Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells

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Abstract

Induction of cell death and inhibition of cell growth are the main targets of cancer therapy. Here we evaluated the role of autophagy on chemoresistance of human hepatocarcinoma (HCC) cell lines, focusing on its crosstalk with cell apoptosis and proliferation. In this study, a chemotherapeutic agent (cisplatin or 5FU) induced the formation of autophagosomes in three human HCC cell lines and upregulated the expression of autophagy protein LC3-II. Inhibition of autophagy by 3-methyladenine or si-beclin 1 increased chemotherapy-induced apoptosis in HCC cells. Meanwhile, increased damage of the mitochondrial membrane potential was also observed in HCC cells when autophagy was inhibited. Furthermore, inhibition of autophagy reduced clone formation and impaired cell growth of HCC cells when treated with chemotherapy. Co-administration of an autophagy inhibitor (chloroquine) and chemotherapy significantly inhibited tumor growth in a mouse xenograft tumor model, with greater extent of apoptosis and impaired proliferation of tumor cells. This study suggests that autophagy is a potential novel target to improve therapy efficiency of conventional chemotherapeutics towards HCC.

1. Introduction

Hepatocarcinoma (HCC) is one of the most common malignant tumors in China [1]. Surgical resection and liver transplantation are potentially curative therapies [2]. Unfortunately, most of hepatocarcinomas are too advanced at the time of diagnosis to benefit from these surgical approaches. Currently, chemotherapy is ineffective for HCC because of the inherent chemoresistance. However, the exact mechanism of chemotherapy resistance in hepatocarcinoma is largely unknown. Recently, accumulated evidence suggested that autophagy can promote cancer resistance to chemotherapy [3–6].

Autophagy is an evolutionarily conserved catabolic process. The autophagic pathway consists of isolation of membrane, formation of autophagosomes and autolysosomes. Isolation membrane engulfs cellular macromolecules and organelles to form autophagosomes. Subsequently, the autophagosome fuses with the lysosome to form autolysosomes, leading to breakdown of macromolecules and organelles by the lysosomal enzymes [7–9]. Dysregulation of autophagy contributes to a number of diseases including tumorigenesis [7]. However, the role of autophagy in cancer is still controversial. Recent studies suggested that autophagy was required for cancer survival [10] and tumorigenesis [11–13]. On the other hand, prolonged autophagy has been suggested to cause non-apoptotic type II programmed cell death [14,15]. Some pharmacologic inhibitors have been used to evaluate the physiologic relevance of autophagy. For example, 3-methyladenine (3-MA), an inhibitor of phosphatidylinositols 3-kinase, blocks autophagosome formation to inhibit autophagy.

Induction of cell death and inhibition of growth are the main targets of cancer therapy. It was found that some types of cancers including hepatocarcinoma were still resistant to chemotherapy. Thus, drugs which reinforce the function of chemotherapy in cell death induction and growth inhibition are beneficial to anti-cancer therapy. Here we assessed the role of autophagy on chemotherapy-induced apoptosis and growth inhibition. We investigated the effect of autophagy on HCC cells exposed to chemotherapeutic agents. Then we detected whether inhibition of autophagy could affect chemotherapy-induced apoptosis and growth inhibition.

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Abbreviations: HCC, hepatocarcinoma; 5FU, 5-fluorouracil; LC3, microtubule-associated protein 1 light chain 3; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; 3MA, 3-methyladenine; GFP-LC3, green fluorescent protein-tagged LC3; PI, propidium iodide; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; siRNA, small interfering RNA; CQ, chloroquine.

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2. Materials and methods

2.1. Cell culture

Human hepatocarcinoma cell lines SMMC-7721, Hep3B and HepG2 were maintained in Dulbecco’s modified Eagle’s medium (high glucose) ( Gibco, Invitrogen) and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator under 5% CO2 at 37 °C.

2.2. Regents

Clasiplatin and 5-fluorouracil (5FU) were purchased from Qilu Pharmaceutical Co., Ltd. ( Jinan, Shandong, China). 3-Methyladenine (3MA, M9281) was obtained from Sigma–Aldrich and dissolved in sterile double distilled water. Chloroquine (CQ, Sigma, C6628) was dissolved as stock solution (400 mM).

