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Hsa-let-7a functions as a tumor suppressor in renal cell carcinoma cell lines by targeting c-myc

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ABSTRACT

Widespread functions of the c-myc pathway play a crucial role in renal cell carcinoma (RCC) carcinogenesis. Thus, we evaluated the connection between proto-oncogenic c-myc and anti-neoplastic hsa-let-7a (let-7a) in RCC cell lines. The levels of c-myc and let-7a in 3 RCC cell lines (769P, Caki-1 and 786O) were measured after transfecting the cells with let-7a mimics or a negative control. The change in c-myc protein level was confirmed by Western blot. The anti-neoplastic function of let-7a was evaluated using cell counting kit-8 (CCK-8) for proliferation analysis and cell flow cytometry for cell cycle analysis. The changes of downstream targets of c-myc were measured using reverse transcription quantitative real-time PCR (qRT-PCR). Our results suggest for the first time that let-7a acts as a tumor suppressor in RCC cell lines by down-regulating c-myc and c-myc target genes such as proliferating cell nuclear antigen (PCNA), cyclin D1 (CCND1) and the miR17–92 cluster, which is accompanied by proliferation inhibition and cell cycle arrest.

1. Introduction

Myc proteins act as important regulators of protein synthesis, cell growth and proliferation in various organisms by binding to the E-box sequence CACGTG that is associated with gene activation. Nearly 10–15% genes are involved in this myc-regulatory network [1]. Myc family members, namely c-myc, n-myc and v-myc, are over-expressed in many human malignancies [2–4]. They share much similarity in their DNA, RNA and protein sequences [2,5]. c-myc was the first myc family member identified and was characterized as a transcription factor influencing cell cycle progression, cell growth, metabolism, apoptosis and angiogenesis [3,6–9]. Many reports, including our own, find that the myc-pathway is activated in RCC with the expression of c-myc correlating with RCC clinical parameters [10–12]. Chromosomal deletion of the region spanning c-myc gain, 3p25.1-25.3, shows a significant correlation with tumor size, indicating an association with tumor progression [13].

Let-7 is a conserved microRNAs (miRNAs) family with 12 members (let-7-a1, a2, a3, b, c, d, e, f1, f2, g, I and miRNA-98) and was one of the first mammalian miRNAs families identified to down-regulate cell cycle regulators and promoters of growth such as RAS and c-myc [14,15]. Although let-7 family members can all function as tumor suppressors [16–18], let-7a is the one that is the most reported to down-regulate c-myc [18–20]. However, no study has yet to investigate the significance of let-7a involvement in RCC.

Together with Oct4, Sox2 and Klf4, c-myc can reprogram differentiated adult fibroblasts into pluripotent stem cells [21]. Let-7a is also involved in the differentiation of stem cells. c-myc has a widespread role in the induction of many genes including Bcl-2, VEGFA and PGK in RCC cell lines [10]. c-myc can be down-regulated by let-7a in many human malignancies [18–20]. Therefore, we hypothesized that there is a significant association of c-myc with let-7a in the development of RCC. If this hypothesis is confirmed, we could study RCC by focusing on c-myc-related biomarkers.

Our study investigated the reported let-7a/c-myc regulatory loop in RCC cell lines over-expressing c-myc. We demonstrated for the first time that let-7a could suppress tumorigenesis by down-regulating the expression of c-myc, resulting in an inhibition of proliferation and cell cycle arrest in three RCC cell lines. c-myc target genes were also significantly decreased after c-myc was down-regulated by let-7a.

2. Materials and methods

2.1. Clinical normal specimens from RCC patients

We collected 10 normal specimens from 10 RCC patients after nephrectomy at Shanghai First People's Hospital of Shanghai

Abbreviations: RCC, renal cell carcinoma; let-7a, hsa-let-7a; CCK-8, cell counting kit-8; qRT-PCR, quantitative real-time PCR; PCNA, proliferating cell nuclear antigen; CCND1, cyclin D1; miRNAs, microRNAs; ATCC, American Type Culture Collection; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

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Jiaotong University. The specimens were collected from the normal region at least 5 cm outside the tumor range from each patient. All the specimens were stored at −80 °C immediately after surgery until RNA extraction. All patients were informed and consent was given. A pathologist confirmed that all specimens were derived from normal tissues.

