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MiRNA-30a-mediated autophagy inhibition sensitizes renal cell carcinoma cells to sorafenib

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ABSTRACT

Chemotherapy-induced autophagy activation often contributes to cancer resistance. MiRNA-30a (miR-30a) is a potent inhibitor of autophagy by downregulating Beclin-1. In this study, we characterized the role of miR-30a in sorafenib-induced activity in renal cell carcinoma (RCC) cells. We found that expression of miR-30a was significantly downregulated in several human RCC tissues and in RCC cell lines. Accordingly, its targeted gene *Beclin-1* was upregulated. Sorafenib activated autophagy in RCC cells (786-0 and A489 lines), evidenced by p62 degradation, Beclin-1/autophagy protein 5 (ATG-5) upregulation and light chain (LC)3B-I/-II conversion. Exogenously expressing miR-30a in 786-0 or A489 cells inhibited Beclin-1 expression and enhanced sorafenib-induced cytotoxicity. In contrast, knockdown of miR-30a by introducing antagomiR-30a increased Beclin-1 expression, and inhibited sorafenib-induced cytotoxicity against RCC cells. Autophagy inhibitors, including chloroquine, 3-methyladenine or Bafliomycin A1, enhanced sorafenib activity, causing substantial cell apoptosis. Meanwhile, knockdown of Beclin-1 or ATG-5 by targeted siRNAs also increased sorafenib-induced cytotoxicity in above RCC cells. These findings indicate that dysregulation of miR-30a in RCC may interfere with the effectiveness of sorafenib-mediated apoptosis by an autophagy-dependent pathway, thus representing a novel potential therapeutic target for RCC.

1. Introduction

Renal cell carcinoma (RCC) accounts for 2–3% of all human cancers, and its incidence is steadily rising [1]. Many RCC patients are found at advanced stages with local or systematic metastasis, these patients are suffering poor prognosis [1]. Due to pre-existing or acquired resistances, chemotherapy, hormonal therapy or radiation only demonstrated limited value for advanced or metastatic RCC [2]. Many factors contribute to the resistance of RCC, and activation of autophagy is one main contributor [3].

Under various stress conditions, autophagy activation helps cells clear damaged proteins or organelles through lysosomal degradation, thus providing energy and nutrients for cells to survive [4]. In cancer cells, autophagy induction is considered as a pro-survival and resistance factor [4,5]. Numerous studies have reported autophagy activation by various anti-cancer therapies in cancer. Accordingly, autophagy inhibition, through genetic or pharmacological means, could sensitize multiple anti-cancer-targeted therapies [4,6]. Our previous study showed that autophagy blockage enhanced AZD-2014, a mTORC1/2 dual inhibitor, -induced anti-RCC activity *in vitro* and *in vitro* [7].

MicroRNAs (miRs) inhibit translation of target mRNAs through binding to their 3′ untranslated regions (UTRs) [8,9]. MiRs dysregulation is a general feature of RCC and many cancers [10,11], which is an important contributor of cancer progression and/or oncogenic activities [10]. Further, MiRs are able to accurately classify RCC samples. Deregulated miRs may cause the high chemo-resistance of RCC [10,12]. Many alterations in miRs have been indentified in different cancers, but their functional significance is largely unknown [13,14]. Studies have identified several autophagy-related miRs (i.e. miR-30a, miR-101) which decrease autophagic activity to exert cancer suppressive roles [11,15].

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Groups including ours [7] are searching for novel and more efficient anti-RCC agents [16,17]. Sorafenib, an orally active multi-kinase inhibitor, has shown significant anti-cancer activities in culture cancer cells and in animal models [18]. Sorafenib is able to inhibit cancer growth, either by inhibiting angiogenesis or by directly inducing cancer cell apoptosis [19]. Sorafenib displayed activities in murine RCC model and in the von Hippel–Lindau (*VHL*) knockout model [20,21]. The aim of this study is to sensitize sorafenib-induced activity in RCC cells through miR-30a-mediated regulation of autophagy.

