission and normalized to respective control group.

**Luciferase reporter assay**

Putative miR-451a binding sites in circ_TNFRSF21 and the 3′-UTR of SLC7A11 were predicted using starBase, respectively. A wild-type (WT) and mutant (MUT) circ_TNFRSF21 sequence and a WT and MUT 3′-UTR fragment of SLC7A11 containing the putative miR-451a binding sites were synthesized at Genechem (Shanghai, China). The constructs were cloned into the psiCHECK-2 luciferase reporter
plasmid (Promega, Madison, WI, USA) to generate luciferase reporter systems including circ_TNFRSF21-WT, circ_TNFRSF21-MUT, SLC7A11-WT and SLC7A11-MUT. After seeded in six-well plates, cells were co-transfected with luciferase reporter plasmids and mimics NC or miR-451a mimics. After 48 h transfection, cells were lysed and accessed by dual-luciferase reporter assay system (Promega). Evaluations were performed on Beckman-Coulter DTX880. Experiments were carried out for at least four replicates for three times independently. Renilla luciferase was used to normalize for transfection efficiency.

**RT-qPCR**

RNA was extracted from cells and tissues with TRizol method (Takara). cDNA was gained by RNA reverse transcription with reverse transcription kits (K1621, Fermentas). Fluorescence quantitative PCR kit (Takara,) was adopted for gene detection by employing RT-qPCR (ABI 7500, ABI). U6 and GAPDH were used as internal control. The relative expression of target genes was calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequences were listed in Table 1.

**Western blot analysis**

Protein was extracted from cell, tissue or exosome by RIPA lysis buffer (Sigma). Supernatant was obtained by centrifugation at 4°C for 15 min at 12,000 rpm. Afterwards, a BCA kit (Beyotime, shanghai, China) was applied to determine the protein concentration. Then protein was separated with SDS-PAGE and removed onto PVDF membrane followed by blocking with 5% skim milk. They were incubated with primary antibodies: anti-SLC7A11 (1: 500, Abcam) and anti-GAPDH (1: 1000, Abcam) at 4°C overnight. Subsequently, they were incubated with horseradish peroxidase (HRP)-labeled second antibodies (1: 1000, Abcam) for 1 h at room temperature. Protein quantitative analysis was performed with Image J 1.48u software. Protein expression was exhibited as ratio of target band gray values to GAPDH.

**Statistical analysis**

All results were accessed with SPSS 22.0 software and consistent with homogeneity variance test and normal distribution. The measurement results were presented as mean ± SD. Differences among multiple groups were evaluated with one-way ANOVA followed
by Tukey’s post hoc test, while differences between two groups were evaluated with independent sample t test. A value of p < 0.05 was considered to indicate a statistically significant.

Results

1. **SLC7A11 was up-regulated and correlated with the declined ferroptosis in cSCC tissues**

To explore the role of SLC7A11 in cSCC, western blotting and RT-qPCR assays were employed. In comparison to adjacent noncancerous tissues (P represents for adjacent tissues), both mRNA and protein levels of SLC7A11 were apparently increased in cSCC tissues (T represents for tumor tissues) (Fig. 1A, B). We then analyzed the association between SLC7A11 level and survival time of cSCC patients. It was found that patients with higher SLC7A11 level exhibited shorter survival time than those with lower SLC7A11 level (Fig. 1C). In comparison with normal tissues, lipid-ROS level was significantly decreased in cSCC tissues, which indicated an inhibited ferroptosis in cSCC (Fig. 1D). SLC7A11 level was correlated negatively with lipid-ROS accumulation presented by correlation analysis (Fig. 1E). These data indicated that SLC7A11 was up-regulated and negatively correlated with ferroptosis in cSCC.