2.2. Cell culture and miRNAs transfection

The human RCC cell line 769P was purchased from American Type Culture Collection (ATCC); 786O and Caki-1 were purchased from Chinese Academy of Sciences (Shanghai, China). ATCC-recommended 1640 cell culture medium (GIBCO, NY, cat. no: 31800-022) was used for 769P and 786O. Caki-1 was cultured in ATCC-recommended McCoy's 5A medium (GIBCO, NY, cat. no: 16600-082). All cultures were supplemented with 10% fetal bovine serum (GIBCO, Newcastle, Australia, cat. no: 10099-141), 50 U/mL Penicillin and 50 μg/mL Streptomycin (GIBCO, NY, cat. no: 15070-063). The cells were incubated at 37 °C in a humid atmosphere with 5% CO_2. Let-7a mimics and the negative control were synthesized by Shanghai GenePharma Co. Cells were transfected with let-7a mimics or the negative control using Lipofectamine2000 Transfection Reagent (Invitrogen, NY, cat. no: 11668-019) at 50 nmol/L according to the manufacturer's instruction. The medium was replaced 6 h after the transfection.

2.3. Western blot analysis

Proteins were harvested for Western blot analysis 48 h after transfecting the cells with the let-7a mimics or negative control. Protein content was determined using the Enhanced BCA protein assay kit (Beyotime, Shanghai, China, cat. no: P0010S). Briefly, protein was denatured at 100 °C for 5 min, electrophoretically separated (30 μg per lane) on 10% SDS–PAGE (Bio-Rad Laboratories, CA, cat. no: 161-0156) and then transferred onto a PVDF Western Blotting Membrane (Roche Applied Science, Mannheim, Germany, cat. no: 3010040001). The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 30 min, incubated with a monoclonal c-myc antibody (Cell Signaling Technology, MA, cat. no: 5605) at 4 °C overnight, and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology, MA, cat. no: 7074). A β-Actin antibody was used to determine loading (Cell Signaling Technology, MA, cat. no: 5125). Protein expression was evaluated using chemiluminescence and exposure to Kodak film.

2.4. Cell proliferation analysis

Cells were seeded into 96-well plates at 1000 cells per well 24 h after transfection. The effects of let-7a on cell proliferation were detected 0, 24, 48, 72 and 96 h after seeding using CCK-8 (Dojindo Laboratories, Kumamoto, Japan, cat. no: CK04) according to the manufacturer's instruction.

2.5. Flow cytometry for analysis of cell cycle

Forty-eight after transfection, cells were harvested, trypsinized, washed in cold PBS and fixed in 70% ethanol at 4 °C overnight. Cells were stained in phosphate buffered saline containing 0.5 mg/mL RNase and 40 μg/mL propidium iodide (Sigma–Aldrich, MO, cat. no: P4864) at 37 °C for 30 min, in the dark, at room temperature, and then analyzed using a FACS-Caliburcytometer (Becton Dickinson, CA). At least 10,000 events were counted for each sample.

2.6. RNA extraction

Total RNA from cultured cells was extracted using the mirVana miRNA isolation kit (Ambion, TX, cat. no: AM1561) according to the provided protocol. Total RNA from tissues was extracted using the TRizol reagent (Invitrogen, CA, cat. no: 15596-018) as described previously [12].

RNA quantity and quality were determined by spectrophotometry at 260 nm and agarose gel electrophoresis.