2. Material and methods

2.1. Reagents and chemicals

Sorafenib was obtained from Selleck China (Shanghai, China). Chloroquine (Cq), 3-methyaldenine (3-MA) and Bafliomycin A1 (Baf-A1) were obtained from Sigma–Aldrich Co. (St. Louis, MO). Anti-light chain 3B (LC3B), Beclin-1, autophagy protein 5 (ATG-5) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cells

Human RCC cell lines 786-0, A498 and SK-RC-44 (SK-RC) were purchased from Shanghai Biological Institute (Shanghai, China). RCC cells were grown in DMEM/RPMI supplemented with 10% fetal bovine serum (FBS). HK-2 cells, an immortalized proximal tubule epithelial cell line from adult human kidney [22], were cultured as described [7].

2.3. Methylthiazol tetrazolium (MTT) assay

Cell survival was assessed through MTT assay with recommended protocol (Roche Diagnostics) as described [7].

2.4. Annexin V assay of cell apoptosis

As reported [7], cells were washed and incubated in 500 μl binding buffer, 5 μl annexin V-FITC and 5 μl of propidium iodide (PI) (Invitrogen, Karlsruhe, Germany) at room temperature for 15 min, which were then detected through fluorescence-activated cell sorting (FACS) with a Becton–Dickinson machine (San Jose, CA). Annexin V positive cells were labeled as apoptotic cells, and its percentage was recorded as the indicator of apoptosis intensity.

2.5. Histone/DNA ELISA for detection of apoptosis

The Cell Death Detection ELISA Kit was used for assessing apoptosis in cancer cells following indicated treatments according to the protocol provided by the manufacturer. Briefly, the cells were lysed and the cell lysates were overlaid and incubated in microplate plate modules coated with anti-histone antibody. Samples were then incubated with anti-DNA peroxidase followed by color development with ABTS substrate. The absorbance of the samples was determined with a microplate reader (Tecan, Durham, NC) at 405 nm.

2.6. Western blots

Cells were lysed in lysis solution (Cell Signaling, Shanghai, China) supplemented with sodium fluoride (10 μM). Lysates were fractionated on polyacrylamide gels and transferred to nitrocellulose. The blots were probed with specific primary antibody, followed by a second antibody-horseradish peroxidase (HRP) conjugate and then incubated with Super-Signal substrate (Pierce). Band intensity was quantified through ImageJ software (NIH, USA), before normalization to each loading control band.

2.7. Real-time PCR assay detecting miR-30a or Beclin-1 mRNA

Total RNA was extracted from cells or tissue samples using TRIzol (Invitrogen) according to the manufacturer's protocol. The RNA concentrations were determined using a biophotometer (Eppendorf), and reverse transcription (RT) was performed using TOYOBO ReverTra Ace-a RT-PCR kit (Toyobo, Osaka) according to the manufacturer's instructions. The real-time PCR was performed on a Bio-Rad IQ5 multicolor detection system (Bio-Rad, Hercules, CA) using 1 μg of starting RNA per sample. The following human *Beclin-1* primers were used: forward: 5′-CAA GAT CCT GGA CCG TGT CA-3′; reverse: 5′-TGG CAC TTT CTG TGG ACA TCA-3′ [15]. The following human *GAPDH* primers were used: forward: 5′- TGC ACC ACC AAC TGC TTA-3′; reverse: 5′- GGA TGC AGG GAT GAT GTT C-3′ [23]. Primers for miR-30a were from Ref. [24]. One RNA sample of each preparation was processed without real time-reaction to provide a negative control in subsequent PCR. After amplification, melt curve analysis was performed to analyze product melting temperature. *GAPDH* gene was chosen as the reference gene for normalization, and the $2^{-\Delta\Delta Ct}$ method was applied to quantify targeted mRNA change within samples [26].