2. **M2 macrophages were increased in cSCC tissues and inhibited ferroptosis through exosomes**

We study the role of M2 macrophages in ferroptosis during cSCC. The data from immunohistochemistry revealed that CD68 expression was higher in cSCC tissues than in adjacent noncancerous tissues (Fig. 2A). The amount of CD11b⁺CD68⁺ macrophages in cSCC tissues was obviously increased in comparison with adjacent noncancerous tissues, which was analyzed from flow cytometry (Fig. 2B). M2 macrophages markers CD163 and CD206 were then detected to be increased in normal tissue macrophages (NTMs) and TAMs (Fig. 2C). RT-qPCR was applied to detect tumor-related M1 and M2 macrophage markers. The mRNA expression levels of M2 macrophage markers, including IL-4, FIZZ-1, IL-10, irf-4 and Arg-1 in TAMs were up-regulated, while the mRNA levels of M1 macrophage markers, that was TNF-α, irf-5, iNOS and IL-6, were declined (Fig. 2D). The mRNA level of M2 macrophage marker CD163 in cSCC tissues was elevated. Furthermore, we found that the CD163 level was correlated negatively with the lipid-ROS (Fig. 2E, F). Moreover, lipid-ROS level in cSCC cells was increased after treated with Erastin. However, M2 macrophages inhibited Erastin-induced ferroptosis, which could be neutralized with exosome inhibitor GW4869 (Fig. 2G). Exosomes were isolated from M2 macrophages and presented as vesicles with double membrane observed under electron microscopy (Fig. 2H). NTA further determined that the diameters of these vesicles ranged from 100 nm to 150 nm (Fig. 2I). Exosome markers, including CD63, CD9, CD81 and TSG101, were enhanced in the extracted exosomes from M2 macrophages (Fig. 2J). PKH67-labeled M2 exosomes were detected with immunofluorescence and the data presented that PKH67 was expressed in cSCC cells (Fig. 2K). The data revealed that M2 exosomes contributed to inhibit ferroptosis of cSCC.

3. **M2 exosomes inhibited ferroptosis of cSCC cells by circ_TNFRSF21**

Given that M2 exosomes inhibited ferroptosis in cSCC, the underlying mechanism is necessary to investigate. Circ_TNFRSF21 expressions in different types of macrophages and M2 exosomes were measured with RT-qPCR, the data indicated that in comparison to those in M0 and M1 macrophages, circ_TNFRSF21 levels were significantly increased in both M2 macrophages and exosomes (Fig. 3A). Circ_TNFRSF21 expression was reduced in exosomes isolated from M2 macrophages transfected with sh-circ_TNFRSF21 (Fig. 3B). In comparison with control group administrated with PBS, SLC7A11 protein level was up-regulated after M2 exosomes treatment in A431 and SCL-1 cells. However, the up-regulated SLC7A11 expression induced by M2 exosomes could be reduced after circ_TNFRSF21 knockdown (Fig. 3C). The data from MTT assay exhibited that Erastin inhibited cell viability, which could be elevated by M2 exosomes. Besides, knockdown of circ_TNFRSF21 in M2 exosomes reversed the effect of M2 exosomes on cell viability (Fig. 3D). Erastin increased iron and Fe⁺⁺ level in cSCC cells, while M2 exosomes decreased iron and Fe⁺⁺. The effect of M2 exosomes on the levels of iron and Fe⁺⁺ were reversed by knocking down of circ_TNFRSF21 in M2 exosomes (Fig. 3E-F). MDA, lipid-ROS and mitochondrial superoxide were elevated after treated with Erastin, which were declined by M2 exosomes. In addition, knockdown of circ_TNFRSF21 in M2 exosomes had the opposite effect (Fig. 3G-I). Compared with control group, Erastin reduced mitochondrial membrane potential. The inhibitory effect of Erastin on mitochondrial membrane potential could be reversed by M2 exosomes,
whereas circ_TNFRSF21 knockdown antagonized the effect of M2 exosomes (Fig. 3J). Collectively, the above results suggested that M2 exosomes inhibited ferroptosis by circ_TNFRSF21 in cSCC cells.