2.7. Reverse transcription and qRT-PCR

Reverse transcription for miRNAs was performed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, cat. no: 4366597). TaqMan microRNA expression assays (Applied Biosystems, CA, ID: let-7a. 000377; RNU6B. 001093) were used to provide specific primers for reverse transcription and quantitation of mature let-7a and RNU6B. Thermal cycling conditions for qRT-PCR were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. RNU6B expression was used as an internal control for let-7a expression.Reverse transcription for mRNAs was performed using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China, cat. no: DRR037A). The cDNA template was amplified by qRT-PCR using the SYBR® Premix EX Taq™ II kit (TaKaRa, Dalian, China, cat. no: DRR081A). The thermal cycling conditions were as follows: 95 °C for 60 s followed by 35 cycles of 94 °C for 10 s, 59 °C for 30 s and 72 °C for 30 s. After amplification, the products were subjected to an increasing temperature gradient from 60 to 95 °C. Plates were held for 1 s and read every 0.4 °C to create a melting curve. PCR products were evaluated by electrophoresis on 1% agarose gels containing ethidium bromide. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was used as an internal control to normalize input mRNA level. The primer sequences are as follows: c-myc-forward, 5′-ACAGCTACGGAACTCTTGTGCGTA-3′, reverse, 5′-GCCCAAAGTCCAATTTGAGGCAGT-3′; PCNA-forward, 5′-AGCCGAAACCAGCTAGACTTTCCT-3′, reverse, 5′-ACGAGTCCATGCTCTGCAGGTTTA-3′; CCND1-forward, 5′-ACACACACACACAAACCTTCTGCC-3′, reverse, 5′-ATCAGATGACTCTGGGAAACGCCA-3′; miR-17–92-cluster-forward, 5′-CAGTAAAGGTAAGGAGAGCTTCATCTG-3′, reverse, 5′-CATACAACCACTAAGCTAAAGAATAATCTGA-3′; p53-forward, 5′-GCCGTCCCAAGCAATGGATGATTT-3′, reverse, 5′-TCTGGCATTCTGGGAGCTTCATCT-3′; GAPDH-forward, 5′-TCGACAGTCAGCCGCATCTTCTTT-3′, reverse, 5′-ACCAAATCCGTTGACTCCGACCTT-3′. All the experiments were performed according to the provided protocol.

The relative expression fold change of miRNAs and target mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method [22]. All reactions were performed in triplicate.

2.8. Statistical analysis

Data were presented as the mean ± SD from at least three separate experiments, and significance was analyzed using Student's *t*-test. A p-value < 0.05 was considered significant.

3. Results

3.1. c-myc was over-expressed in RCC cell lines

To evaluate c-myc expression levels in 769P, Caki-1 and 786O RCC cell lines, equal amounts of total RNA from the normal tissues of 10 RCC patients were pooled together as a control. We compared the c-myc mRNA level of each RCC cell line with the pool of 10 normal specimens by qRT-PCR. We found that all cell lines over-expressed c-myc (Fig. 1A), with 769P displaying the highest level of c-myc.