2.8. Recombinant expressing miR-30a or antagomir-30a in RCC cells

The recombinant miR-30 or antagomir-30a was generated by PCR (see Ref. [25]) and obtained from Kaiji Biotech (Shanghai, China). MiRNA precursor sequences were amplified using the primers listed in Ref. [25]. The fragments were cloned into a p-Super-GFP-puro driven vector. Empty p-Super-GFP-puro was used as the control construct. LipofectamineTM and PLUS reagent (Invitrogen, Carlsbad, CA) were applied to transfect miR-30a/antagomir-30a plasmid or the vector (1 μg/ml) into RCC cells according the manufacturer's protocol. The stable clones were selected by puromycin (0.5 μg/ml medium) for a total of 4 days.

2.9. RNA interference

For transient inhibition of Beclin-1, 786-0 cells or A489 cells were transfected with the commercially available 20 to 25 nucleotide-long siRNAs designed to “knockdown” Beclin-1 (Beclin-1 siRNA-1 from Cell Signaling Tech [7], Beclin-1 siRNA-2 from Santa Cruz). For siRNA knockdown of ATG-5, ATG-5 siRNAs were designed by Kaiji Biotech. ATG-5 siRNA-1 sequence: GCAACUCUGG AUGGGAUUGdTdT [26], ATG-5 siRNA-2 sequence: GACGUUGGUAA CUGACAAAdTdT [26]. SiRNA (200 nM each) transfection was performed through Lipofectamine 2000 (Invitrogen) according to the procedure [7]. The transfection took 24 h, and whole transfection was repeated again after 24 h, expressions of targeted protein (ATG-5 and Beclin-1) and GAPDH (loading) were tested by Western blots.

2.10. Patient's RCC tissues isolation and preparation

Surgery-isolated fresh human RCC tissues of different-grades (AJCC stages 1–4) and their surrounding normal tissues were homogenized and lysed, proteins and RNAs were isolated and were examined by Western blots and Real time-PCR, respectively. The study was approved by the institutional review board of all authors' affiliations, and written informed consent was obtained from each participating patient. All clinical investigations were conducted

Table 1
Basic information of the patients with renal cell carcinoma of this study.

Stages	AJCC stages 1–2					AJCC stages 3–4				
Patient ID	1	2	3	4	5	6	7	8	9	10
Gender (M/F)	M	M	F	M	F	M	F	M	M	F
Age/year	68	58	59	62	72	65	68	79	64	75

according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The privacy rights of human subjects are always observed.

2.11. Statistical analyses

Statistical analyses were performed as described [7]. A p value <0.05 was considered statistically significant.

3. Results

3.1. MiR-30a downregulation correlates with Beclin-1 upregulation in human RCC tissues and cell lines

We first examined the expression of miR-30a in human RCC tissues ("T"), and compared its level with that in normal surrounding kidney tissues ("N"). The basic information of RCC patients of this study was summarized in Table 1. Total ten RCC samples were analyzed, five of them were AJCC stages 1–2, the other five were AJCC stages 3–4 (Table 1). Real-time PCR results clearly showed that miR-30a is downregulated in RCC tissues (Fig. 1A). MiR-30a downregulation in RCC tissues was correlated with cancer progression, and high-grade cancers (AJCC stages 3–4) had lesser miR-30a than low-grade cancers (AJCC stages 1–2) (Fig. 1A). On the other hand, mRNA expression of Beclin-1, the miR-30a target gene [15,25], was increased in human RCC tissues (Fig. 1B). Western blot results in Fig. 1C confirmed Beclin-1 upregulation in RCC tissues.

The expression of miR-30a and Beclin-1 in human RCC cell lines was also tested. Results again demonstrated miR-30a downregulation (Fig. 1D) and Beclin-1 upregulation (Fig. 1E and F) in several human RCC cell lines (A498, SK-RC-44 and 786-0), as compared to HK-2 cells, the latter is an immortalized proximal tubule epithelial cell line (non-cancerous cell) [22]. Together, these results show miR-30a downregulation and Beclin-1 upregulation in human RCC tissues and cells.