2.3. Cell viability assay

The measurement of viable cell mass was assessed by a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan), as previously described [16].

2.4. Cell growth inhibition assay

Cells, seeded at 2 × 10^4 in each well of six-well plates, were pretreated with 10 mM 3MA for 1 h or transfected with si-beclin1, and then treated with chemotherapeutic agents for 24 h. At the end of treatment, cells were washed with phosphate buffered saline and incubated at 37 °C in 5% CO2 humidified atmosphere for an additional 1–4 days. Adherent cells were then trypsinized and counted. Each experimental sample was run in triplicate.

2.5. Cell apoptosis assay

Apoptosis detection by DAPI staining were performed as described [17].

2.6. Colony formation assay

SMMC-7721 and HepG2 cells were seeded in six-well plate (500 cells/well) and pretreated with 10 mM 3MA for 1 h or transfected with si-beclin1, and then treated with chemotherapeutic agents for 24 h. After that, cells were allowed to grow in complete medium without any drugs treatment for 14 days. The colonies were fixed in methanol, stained with 0.1% crystal violet and counted.

2.7. Transient transfection

GFP-LC3 expressing plasmids transiently transfecting into HCC cells were performed as described [17].

2.8. Electron microscopy

Cells were fixed with 2.5% glutaraldehyde in phosphate buffer and stored at 4 °C until embedding. Cells were postfixed with 1% osmium tetroxide followed by an increasing gradient dehydration step using ethanol and acetone. Cells were then embedded in araldite, and ultrathin section were obtained (50–60 nm), placed on uncoated copper grids, and stained with 3% lead citrate–uranyl acetate. Images were examined with a CM-120 electron microscope (PHILIPS).

2.9. siRNA

The Stealth™ RNAi negative control duplex (Invitrogen, Cat. 12935-200) and Stealth RNAi™ siRNA duplex oligonucleotides targeting human beclin 1 ( Invitrogen, Cat. 1299003) were obtained from Invitrogen. The siRNA was transfected into HCC cells using siRNA transfection reagent (Santa Cruz, Cat. sc-29528) according to the manufacturer’s protocol.

2.10. Western blot analysis

Western-blot analysis were performed as described [18]. Antibodies were specific for LC3 (Novus Biologicals, Cat. NB100-2220H) and β-actin (Santa Cruz, Cat. sc-47778).

2.11. TUNEL assay

Analysis of apoptotic cells in tumor tissue was performed by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using an apoptotic cell detection kit following the manufacturer’s directions (Merck Corp. Cat. QIA39). TUNEL-positive (apoptosis) cells had pyknotic nucleus with dark green fluorescent staining. Images of the sections were taken by a fluorescence microscope (Olympus IX71). Each sample was observed at a magnification of ×400 and TUNEL-positive cells were calculated in five random fields per tumor.

2.12. BrdU incorporation assay

BrdU (Sigma, Shanghai, China) was used to evaluate the synthesis of DNA. SMMC-7721 and HepG2 cells were seeded in six-well plate (5 × 10^4 cells/well) and pretreated with 10 mM 3MA for 1 h, and then treated with chemotherapeutic agents for 24 h. Cells were incubated with BrdU 30 μM for 1 h (from 23 to 24 h after treatment). After this, cells were washed with PBS, followed by fixation in cold ethanol 70% for 1 h. Thereafter, cells were washed twice with PBS and incubated with HCl 2 N for 30 min at room temperature. Then cells were incubated with the anti-BrdU FITC-conjugated antibody, diluted 1:100 in a PBS-T solution (1% bovine serum albumin (BSA)/PBS and 0.5% Tween 20), for 1 h, in the dark, under agitation. Finally, cells were washed once with PBS-T and incubated with the same solution, followed by flow cytometry.