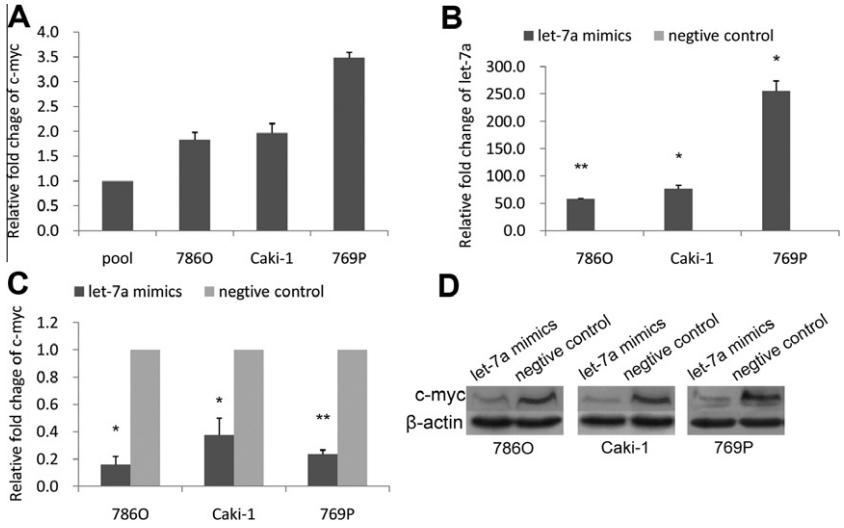

Fig. 1. Let-7a down-regulated c-myc in RCC cell lines. (A) The c-myc mRNA levels of 786O, Caki-1 and 769P compared the pool of 10 normal specimens from 10 RCC patients. (B) Forty-eight hours after transfection, let-7a was enhanced compared with corresponding negative control group in each RCC cell line. (C) Forty-eight hours after transfection, the c-myc mRNA level was down-regulated by let-7a in each RCC cell line. (D) Forty-eight hours after transfection, the c-myc protein level was down-regulated by let-7a in each RCC cell line. The data are shown as the mean ± SD. * Represents a significant difference between the let-7a-mimics-transfected group and the negative control group ($p < 0.05$). ** Represents a significant difference between the let-7a-mimics-trnsfected group and the negative control group ($p < 0.001$). All experiments were performed three times independently.

3.2. c-myc was down-regulated by let-7a in RCC cell lines

Forty-eight hours after transfection, the cells transfected with let-7a mimics displayed increased expression of let-7a (Fig. 1B) and decreased c-myc mRNA level (Fig. 1C) in comparison to those transfected with the negative control. c-myc expression levels of all three RCC cell lines were also significantly down-regulated (Fig. 1D).

3.3. Downstream effects of let-7a-mediated down-regulation of c-myc

We compared the proliferation rates of each RCC cell line transfected with the let-7a mimics or negative control 24 h after transfection and for the following five consecutive days. Each RCC cell line displayed a decreased proliferation rate (Fig. 2). The cell cycle change varied in different RCC cell lines (Fig. 3 and Supplementary materials). Forty-eight hours after transfection, all three RCC cell lines showed significant decreases in the abundance of cells in S phase compared to the negative control group. A significant G0–G1 arrest in 786O and G2–M arrest in 769P was also observed in cells transfected with let-7a mimics in comparison to the negative control. To analyze the detailed mechanism of down-stream effects due to c-myc reduction imposed by let-7a, the mRNA levels of c-myc target genes were measured by qRT-PCR in 786O, 769P and Caki-1 cells transfected with let-7a mimics or the negative control. As shown in Fig. 4, the miR-17–92 cluster, PCNA and CCND1 were all significantly down-regulated in three RCC cells 48 h after transfection. p53 was up-regulated in 769P and Caki-1 cell lines but down-regulated in the 786O cell line (Fig. 4A).

4. Discussion

Many studies, including our own [12], have reported that c-myc is up-regulated in RCC [10,11]. In agreement with Tang et al. [10], we showed that c-myc was over-expressed in 769P, Caki-1 and 786O RCC cell lines (Fig. 1A). c-myc protein and mRNA levels were decreased compared to the negative control group (Fig. 1C and D) after let-7a levels were enhanced by transfection (Fig. 1B). These results suggest that c-myc may be a target of let-7a in RCC cells. miRNAs regulate target mRNAs by binding to the complementary seed sequence and either signal for cleavage/degradation or inhibit translation of the target mRNAs [23]. In the case of the former, the mRNA level of miRNA-targeted genes will decrease, while the miRNAs are up-regulated. In the latter, there is no change in the abundance of the target mRNA after miRNA up-regulation, but a decrease in protein level will be observed. We observed that let-7a down-regulated the c-myc protein level by both inhibiting translation and causing mRNA cleavage/degradation as the mRNA level of c-myc showed significant down-regulation in three cell lines (Fig. 1C). This result is in accordance with the observations made by He et al. [19] in lung cancer.