4. Circ_TNFRSF21 targeted miR-451a while miR-451a bound to SLC7A11

Previous studies suggested that circRNAs can interact with miRNAs to regulate the expression of its target gene. We then explored whether circ_TNFRSF21 regulated cSCC ferroptosis by interacting with miRNAs. Bioinformatic analysis with Starbase revealed potential binding sites between circ_TNFRSF21 and miR-451a (Fig. 4A). Compared to the adjacent noncancerous tissues, miR-451a in cSCC tissues was down-regulated (Fig. 4B). Dual luciferase assay was applied to evaluate binding relationship between circ_TNFRSF21 and miR-451a. Luciferase activity in miR-451a mimics group was significantly declined after co-transfected with circ_TNFRSF21 WT, whereas there was no change after transfected with circ_TNFRSF21 MUT. These results indicated that circ_TNFRSF21 directly targeted miR-451a (Fig. 4C). It was forecasted that SLC7A11 bound to miR-451a (Fig. 4D). Dual-luciferase reporter gene assay revealed that the luciferase activity of cells co-transfected with miR-451a mimics and SLC7A11-WT decreased remarkably, while no obvious difference was found in luciferase activity of cells co-transfected with miR-451a mimics and SLC7A11-MUT, indicating that miR-451a specifically bound to SLC7A11 (Fig. 4E). These observations suggested that circ_TNFRSF21 potentially targeted miR-451a to regulate SLC7A11 expression.

5. MiR-451a promoted ferroptosis of cSCC cells through regulation of SLC7A11

To explore cellular functions of miR-451a in ferroptosis of cSCC cells, cSCC cells were transfected with OE-SLC7A11 or miR-451a mimics. The transfection efficiency of miR-451a mimics and OE-SLC7A11 was confirmed by RT-qPCR (Fig. 5A, B). We performed western blot assay to determine SLC7A11 expression in cSCC cells. The results illustrated that SLC7A11 declined in miR-451a mimics group in comparison by the mimics NC group, whereas SLC7A11 was elevated in cells co-transfected with miR-451a mimics and OE-SLC7A11 (Fig. 5C). Relative to the Erastin induction, cell viability was reduced by miR-451a mimics, whereas OE-SLC7A11 reversed the effect of miR-451a mimics (Fig. 5D). Iron and Fe$^{2+}$ levels in Erastin + miR-451a mimics group rose markedly compared with Erastin treatment group. Besides, Iron and Fe$^{2+}$ levels were down-regulated after overexpression of SLC7A11 (Fig. 5E, F). The findings illustrated that MDA, lipid-ROS and mitochondrial superoxide levels in cSCC cells co-cultured with Erastin and miR-451a mimics were elevated while SLC7A11 overexpression restored effects of miR-451a mimics on MDA, lipid-ROS and mitochondrial superoxide levels (Fig. 5G-I). In comparison to that in cSCC cells treated with Erastin and mimics NC, mitochondrial membrane potential was decreased by miR-451a mimics, which could be restored by OE-SLC7A11 treatment (Fig. 5J). Altogether, the above data demonstrated that miR-451a promoted ferroptosis through regulation of SCL7A11 in cSCC cells.

6. M2 exosomes regulated miR-451a and SLC7A11 to inhibit ferroptosis of cSCC cells by circ_TNFRSF21

To investigate molecular mechanisms of M2 exosomes on ferroptosis, cSCC cells were transfected with sh-SLC7A11 or miR-451a mimics. The results showed that compared with that in Erastin + PBS group, M2 exosomes elevated the cell viability in the cSCC cells incubated with Erastin, which was declined markedly by overexpression of miR-451a or inhibition of SLC7A11 (Fig. 6A). Compared with the levels in Erastin + PBS group, iron and Fe$^{2+}$ concentrations in the Erastin + M2 exosomes group diminished noticeably, and overexpression of miR-451a or inhibition of SLC7A11 reversed the effects of M2 exosomes (Fig. 6B, C). We then examined the impact of sh-SLC7A11 or miR-451a mimics on MDA, lipid-ROS and mitochondrial superoxide in cSCC cells. Versus the Erastin + PBS group, the expressions of MDA, lipid-ROS and mitochondrial superoxide in Erastin + M2 exosomes group fell dramatically, while the effects of M2 exosomes could be neutralized by inhibition of SLC7A11 or overexpression of miR-451a (Fig. 6D-F). Subsequently, we further observed that M2 exosomes up-regulated the mitochondrial membrane potential in cSCC cells compared to Erastin + PBS group. The effect of M2 exosomes on the mitochondrial membrane potential could be antagonized by sh-SLC7A11 or miR-451a mimics (Fig. 6G). Based on these results, we concluded that M2 exosomes inhibited ferroptosis in cSCC cells by circ_TNFRSF21/ miR-451a/SCL7A11 axis.