2.13. Studies in vivo

Male athymic BALB/c nu/nu mice (5-week-old) were obtained from the Shanghai Experimental Animal Center, Chinese Academy of Science, maintained under specific pathogen-free conditions, and acclimated before the experiment. For establishing a hepatocarcinoma xenograft tumor model, SMMC-7721 (2.0 × 10^6 cells) in 0.2 ml of serum-free DMEM medium were injected subcutaneously into the right back of BALB/c nude mice, and the tumor growth was monitored with electronic calipers using the formula: Volume = a × b²/2, where a was the width at the widest point of the tumor and b was the maximal width. When the tumors reached a mean tumor volume of 150–160 mm³, mice were randomly separate to five groups (each group has five mice); then the treatment was initiated as follows: (a) control group, animals received no treatment; (b) PBS group, animals received i.p. injections of 100 μl of PBS thrice weekly; (c) cisplatin or 5FU group, animals received i.p. injections of 3 mg/kg cisplatin or 30 mg/kg 5FU in 100 μl of PBS thrice weekly; (d) Autophagy inhibition group, animals received i.p. injections of 60 mg/kg chloroquine (CQ) in 100 μl of PBS thrice weekly; (e) combination group, animals received i.p. injections of 3 mg/kg cisplatin (or 30 mg/kg 5FU) and 60 mg/kg CQ in 100 μl of PBS thrice weekly. After 2 weeks of treatment, all nude mice were sacrificed, and tumors were excised for histological and western blot study.

2.14. Immunohistochemical analysis

Immunohistochemical analysis was performed as previously described [19]. Ki67 antibody (1:200, abcam, ab15580) was used to detect proliferation. HRP-la-beled goat anti-rabbit immunoglobulin (1:500, Santa Cruz, Cat. sc-2030) was applied as the secondary antibody.

2.15. Statistical analysis

All of the experiments were repeated at least three times. The data were expressed as means ± SD. Statistical analysis was performed by using Student’s t-test (two-tailed). The criterion for statistical significance was taken as p < 0.05.

3. Results

3.1. Chemotherapeutic agents induces autophagy in hepatocarcinoma cells

Autophagy occurs at low basal levels in all cells and will be rapidly upregulated when cell faces stress. To determine the role of autophagy on chemosensitivity, we first examined the effect of chemotherapy on autophagy. Hepatocarcinoma cells were cultured for 24 h with or without cisplatin (8 μg/ml) or 5FU (120 μg/ml). LC3-I to LC3-II protein processing is considered a hallmark of autophagy. As shown in Fig. 1A, expression levels of endogenous LC3-II were markedly increased in hepatocarcinoma (HCC) cells by chemotherapeutic agents. To further confirm the activation of autophagy, three HCC cell lines (SMMC-7721, HepG2 and Hep3B) were transfected with GFP-LC3 plasmid. Fluorescent microscopy results showed cells treated with cisplatin or 5FU exhibited significantly higher percentage of punctate GFP (green dot), while untreated cells showed primarily diffusion (Fig. 1B). Meanwhile, treatment with the lysosome-inhibitor chloroquine (CQ) further increased the levels of the LC3-II (Supplementary Fig. S1A) and GFP-LC3 puncta (Supplementary Fig. S1B) in chemotherapy treated
SMMC-7721 cells, suggesting that the autophagy flux in this study is intact. Furthermore, electron microscopy analysis demonstrated that increased autophagosomes were observed in chemotherapeutic agents-treated HCC cells (Fig. 1C). Taken together, these findings suggested autophagy was activated by chemotherapeutic agents in HCC cells.

3.2. Inhibition of autophagy by 3-MA or knockdown of beclin1 enhances chemotherapeutic agents-induced apoptosis in hepatocarcinoma cells

To determine whether inhibition of autophagy enhanced the chemosensitivity of HCC cells, HCC cells were pretreated with 3-MA for 1 h or transfected with si-beclin1 (Supplementary Fig. S2) [17], and then cells were incubated with cisplatin (8 µg/ml) or 5FU (120 µg/ml) for 24 h. WST-8 assay showed that combined treatment (3-MA or si-beclin1 and cisplatin or 5FU) caused much greater extent of cell death (Fig. 2A–C). As shown in Fig. 3A, a significantly increased amount of cell death was observed in combination group by cell morphology detection. The dead cells showed typical apoptotic changes including marked rounding, shrinkage and detachment from the culture dish. Similar effects were further confirmed by DAPI staining that 3-MA or si-beclin1 treatment markedly induced chromatin condensation or fragmentation in HCC cells with cisplatin or 5FU (Fig. 3B). These results suggested that inhibition of autophagy increased chemotherapy-
induced apoptosis. Thus, autophagy contributed to chemotherapy insensitivity in HCC cells by reducing apoptosis potency.

