Identification of microRNAs and mRNAs associated with multidrug resistance of human laryngeal cancer Hep-2 cells

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Abstract

Multidrug resistance (MDR) poses a serious impediment to the success of chemotherapy for laryngeal cancer. To identify microRNAs and mRNAs associated with MDR of human laryngeal cancer Hep-2 cells, we developed a multidrug-resistant human laryngeal cancer subline, designated Hep-2/v, by exposing Hep-2 cells to stepwise increasing concentrations of vincristine (0.02-0.96 μM). Microarray assays were performed to compare the microRNA and mRNA expression profiles of Hep-2 and Hep-2/v cells. Compared to Hep-2 cells, Hep-2/v cells were more resistant to chemotherapy drugs (–45-fold more resistant to vincristine, 5.1-fold more resistant to cisplatin, and 5.6-fold more resistant to 5-fluorouracil) and had a longer doubling time (42.33±1.76 vs 28.75±1.12 h, P<0.05), higher percentage of cells in G0/G1 phase (80.98±0.52 vs 69.14±0.89, P<0.05), increased efflux of rhodamine 123 (95.97±0.56 vs 12.40±0.44%, P<0.01), and up-regulated MDR1 expression. A total of 7 microRNAs and 605 mRNAs were differentially expressed between the two cell types. Of the differentially expressed mRNAs identified, regulator of G-protein signaling 10, high-temperature requirement protein A1, and nuclear protein 1 were found to be the putative targets of the differentially expressed microRNAs identified. These findings may open a new avenue for clarifying the mechanisms responsible for MDR in laryngeal cancer.

Key words: Multidrug resistance; Laryngeal cancer; Hep-2 cells; mRNA; microRNA

Introduction

Laryngeal cancer (LC) is one of the most common head and neck cancers. Surgery-based multimodality therapy is currently the most effective treatment for LC. Chemotherapy, as an important therapeutic modality for LC, plays a crucial role in killing residual tumor cells, preventing tumor micrometastasis, and lowering the incidence of local recurrence or distant metastases (1). However, multidrug resistance (MDR) poses a serious impediment to the success of chemotherapy for LC (2).

The acquisition of MDR by tumor cells is a complicated, multifactorial process. Several interrelated mechanisms have been proposed for the explanation of the emergence of MDR (3,4). These mechanisms include i) reduced drug accumulation due to increased expression of efflux drug transporters, such as MDR1 and MDR-associated proteins (5), ii) increased drug inactivation resulting from metabolic alterations (6), iii) increased ability to repair and/or tolerate DNA lesions due to increased expression of DNA topoisomerases (7), and iv) inhibition of apoptosis by alteration in the expression of apoptosis-associated genes or proteins, such as p53 and Bcl-2 (8,9). Understanding the precise mechanisms by which tumor cells acquire MDR is imperative for the discovery of novel anti-MDR agents.

MicroRNAs (miRNAs) are a class of recently discovered small non-coding RNAs that play important regulatory roles in cell proliferation, differentiation, and apoptosis by targeting mRNAs for degradation or translational repression (10,11). Deregulated miRNA expression has been linked to the development and progression of a variety of cancers, including LC (12-14). miRNAs have also been strongly implicated in the modulation of MDR in many different types of cancer, such as breast, gastric, ovarian, and prostate cancer (15-19). Multiple MDR-associated miRNA targets, such as Bcl-2 (17), PTEN (18), and MDR1 (20), have been identified. However,
there have been no reports of miRNAs involved in MDR in LC.

To better understand the mechanism underlying MDR in LC and gain an insight into the role of miRNAs in MDR of LC cells, we developed a multidrug resistant variant (designated Hep-2/v) of the human LC line Hep-2 in the present study. Given that vincristine (VCR) is an important component of many combination chemotherapy regimens for treating LC and that MDR of VCR-resistant cell lines has been demonstrated in previous studies (21,22), we selected VCR to induce MDR of Hep-2 cells. By comparing the expression profiles of miRNAs and mRNAs of Hep-2 cells and Hep-2/v cells using microarray assays, we identified multiple miRNAs and mRNAs that are potentially involved in MDR of LC cells. Furthermore, the association between these miRNAs and mRNAs was analyzed using an miRNA target prediction program. The results obtained provide new clues into the molecular mechanisms behind MDR in LC.

Material and Methods

Cell culture and treatment

The human LC cell line Hep-2 was provided by the Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. VCR (Sigma, USA) was added to Hep-2 cells in stepwise increasing concentrations (from 0.02 to 0.96 μM) to develop a drug-resistant cell variant (Hep-2/v). The Hep-2/v cell line was obtained after a total of 40 passages in the presence of VCR. The MDR phenotype of Hep-2/v cells was determined by incubating the cells with serially diluted VCR (8-128 μM), cisplatin (DDP, 0.625-10 μM), or 5-FU (1,000-16,000 μM) for 72 h, followed by incubation with MTT solution for 4 h. RPMI 1640 medium was used as a blank control. At the end of the incubation period, dimethyl sulfoxide was added at 200 μL/well and the plates were incubated in an air bath shaker at 37°C for 5 min. Absorbance at 490 nm (A₄₉₀) was measured using a microplate reader to assess cell viability. The dose-response curve was then plotted to determine the half-maximal-inhibitory concentration (IC₅₀). The initial concentration of VCR was equal to half of the IC₅₀ value. The resistance index (RI) was calculated by dividing the IC₅₀ values obtained for Hep-2/v cells by those for Hep-2 cells.