Discussion

The cSCC has become the second most frequent cancer with the increasing incidence [3]. Although surgical excision can successfully eradicated most cases of cSCC, cSCC have the characters of higher probability of recurrence, metastasis and death, [1]. Recently, M2
phenotype of TAMs exhibits pro-tumoral and anti-inflammatory effects [27]. Nevertheless, the role of M2 macrophage derived exosomes in cSCC remains to be elucidated. The purpose of this research was to study the effect of M2 exosomes on ferroptosis of cSCC cells. Our results proved that circ_TNFRSF21 knockdown in M2 exosomes up-regulated miR-451a level to suppress SLC7A11 expression, which further promoted ferroptosis in cSCC. The present study is the first study regarding the relationship between M2 macrophage exosomes and ferroptosis in cSCC.

Accumulating evidence has reported that ferroptosis is highly related with cancer occurrence and development[28]. The fast-growing researches on ferroptosis in cancer have improved an insight for its applying for cancer treatment [29]. Ferroptosis induction has presented perspective anti-tumour actions in cancer [30]. However, the role of ferroptosis in cSCC is still not fully elucidated. We found that ferroptosis declined in cSCC, as indicated by the significantly decreased lipid-ROS level in cSCC tissues. SLC7A11, the cystine/glutamate antiporter, has been demonstrated to import cystine for glutathione biosynthesis and antioxidant defense [31]. SLC7A11 is up-regulated in many cancers and intimately related with poor prognosis of patients [32]. Recent studies have revealed that SLC7A11 up-regulation suppresses ferroptosis to facilitate the growth of tumor [31]. For instance, targeting SLC7A11 specifically inhibits colorectal cancer stem cell progression through regulating ferroptosis [33]. OTUB1 inactivation destabilizes SLC7A11, resulting the inhibition of tumor xenographs growth in mice, which is related with ferroptosis suppression [34]. SLC7A11 expression can be declined by benzopyran derivative 2-imino-6-methoxy-2H-chromene-3-carbothioamide (IMCA), contributing to ferroptosis and oxygen species accumulation in colorectal cancer [35]. In lung cancer, SLC7A11 is involved in ferroptosis regulation of dihydroartemisinin on repressing cell colony formation and proliferation [36]. In this context, we discovered that SLC7A11 was elevated in cSCC tissues, and SLC7A11 was negatively correlated with patient survival. Furthermore, SLC7A11 was negatively correlated with lipid-ROS accumulation. These results suggested that SLC7A11 acted as a target for regulation of ferroptosis in cSCC cells.

In parallel with regulatory T cells (Tregs), higher numbers of CD163+ M2 macrophages were detected only in cSCC [37]. We found that CD11b*CD68+ macrophages in cSCC tissues were significantly abundant, M2 macrophages markers CD163 and CD206 were increased, mRNA levels of M2 macrophage markers including IL-4, FIZZ-1, IL-10, irf-4 and Arg-1 in cSCC tissues were up-regulated. M2 exosomes play a significant role in facilitating the growth and angiogenesis in pancreatic ductal adenocarcinoma [15]. Macrophage-derived exosomal miR-501-3p accelerates pancreatic ductal adenocarcinoma development [38]. Cancer-associated fibroblasts-derived exosomal miR-522 inhibits ferroptosis in gastric cancer [39]. We also demonstrated erastin-induced ferroptosis, as indicated by elevated lipid-ROS level, could be inhibited by M2 macrophages, whereas reversed by exosome inhibitor GW4869. These results provided evidence that exosomes secreted by M2 macrophages could inhibit ferroptosis in cSCC. Migration and metastasis in esophageal cancer can be modulated by M2 macrophage-derived exosomal IncRNA AFAP1-AS1 and MiR-26a [40]. Reduction of IncRNA SBF2-AS1 in M2 exosomes up-regulates miR-122-5p to inhibit XIAP, thus suppressing the development of pancreatic cancer [41]. Circ_TNFRSF21 is up-regulated in cSCC compared to healthy skin [26]. Overexpression of circ_TNFRSF21, which is highly expressed in non-small cell lung cancer cells, induces a significant increase in colony formation [42]. Circ_TNFRSF21 facilitates endometrial carcinoma development via modulating miR-1227-MAPK13/ATF2 axis [25]. We found that circ_TNFRSF21 expression were increased in both M2 macrophages and M2 exosomes. Erastin treatment reduced cell viability, increased iron and Fe2+, up-regulated MDA level, lipid-ROS and mitochondrial superoxide, as well as declined mitochondrial membrane potential, which could be reversed by M2 exosomes. Interestingly, circ_TNFRSF21 knockdown antagonized these effects of M2 exosomes. These findings provide evidence that M2 exosomes inhibited ferroptosis of cSCC cells via regulating circ_TNFRSF21.