3.3. Effect of autophagy on mitochondrial membrane potential in HCC cells

Previous report has shown that decreased mitochondrial mass by autophagy may accompany with reduced levels of apoptosis induced by pro-apoptotic agents [20–22]. To examine the change of mitochondrial membrane potential by autophagy inhibition, SMMC-7721 and Hep3B cells were treated with cisplatin or 5FU. As shown in Fig. 4A, cisplatin treatment caused loss of mitochondrial membrane potential in about 22.1% SMMC-7721 and 21.8% Hep3B cells; while about 35.8% SMMC-7721 and 34.2% Hep3B cells lost their mitochondrial membrane potential with combined treatment of 3MA and cisplatin. The similar results were also obtained by 5FU treatment (Fig. 4B). Interestingly, 3MA caused slight change of mitochondrial membrane potential (2.5–3.8% in SMMC-7721 and 2.7–4.7% in Hep3B). Taken together, these findings suggested inhibition of autophagy by 3MA promoted loss of mitochondrial membrane potential.

3.4. Inhibition of autophagy impairs cell proliferation in hepatocarcinoma cells during chemotherapy treatment

To evaluate the effect of combined autophagy inhibition and chemotherapeutic agents on HCC cells survival, colony formation was performed to confirm the long-term cell viability. SMMC-7721 and HepG2 cells were pretreated with 3MA for 1 h or transfected with si-beclin1, and then incubated with cisplatin or 5FU for 24 h. After that, cells were allowed to grow in complete medium without any drugs treatment for 14 days. As shown in Fig. 5A and D, combined treatment with autophagy inhibitor and chemotherapy caused a dramatic inhibition of the colony-forming of HCC cells compared with chemotherapy treatment alone. Autophagy inhibitor alone had no effect on colony-forming ability of cells. We also determined the capacity of cells to resume proliferation upon reseeding equal number of cells (2×10⁶) into complete medium after being treated. SMMC-7721 and HepG2 cells which were treated with autophagy inhibitor and chemotherapy failed to show proliferation when reseeded (Fig. 5B, E, C, and F). Furthermore, the results of BrdU incorporation assay also showed that combined treatment with chemotherapy and autophagy inhibitor (3MA) significantly inhibited DNA synthesis (Fig. 5G–I). Taken together, those results suggested inhibition of autophagy could increase the proliferation inhibition effect of chemotherapy.

3.5. Inhibition of autophagy enhances growth inhibition effect of chemotherapeutic agents in vivo

To test the in vivo efficacy of combined treatment of autophagy inhibition and chemotherapeutic agents in hepatocarcinoma, SMMC-7721 cells were injected into the right back of BALB/c nude mice. Thirteen days later, chloroquine (60 mg/kg in100 µl of PBS) and cisplatin (3 mg/kg/100 µl/time) or 5FU (30 mg/kg/100 µl/time) was intraperitoneally injected thrice weekly. On day 27, mice were sacrificed, the xenograft tumors were excised and the tumor weights were measured. As shown in Fig. 6A, CQ or cisplatin treatment alone could suppress tumor growth. Furthermore, compared with cisplatin group, the combined group showed 28.57% (p < 0.05) reduction in mean tumor weight and 33.4% (p < 0.05) reduction in mean tumor volume (Fig. 6A and B). Similar results were also obtained in 5FU and CQ combined treatment group (Fig. 6C and D).