3.2. MiR-30a regulation of Beclin-1 and sorafenib resistance in cultured RCC cells

Above results showed miR-30a down-regulation in RCC tissues and cell lines. We next tested the potential role of miR-30a in sorafenib's activity. Results showed that exogenously expressing of miR-30a significantly reduced mRNA and protein expression of Beclin-1 in 786-0 cells (Fig. 2A and B). On the other hand, inhibition of miR-30a through introducing antagomir-30a led to Beclin-1 upregulation in 786-0 cells (Fig. 2A and B). Since Beclin-1 is a key player in autophagy, and plays significant roles in chemo-resistance [27], we thus tested the potential role of miR-30a in sorafenib-mediated actions in 786-0 cells. Results showed that sorafenib-induced viability reduction (Fig. 2C) and apoptosis (Fig. 2D and E) in 786-0 cells were enhanced by miR-30a over-expression.

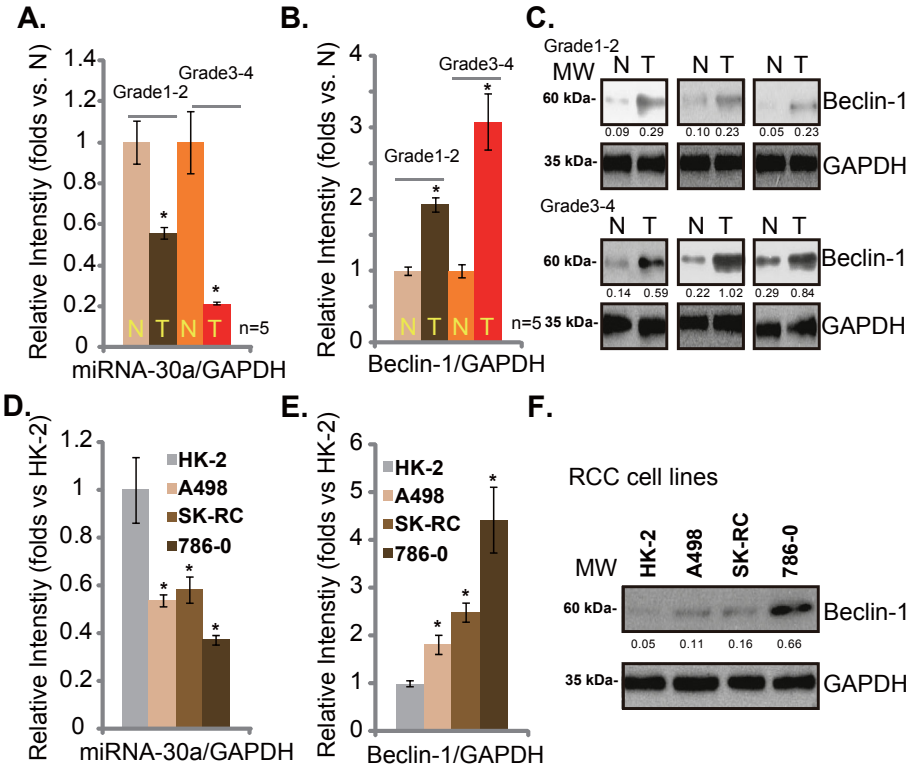

Fig. 1. ***MiR-30a downregulation correlates with Beclin-1 upregulation in human RCC tissues and cell lines***-Relative miR-30a (**A**) and Beclin-1 (**B**) mRNA expression (vs. GAPDH) in human RCC tissues ("T") and surrounding normal kidney tissues ("N") was shown. Beclin-1 and GAPDH protein expression in above tissues (patient ID 1-3, upper panel and patient ID 6-8, lower panel) was also shown (**C**). Relative miR-30a (**D**) and Beclin-1 (**E**) mRNA expression (vs. GAPDH) in human RCC cells (A498, 786-0 and SK-RC-44) and in HK-2 non-cancerous cells was shown. Beclin-1 and GAPDH protein expression in above cell lines was also shown (**F**). Beclin-1 protein expression was quantified (vs. GAPDH) (**C** and **F**).*$p < 0.05$ vs. "N" tissues (**A** and **B**). *$p < 0.05$ vs. HK-2 cells. (**D** and **E**). "MW" stands for molecular weight (for all figures).