Myc family proteins promote proliferation, growth, apoptosis [24,25] and cell cycle progression [26]. The proliferation rate was decreased in each RCC cell lines (Fig. 2), which was characterized

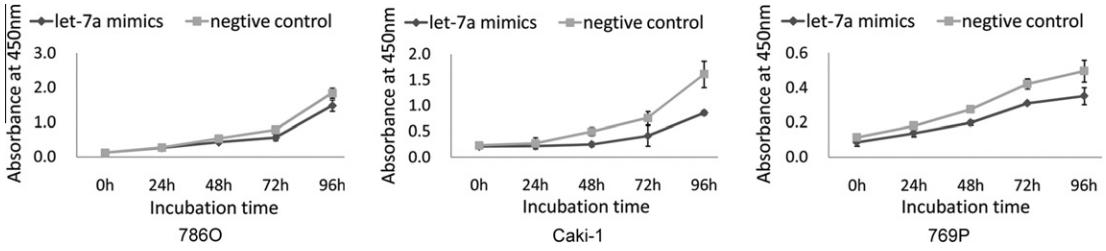

Fig. 2. Inhibitory effects of let-7a on growth ability of RCC cell lines. Twenty-four hours after transfection, cells were seeded into 96-well plates at 1000 cells per well. The effect of let-7a on cell proliferation was detected 0, 24, 48, 72 and 96 h after seeding. The data are shown by the mean ± SD. All experiments were performed three times independently.

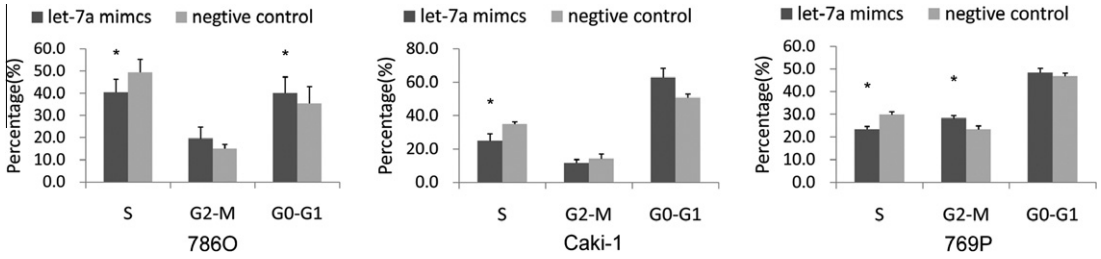

Fig. 3. Cell cycle arrest caused by let-7a in three RCC cell lines. Cell cycle arrest varied in different RCC cell lines. The percentages are shown as the mean ± SD. ∗ Represents a significant difference between the let-7a mimics-transfected group and the negative control group ($p < 0.05$). ∗∗ Represents a significant difference between the let-7a mimics-transfected group and the negative control group ($p < 0.001$). All experiments were performed three times independently.

as an S phase delay (Fig. 3 and Supplementary materials). In addition, one c-myc target gene, PCNA, is reported to play a role in cancer cell proliferation by contributing to DNA synthesis in S phase [27–29]. Our study revealed that the decreased PCNA level might be caused by down-regulated c-myc (Fig. 4B). This down-regulation partly explains why the percentage of cells in S phase was significantly lower in cells transfected with let-7a mimics than in the three RCC cell lines (Fig. 3 and Supplementary materials).c-myc, which is able to bind the E box that is present in the CCND1 promoter, is an upstream regulator of CCND1 [30]. c-myc and CCND1 are involved in the Wnt pathway, which regulates cell growth and proliferation. There is a chronological order of target gene expression in this pathway when c-myc is activated earlier than CCND1 [31]. In our study, let-7a functioned as a tumor suppressor by down-regulating c-myc and its downstream target CCND1 (Fig. 4C). We found that the decreased CCND1 level might be a potential mechanism for the arrest of 786O observed in G0–G1. However, according to Wang et al. [6], c-myc depletion inhibits proliferation of human tumor cells at various stages of the cell cycle, causing the cell cycle arrest to arrest at multiple stages depending upon the p53 levels. We observed that cells arrested in different phases of the cell cycle, for example, 786O arrested in the G0–G1/S phase, 769P arrested in the G2–M/S phase and Caki-1 arrested only in S phase (Fig. 3 and Supplementary materials). p53 is a regulator in the G2–M transition [32]. Therefore, decreased p53 levels may explain why 786O arrested in the G0–G1 transition while increased p53 levels in 769P may have caused an arrest at the G2–M transition (Fig. 3). Although, p53 was up-regulated in Caki-1 after transfection (Fig. 4A), we did not see any significant change in the G0–G1 or G2–M cell cycle distribution in Caki-1 (Fig. 3). Our results can only partially explain the various cell cycle changes observed in different RCC cell lines, suggesting that additional factors within the complicated network regulate cell cycle progression and might contribute to the arrests that we observed.