3.6. Combination treatment with chemotherapy and chloroquine cause increased apoptosis and reduced proliferation in xenografted tumor

Our results demonstrated that combined treatment with chemotherapy and autophagy inhibit or increased HCC cells growth inhibition in vitro, and decreased the growth of xenografted tumor in mice. We next evaluated the effect of combination treatment with chemotherapy and chloroquine (CQ) on apoptosis and proliferation of xenografted tumor in mice. The results of TUNEL assay demonstrated that cisplatin and CQ combined treatment caused high level of apoptosis (with green nuclei) (Fig. 7A-upper and C). The results of immunoblotting showed that ki-67 expression (with brown nuclei) was significantly reduced in cisplatin and CQ combined treatment group (Fig. 7A-lower and E). Similar results can also be observed in 5FU and CQ combined treatment group (Fig. 7B, D, and F). These results suggested that increased cell apoptosis and impaired cell proliferation contributed to the tumor suppressive function of chemotherapy and CQ combination treatment.
4. Discussion

The results described here demonstrated that autophagy functions as a chemoresistant mechanism in HCC cells. We report here that chemotherapeutic agents (cisplatin or 5FU) could induce autophagy and inhibition of autophagy rendered HCC cells susceptible to chemotherapy-induced apoptosis and cell growth inhibition. Meanwhile, inhibition of autophagy increased damage of the mitochondrial membrane potential in HCC cells. Furthermore, combined treatment of autophagy inhibitor and cisplatin or 5FU markedly inhibited the xenograft tumor growth with enhanced apoptosis and impaired proliferation. Our data suggest inhibitor of autophagy is a novel sensitizer to increase efficiency of conventional chemotherapeutic agents in HCC.

Autophagy occurs at low basal levels in most cells to establish homeostasis and may rapidly upregulate when cells suffer intensive and changed stress. We suspect that the upregulated autophagy may help cells to adapt to the change of exterior and interior environment and may serve as an effective survival strategy by providing more metabolic energy or accelerating clearance of damaged organelles. In this study, we showed that autophagy in HCC cells was upregulated when treated with cisplatin or 5FU. It is suggested that BNIP3 was required for induction of autophagy by 5FU, and cisplatin-triggered autophagic response was through activation of AMPK and subsequent suppression of mTOR activity [23,24]. Furthermore, in this study, we also demonstrated that inhibition of autophagy increased sensitivity of HCC cells to chemotherapy by inducing apoptosis and inhibiting growth of tumor.
Fig. 5. Inhibition of autophagy impairs cell proliferation in hepatocarcinoma cells during chemotherapy treatment. (A and D) Long-term cell viability was evaluated by colony formation. (B, C and E, F) SMMC-7721 and HepG2 cells were pretreated with 10 mM 3MA for 1 h or transfected with si-beclin1, and then treated with chemotherapeutic agents for 24 h. After that, cells were allowed to grow in complete medium without any drugs treatment. Twenty-four hours later, cells were trypsinized and continued counting for 4 days in a successive. \( p < 0.05 \) compared to cells with cisplatin or 5FU treatment alone. (G–I) Quantification of BrdU incorporation by flow cytometry. SMMC-7721 and HepG2 cells were treated with 3MA and/or chemotherapeutic agents for 24 h, as indicated. From 23 to 24 h after treatment, cells were incubated with 30 \( \mu \)M BrdU. Thereafter, cells were fixed, stained and analyzed by flow cytometry (G). Graph indicates the percentage of BrdU-positive cells (Mean ± SD); **\( p < 0.01 \) (H and I).
Consistent with our findings, previous reports suggested that several cytotoxic chemotherapeutic agents could induce autophagy, and inhibition of autophagy enhanced efficiency of therapy \cite{4,25}. Thus, upregulated autophagy may serve as a chemotherapy defense mechanism for tumor cells.