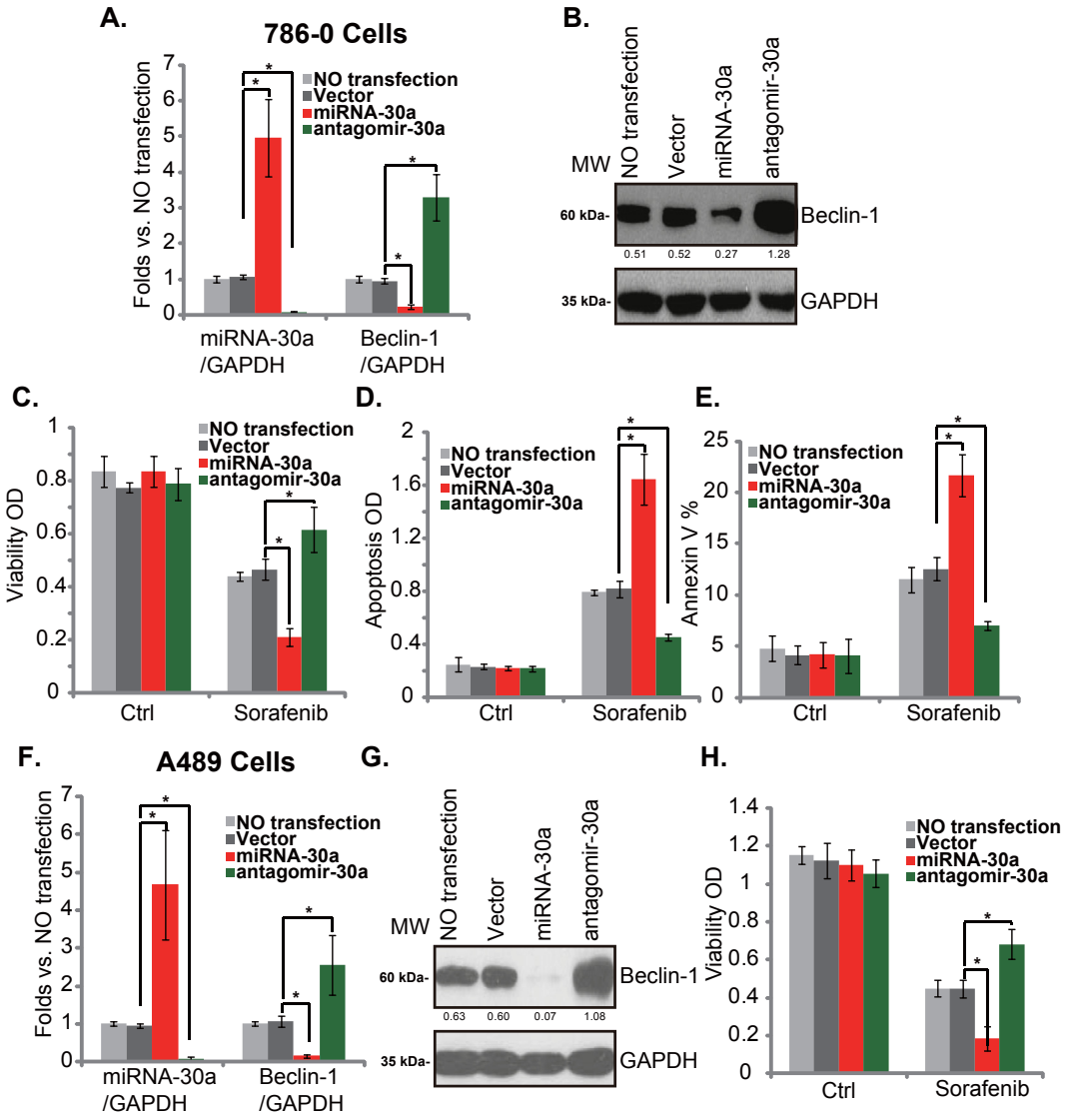

Fig. 2. ***MiR-30a regulation of Beclin-1 and sorafenib resistance in cultured RCC cells***-Un-transfected control 786-0 cells or A489 cells, or puromycin-selected stable 786-0/A489 cells with empty vector, miR-30a or antagomir-30a were either left untreated or treated with sorafenib (10 μM) for 48 h, relative mRNA expression of Beclin-1 (vs. GAPDH) and miR-30a (vs. GAPDH) was tested by real-time PCR (**A and F**); Beclin-1 and GAPDH protein expression was tested by Western blots (**B and G**), cell survival was tested by MTT assay (**C and H**), and cell apoptosis was tested by Histone DNA ELISA assay (**D**, for 786-0 cells) or Annexin V FACS assay (**E**, for 786-0 cells). Experiments in this figure were repeated three times, and similar results were obtained. Beclin-1 protein expression (vs. GAPDH) was quantified (**B** and **G**). $^{*}p < 0.05$.

Reversely, antagomir-30a inhibited sorafenib-induced cytotoxicity in 786-0 cells (Fig. 2C–E). We also repeated above experiments in A489 RCC cells. As demonstrated, over-expression of miR-30a (Fig. 2F) inhibited Beclin-1 expression (Fig. 2G), while increasing sorafenib's cytotoxicity (Fig. 2H). On the other hand, antagomir-30a caused Beclin-1 upregulation, and increased sorafenib's resistance (Fig. 2F–H). Together, these results indicate that miR-30a is important for Beclin-1 expression and sorafenib resistance in RCC cells.