The human miR-17–92 cluster is located in the third intron of a ~7 kb primary transcript known as C13orf25, yielding the 6 mature miRNAs miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a [33]. This cluster is located at chromosome 13q31.3 and can be regulated by c-myc [34]. The single function of this transcript is to produce these miRNAs [35]. The miR-17–92 cluster can contribute to carcinogenesis by promoting proliferation and angiogenesis and inhibiting apoptosis through suppression of the expression of several tumor suppressor genes [8,36]. In RCC, the miR-17–92 cluster is over-expressed and functions as an oncogene [37,38]. We observed that the levels of the miR-17–92 cluster transcript were significantly decreased in three RCC cell lines upon let-7a-mediated down-regulation of c-myc, (Fig. 4D), which also contributed to the decreased proliferation rate.

Therefore, in RCC cell lines, let-7a can function as a tumor suppressor by down-regulating c-myc and c-myc target genes such as PCNA, CCND1 and the miR17–92 cluster. Let-7a decreases the

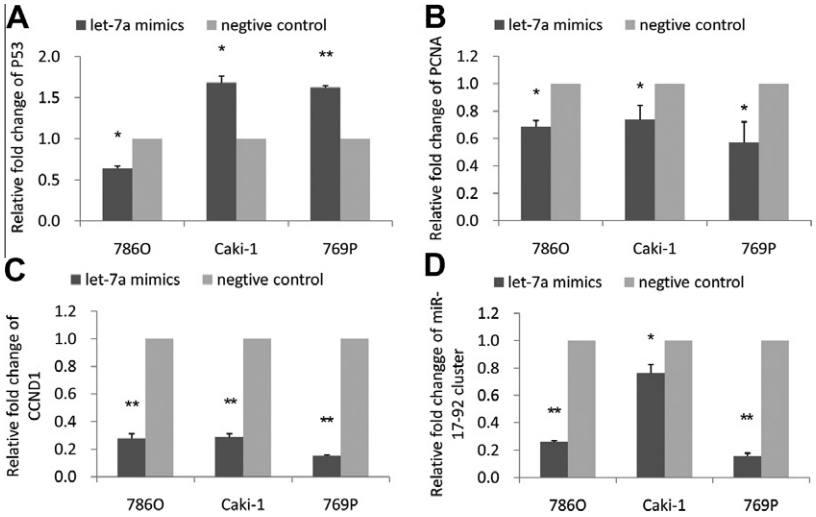

Fig. 4. The down-stream effects of let-7a on mRNAs in RCC cell lines. (A) p53 expression levels were down-regulated in 786O and up-regulated in Caki-1 and 769P. (B) PCNA expression levels were down-regulated in 786O, Caki-1 and 769P. (C) CCND1 expression levels were down-regulated in 786O, Caki-1 and 769P. (D) MiR-17–92 cluster expression levels were down-regulated in 786O, Caki-1 and 769P. Data are shown as the mean ± SD. ∗ Represents a significant difference between the let-7a mimics-transfected group and the negative control group ($p < 0.05$). ∗∗ Represents a significant difference between the let-7a mimics-transfected group and the negative control group ($p < 0.001$). All experiments were performed three times independently.

proliferation rate and causes a cell cycle arrest at different stages. We believe that let-7a has a widespread effect on RCC cell lines by down-regulating c-myc because of the broad regulatory network of c-myc. c-myc can regulate more than the three above-mentioned genes. It is not only important in RCC, as we have presented, but also in many other human malignancies [2]. We speculate that studying any factor that can directly act with c-myc could provide significant insight into carcinogenesis, or at least into the carcinogenesis of RCC. Further studies are needed to discover the mechanism of the anti-neoplastic effects of let-7a and to investigate c-myc-related biomarkers in RCC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.119.

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