CircRNAs competes with miRNAs to affect the target RNAs, modulating gene expression at transcriptional level [43]. For example, CircIL4R modulates miR-541-3p/GPX4 pathway to suppress ferroptosis and promote tumorigenesis in hepatocellular carcinoma [44]. Circ_0008035 regulates miR-599/Eif4A1 axis to suppress ferroptosis and induce cell proliferation in gastric cancer[45]. MiR-451a can suppress proliferation and promote apoptosis in colorectal cancer [46]. MiR-451a reduced the aggressiveness of cancer cell in lung squamous cell carcinoma [47]. MiR-451a regulates PI3K/AKT pathway mediated by 3-phosphoinositide-dependent protein kinase-1, thereby inhibiting cSCC progression [48]. It remained to be elucidated whether miR-451a was involved in regulating ferroptosis. In the research, the results revealed that circ_TNFRSF21 targeted miR-451a, and miR-451a bound to SLC7A11 and negatively regulated SLC7A11 expression. These observations identified miR-451a as a regulatory link between circ_TNFRSF21 and SLC7A11. In addition, miR-451a overexpression facilitated Erastin-induced alterations on ferroptosis, which was antagonized by up-regulation of SLC7A11. MiR-451a can be sponged by IncRNA SNHG12 to modulate cell proliferation, migration and invasion in breast cancer [49]. In human colorectal cancer, miR-451a is targeted by long intergenic non-protein coding RNA-467 [50]. Erastin-induced ferroptosis was reversed by M2 exosomes. Interestingly, SLC7A11 knockdown or miR-451a overexpression eliminated these effects of M2 exosomes. Therefore, we concluded that M2 exosomes regulated miR-451a and SLC7A11 to inhibit ferroptosis of cSCC cells through circ_TNFRSF21.
In conclusion, our research presented that circ_TNFRSF21 knockdown in M2 exosomes increased miR-451a expression to reduce SLC7A11 level, thus promoting ferroptosis in cSCC. This research provides novel clues for the role of circ_TNFRSF21/miR-451a/SLC7A11 axis in cSCC, more importantly, a novel therapeutic approach for cSCC, which is significant to human beings.

**Declarations**

**Ethical approval and consent to participate**

The study methods have been approved by the Ethics Committee of Nanfang Hospital Southern Medical University. Investigational methods used in this study were conducted according to the standards set out in the Announcement of Helsinki and related guidelines in China.

**Conflict of interest**

The authors in this study have declared no potential conflict of interest.

**Availability of data and material**

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Funding**

This work was supported by the Natural Science Foundation of Guangdong Province [No.2020A151501107], the Guangdong Province Key Field R&D Program Project [No.2020B1111150001], the Science and Technology Innovation Project of Guangdong Province [No.2018KJYZ005], the Natural Science Foundation of Tibet Autonomous Region [No. XZ2017ZR-ZY021].

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**References**


Itraconazole Inhibits the Growth of Cutaneous Squamous Cell Carcinoma by Targeting HMGCS1/ACSL4 Axis. Front Pharmacol 2022, 13:828983.


M2 Macrophage-Derived Exosomes Facilitate HCC Metastasis by Transferring alphaM beta2 Integrin to Tumor Cells. Hepatology 2021, 73:1365-1380.


GATNNCDA: A Method Based on Graph Attention Network and Multi-Layer Neural Network for Predicting circRNA-Disease Associations. Int J Mol Sci 2022, 22.


Circular RNA promotes endometrial carcinoma pathogenesis through regulating miR-1227-MAPK13/ATF2 axis. Aging (Albany NY) 2020, 12:6774-6792.


Figures

Figure 1

SLC7A11 was associated with ferroptosis in cSCC.