3.3. Sorafenib induces cytoprotective autophagy in RCC cells

Above results showed that miR-30a downregulation is associated with Beclin-1 upregulation and sorafenib resistance in 786-0 cells, indicating a role of autophagy in sorafenib actions. Western blot results showed that sorafenib treatment in 786-0 cells induced autophagy activation, which was evidenced by Beclin-1 and ATG-5 upregulation, LC3B-I to LC3B-II conversation as well as p62 degradation (Fig. 3A). Chloroquine (Cq), 3-methyladenine (3-MA), and bafliomycin A1 (Baf-A1), three different autophagy inhibitors, all enhanced sorafenib-induced viability reduction (Fig. 3B) and apoptosis (Fig. 3C and D) in 786-0 cells, suggesting that sorafenib-activated autophagy is anti-apoptosis and pro-survival. We also repeated above experiments in A489 RCC cells, and similar results were obtained (Data not shown).

3.4. SiRNA knockdown of Beclin-1 or ATG-5 increases sorafenib sensitivity in RCC cells

To further confirm the role of autophagy in sorafenib's actions, siRNA-based strategy was applied to knockdown Beclin-1 and ATG-5, two key autophagy genes, in RCC cells. As demonstrated in Fig. 4A, two non-overlapping Beclin-1 siRNAs significantly down-regulated Beclin-1 expression in 786-0 cells. Similar to the inhibitors' results, sorafenib-induced viability reduction (Fig. 4B) and apoptosis (Fig. 4C and D) were dramatically enhanced by siRNA-