How autophagy promotes cell resistance to chemotherapy remains debated. In this study, we demonstrated that autophagy may contribute to chemoresistance in HCC cells, since inhibition of autophagy increased the chemotherapy-induced apoptosis in vitro and in vivo. However, the relationship between autophagy and apoptosis is quite complicated. In some cases, apoptosis and autophagy could be simultaneously induced by the same stimulus and had no connection \cite{26,27}. Meanwhile, report also demonstrated that autophagy might promote apoptosis \cite{28}. Moreover, autophagy could restrain apoptosis in some cellular context \cite{4,20,29}. Thus, the role of autophagy on cell apoptosis needs carefully examined. Actually, the molecular mechanism which determined the interaction between autophagy and apoptosis are quite complex. Similarly, how autophagy helps tumor cells resist to apoptosis remains poorly defined. It has been suggested autophagy could prevent the diffusion of pro-apoptotic factors in response to cell death stimuli \cite{30–32}. In this study, we observed that 3MA treatment induced significant damage of mitochondrial membrane potential in chemotherapy-treated HCC cells. These results suggest that activation of autophagy by therapeutic stress helps to reduce the damage of mitochondria membrane potential. This effect may due to the function of autophagy on clearance of the damaged mitochondrial. Furthermore, recent reports showed that autophagy can reduce metabolic and therapeutic stress-induced DNA damage responses \cite{30,31} or ROS accumulation \cite{32}. Thus, autophagic response may act as self-help mechanism to promote cell survival by multi-mean under stresses. Although it is possible
that there may be other factors that contribute to the role of autophagy in reducing chemosensitivity, we provide a persuasive data to suggest that autophagy may promote chemoresistance in HCC cells by reducing damaged mitochondrial potential.

Besides inducing tumor cell apoptosis, inhibition of tumor proliferation is also another effective anti-cancer strategy. In the present study, we demonstrated inhibition of autophagy enhanced chemotherapy-induced cell growth. Furthermore, combined treatment with chemotherapeutic agents and CQ led to dramatically inhibited tumor growth and impaired cell proliferation in xenografted animal models. It is widely demonstrated that autophagy contributes to cell metabolism. Indeed, it was suggested that in early or later stages of tumor progression, autophagy was activated in metabolically stressful regions of tumor mass [33]. Thus, we suspect that, in our present study, inhibition of autophagy may not only impair the adaptive responses of tumor cells to therapeutic stress, but aggravate the metabolic stress in tumor cells, and eventually lead to cell death and inhibition of cell proliferation. Meanwhile, it has been suggested that inhibition of autophagy rendered cell cycle arrest [12]. In Ras activated cancer, the high basal level of autophagy facilitated tumor growth. The underlying mechanism was that autophagy preserve the pool of functional mitochondria which is required to support growth of Ras-driven tumors [13]. Moreover, dysfunctions of mitochondria might cause an overall delay in cell cycle progression [34]. Autophagy is involved in quality control of mitochondria and inhibition of autophagy leads to accumulation of damaged mitochondria. As damaged mitochondria are a known source to promote apoptosis, this may increase the idea that the interaction between autophagy, apoptosis, and cell proliferation may be mediated by mitochondria [35].

Many studies, including ours, support that autophagy promotes cell survival under chemotherapy. However, sustained autophagy could also lead to autophagic cell death [36]. Under some circumstances (especially apoptosis deficiency), autophagy would contribute to chemotherapy-induced cell death [37]. Thus, the role of autophagy on cell fate decision is paradox and need to be carefully examined. However, the “autophagic cell death” is strictly defined and should meet some requirements [37]. Additionally, the autophagic cell death or autophagy companying cell death is still debated [38]. Recently, it has become clear that autophagy usually acts as a protective mechanism of cells to adapt to adverse stress rather than a mechanism to promote cell death [39]. And research has also shown that some cancers, especially in Ras-driven tumors, are autophagy addiction [40]. In this case, inhibition of autophagy is a novel cancer therapy strategy. This study, our data suggest that autophagy is required by HCC cells to endure chemotherapy. Therefore, autophagy inhibition is a novel way to increase therapy efficiency of conventional chemotherapeutics in HCC. It is conceivable that chemoembolization containing autophagy inhibitor will be benefit for HCC treatment. However, the autophagy-targeting strategies for cancer therapy need more clinical trial testing. The successful development and application of autophagy regulators under clinical settings is important.

Identification of the mechanism underlying chemoresistance will help to increase chemotherapy efficacy. In this study, our data suggest that autophagy is a mechanism utilized by HCC cells to tolerate chemotherapy. These results further expand our understanding of the role of autophagy in cancer formation and progression. Further studies on the molecular mechanism in which autophagy promotes chemoresistance would be important, and may be helpful to HCC or other cancer therapy.

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Appendix A. Supplementary material

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References