Tumor samples (T) and adjacent normal tissues (P) were obtained from 12 cSCC patients. (A, B) SLC7A11 level was measured with western blot and RT-qPCR. (C) Relationship between SLC7A11 level and the survival time of patients. (D) The level of lipid-ROS was detected. (E) Correlation analysis of SLC7A11 and lipid-ROS. *P<0.05, **p<0.01, ***P<0.001.
Figure 2

M2 macrophages were increased in cSCC tissues and inhibited ferroptosis via exosomes.

(A) The expression of CD68 detected by immunohistochemistry. (B) Flow cytometry was applied to analyze the enrichment of CD11b+CD68+ macrophages in CD45+ cells. (C) The number of M2 macrophages (CD163+CD206+) in total macrophages was evaluated with flow cytometry. (D) mRNA expression of M2 macrophages markers (IL-4, FIZZ-1, IL-10, irf-4, and Arg-1) and M1 macrophages markers (TNF-α, irf-5, iNOS, IL-6) accessed by RT-qPCR. (E) CD163 expression was determined with RT-qPCR. (F) Correlation analysis of CD163 and lipid-ROS. (G) The level of lipid-ROS. (H) Typical exosomes were detected under electron microscopy. (I) NTA for determination of vesicle diameter. (J) Exosome markers CD63, CD9, CD81, TSG101 analyzed with western blotting. (K) Immunofluorescence analysis of PKH67 labeled M2 macrophage exosomes. *P<0.05, **P<0.01, ***P<0.001.
Figure 3

M2 exosomes inhibited ferroptosis of cSCC by circ_TNFRSF21.

(A) The expressions of circ_TNFRSF21 in M0, M1, M2 and M2 exosomes were evaluated by RT-qPCR. (B) Circ_TNFRSF21 level in exosomes after knockdown of circ_TNFRSF21 in M2 macrophages detected by RT-qPCR. (C) SLC7A11 expression after treatment with M2 exosomes in cSCC. (D-I) A431 and Scl-1 cells were cultured and incubated with erastin, exosomes from M2 macrophage, or exosomes from M2 macrophage transfected with sh-circ_TNFRSF21, alone or in combination. Then we evaluated the effects of exosomes from sh-circ_TNFRSF21-transfected M2 macrophage on ferroptosis in cSCC cells. (D) Cell activity was detected by MTT. (E, F) Analysis of total iron and Fe^{2+} levels. (G) Detection of MDA expression. (H) Lipid-ROS measurement. (I) Mitochondrial superoxide detection. (J) Mitochondrial membrane potential. *P<0.05, **P<0.01, ***P<0.001.
Circ_TNFRSF21 targeted miR-451a and miR-451a bound to SLC7A11.

(A) Forecasted binding sequences between circ_TNFRSF21 and miR-451a. (B) miR-451a level in cSCC tissues measured with RT-qPCR. (C) The relationship between circ_TNFRSF21 and miR-451a was detected with dual luciferase assay. (D) Predicted binding sequences between miR-451A and SLC7A11. (E) The association between miR-451A and SLC7A11 was determined by dual luciferase assay.

*P<0.05, **P<0.01, ***P<0.001.
miR-451a promoted ferroptosis of cSCC cells through SCL7A11.

(A, B) The overexpression efficiency of miR-451a and SCL7A11 was evaluated with RT-qPCR. (C) SCL7A11 level after cSCC cells co-transfected with miR-451a mimics and OE-SCL7A11 was detected by western blot. (D) Cell viability was determined by MTT. (E, F) Analysis of total iron (E) and Fe$^{2+}$ (F) levels. (G-I) MDA (G), lipid-ROS (H) and mitochondrial superoxide (I) detection. (J) Mitochondrial membrane potential measurement. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. 

Figure 5
Figure 6

M2 exosomes regulated miR-451a and SLC7A11 to inhibit ferroptosis of cSCC cells through circ_TNFRSF21.

Cells were cultured and treated with Erastin, M2 exosomes, miR-451a mimics or sh-SLC7A11, alone or in combination. (A) Cell viability was evaluated with MTT. (B, C) Total iron and Fe^{2+} levels were analyzed. (D) MDA level detection. (E) Determination of lipid-ROS level. (F) Mitochondrial superoxide was assessed. (G) Measurement of mitochondrial membrane potential. *P<0.05, **P<0.01, ***P<0.001.