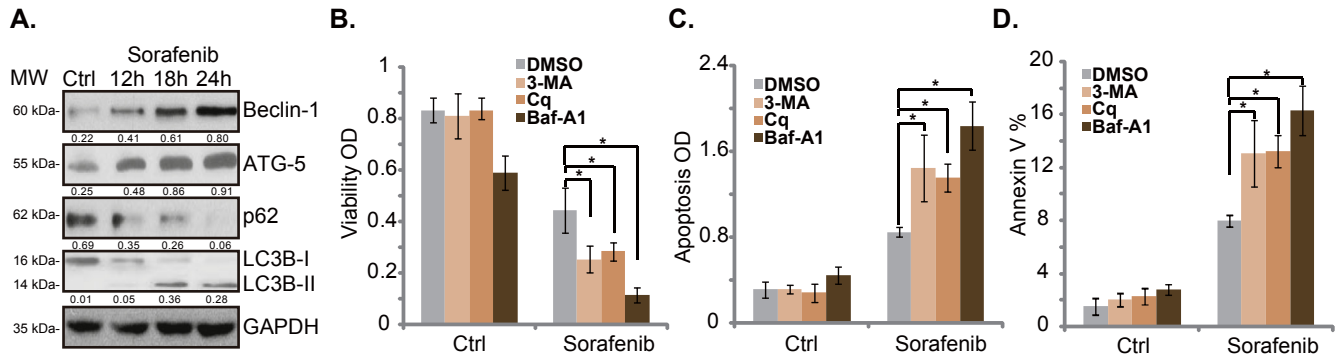

Fig. 3. ***Sorafenib induces cytoprotective autophagy in RCC cells***-786-0 cells were either left untreated ("Ctrl"), or treated with sorafenib (10 μM) for 12, 18 or 24 h, expressions of indicated proteins were tested by Western blots (**A**), protein expression (vs. GAPDH) was quantified (**A**). 786-0 cells were pre-added with 3-methyladenine (3-MA, 0.5 mM), chloroquine (Cq, 10 μM) or bafilomycin-A1 (Baf-A1, 200 nM) for 1 h, followed by sorafenib (10 μM) stimulation, cells were further cultured in drug-containing medium for 48 h, cell viability was tested by MTT assay (**B**), and cell apoptosis was tested by Histone DNA ELISA assay (**C**) or Annexin V FACS assay (**D**). Experiments in this figure were repeated three times, and similar results were obtained. $^{*}p < 0.05$.

knockdown of Beclin-1. At the mean time, siRNA-mediated knockdown of ATG-5 (Fig. 4E) also increased sorafenib-induced cytotoxicity, resulting in substantial 786-0 cell viability reduction (Fig. 4F). Again, two non-overlapping siRNA against ATG-5 achieved similar results (Fig. 4E–F). In A489 cells, sorafenib-induced cell death (viability reduction) was enhanced by siRNA-mediated knockdown of Beclin-1 or ATG-5 (Fig. 4G and H). These results further confirmed that autophagy inhibition could sensitize the activity of sorafenib in cultured RCC cells.

4. Discussion

Autophagy, an intracellular self-defense mechanism, not only prevents accumulation of damaged or unnecessary components, but also recycles the degraded components to sustain metabolic homoeostasis [28,29]. Beclin-1 plays an important role in the formation of Beclin-1-Vps34-Vps15 core complexes, which is required for autophagy induction [27]. MiRs can modulate the autophagic pathways [11]. For example, miR-30a and miR-376b were shown to inhibit Beclin-1 activity, thereby blocking autophagic vesicle nucleation and autophagy initiation [15,30]. In addition, miR-101 is a potent inhibitor of autophagy though suppressing RAB5A, a small GTPase that regulates early autophagosome formation [31]. In the current study, we found that autophagy-associated miR-30a was downregulated in several human RCC tissues and cell lines, likely causing Beclin-1 upregulation in RCC.

High level of autophagy activation has been found in cancer cells with or without chemo-drug stimulation, which participates chemo-resistance [28]. Accordingly, blocking autophagy is emerging as a novel strategy to enhance the sensitivity of chemotherapy in various cancers [4,6]. For example, Yu et al., showed that

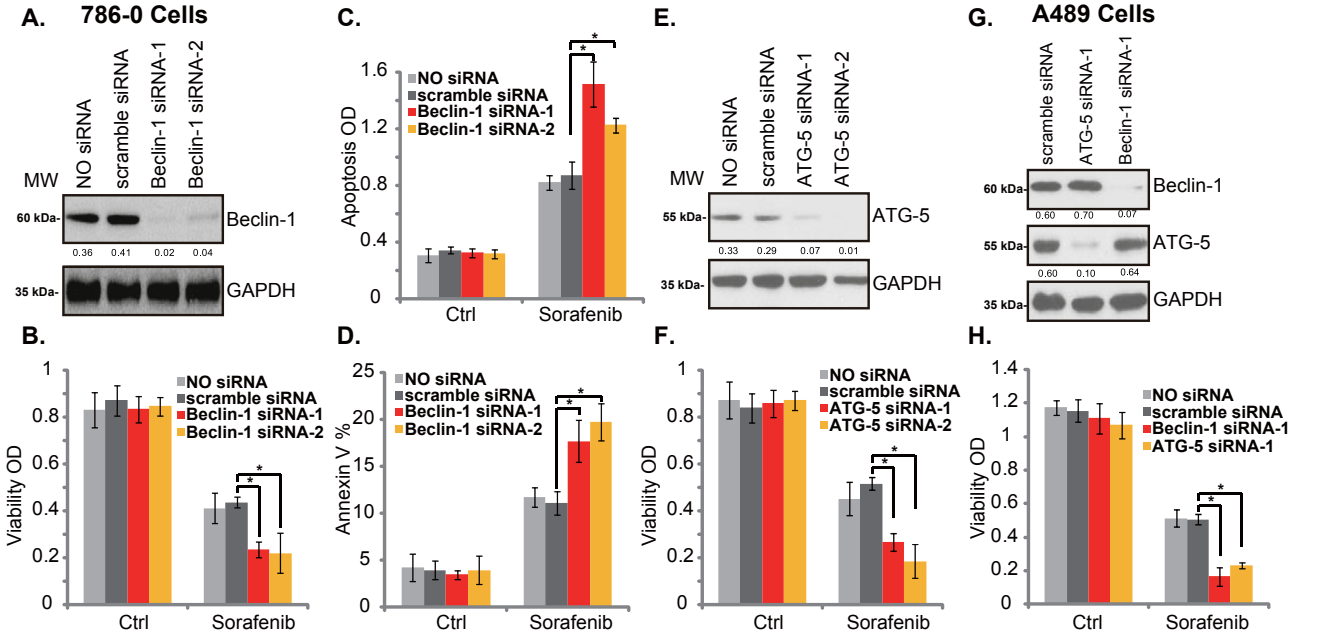

Fig. 4. ***SiRNA knockdown of Beclin-1 or ATG-5 increases sorafenib sensitivity in RCC cells***- 786-0 cells or A489 cells, transfected with or without indicated siRNA, were stimulated with sorafenib (10 μM) for 48 h, cells were further cultured in drug-containing medium for 48 h, expression of indicated proteins was examined by Western blots (**A**, **E** and **G**), cell viability was tested by MTT assay (**B, F** and **H**), cell apoptosis was tested by Histone-DNA ELISA assay (**C**, for 786-0 cells) and Annexin V FACS assay (**D**, for 786-0 cells). Beclin-1/ATG-5 expression (vs. GAPDH) was quantified (**A**, **E** and **G**). "Ctrl" stands for untreated control group. Experiments in this figure were repeated three times, and similar results were obtained. $^{*}p < 0.05$.

miR-30a-mediated autophagy inhibition could significantly enhance Imatinib-induced activity against human chronic myeloid leukemia cells [25]. Here, we demonstrated that treatment with sorafenib in RCC cells induced autophagy activation, which was also involved in chemo-resistance. Blockage autophagy utilizing pharmacological or genetic approaches could significantly enhance sorafenib-induced cytotoxicity against RCC cells.

Chloroquine (Cq) is a lysosomotropic drug that increase intralysosomal pH to disrupts autophagic protein degradation. 3-methyladenine (3-MA) is known to block LC3B-I to LC3B-II conversion, and inhibits autophagosome formation [32]. Bafliomycin A1 (Baf-A1) prevents the maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes [33]. These different inhibitors, targeting different stages of autophagy, all enhanced sorafenib activity against RCC cells, suggesting that autophagy activation by sorafenib is pro-survival and is an important chemo-resistance factor. This conclusion was further support by the fact that RNAi silencing of Beclin-1 or ATG-5 sensitized sorafenib-induced cytotoxicity in RCC cells. In summary, these findings indicate that dysregulation of miR-30a in RCC may interfere with the effectiveness of sorafenib-mediated apoptosis by an autophagy-dependent pathway, thus representing a novel potential therapeutic target in RCC.

Conflict of interest

There are no conflicts of interest.

Acknowledgments

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Transparency document

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