Rhodamine 123 retention assay and cell cycle analysis by flow cytometry

After Hep-2 or Hep-2/v cells (2 × 10⁶) were harvested to prepare single cell suspensions, 2.5 μL rhodamine 123 (5 mM; Sigma) was added and incubated at 37°C for 30 min. The cells were then centrifuged at 60 g to remove the supernatant, washed with fresh medium, and then incubated at 37°C for an additional 10 min. After washing the cells again with fresh medium, the cells were resuspended in precooled medium and subjected to flow cytometric measurement of rhodamine 123 retention. For cell cycle analysis, Hep-2 or Hep-2/v cells (2 × 10⁵) were harvested and fixed with a mixture of 300 μL phosphate-buffered saline (PBS) containing 5% FCS and 700 μL 70% ethanol at -20°C for 24 h. After washing twice with PBS, the cells were incubated with 100 μL RNase A (1 mg/mL) at 37°C for 30 min, stained with 300 μL propidium iodide (100 μg/mL) at room temperature for 20 min, and analyzed by flow cytometry. A BD FACS Aria flow cytometer (BD Biosciences, USA) was used, and data were analyzed using the QuantiCALC software (BD Biosciences).

miRNA microarray analysis

Total RNA was prepared from Hep-2 or Hep-2/v cells using Trizol reagent (Invitrogen), purified using the mirVana miRNA Isolation kit (Ambion, USA), tailed with polyadenylation polymerase, ligated with biotinylated 3DNA dendrimers, and hybridized to Affymetrix GeneChip miRNA arrays using the FlashTag™ Biotin...
RNA labeling kit (Genisphere, USA) according to the manufacturer’s instructions. Slides were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, USA), and miRNA data were analyzed using the miRNA QC Tool (Affymetrix). Differentially expressed miRNA genes were identified using the significance analysis of microarrays (SAM) program. The miRNAs with a q-value < 5% and a fold-change > 2 were considered to be significantly differentially expressed. Hierarchical clustering of differentially expressed miRNAs was performed using the Cluster 3.0 program.

mRNA microarray analysis and miRNA target prediction

Total RNA was prepared from Hep-2 or Hep-2/v cells as described above. RNA purification and cDNA synthesis as well as cRNA synthesis, purification, labeling, and hybridization to Human Genome v2.0 oligonucleotide microarrays were performed using the cRNA Amplification and Labeling Kit (CapitalBio, China) according to the manufacturer’s instructions. Slides were scanned with the LuxScan 10 KA microarray scanner (CapitalBio), and mRNA data were analyzed using the LuxScan3.0 image analysis software (CapitalBio). Differentially expressed mRNAs were detected using the SAM program. Genes with a q-value < 5% and a fold-change > 2 were considered to be significantly differentially expressed. Hierarchical clustering of differentially expressed genes was performed using the Cluster 3.0 program. Based on the data obtained from differentially expressed miRNA genes and mRNAs, miRNA target prediction was performed. Putative target genes of each miRNA were predicted using the miRanda software. We then analyzed the mRNA and miRNA data to select the differentially expressed genes based on the direction of expression change, which was where the up-regulated miRNA was associated with a down-regulated, predicted mRNA target, or otherwise.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA and miRNA expression

Total RNA (the same samples for microarray analysis) was reversely transcribed into cDNA using M-MLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The mRNA and miRNA amplification, a universal sense primer (5'-GGT CGG TGC CCG AGG TTT CCA-3') and an miRNA-specific antisense primer (has-miR-210: 5'-TGT TGC TGC TGG TGG GC-3', 5'-AGC GGC TGG ACT CAG GGA CCG ACT-3'), beta-actin (250 bp): 5'-CAT GTA CGT TGC TAT CCA GGC-3', 5'-CTC TTT AAT GTC CCA-3'; GAPDH (306 bp): 5'-GGG ACT CAG GGA CGG ACT-3', 5'-TCC GGC CTC CAG CCA-3'; GAPDH (306 bp): 5'-GGG ACT CAG GGA CGG ACT-3', 5'-TCC GGC CTC CAG CCA-3'; GAPDH (306 bp): 5'-GGG ACT CAG GGA CGG ACT-3', 5'-TCC GGC CTC CAG CCA-3'; has-miR-210: 5'-TGT TGC TGC TGG TGG GC-3', 5'-AGC GGC TGG ACT CAG GGA CCG ACT-3'. For miRNA amplification, a universal sense primer (5'-GTG CAG TGT CCG AGG T-3') and an miRNA-specific antisense primer (has-miR-210: 5'-TGT TGC TGC TGG TGG GC-3' AGC CAC GAT-3'; GAPDH (306 bp): 5'-TGA ACG GGA AGC TCA CTG G-3', 5'-TCC ACC ACC CTG TG TCT GA-3'; U6 snRNA (94 bp): 5'-GCT CCG ACG TCT CGA-3', 5'-AAC GCT TCA CGA ATT TGC GT-3'. For miRNA amplification, a universal sense primer (5'-GGT CGG TGC CCG AGG TTT CCA-3') and an miRNA-specific antisense primer (has-miR-210: 5'-TGT TGC TGC TGG TGG GC-3', 5'-AGC GGC TGG ACT CAG GGA CCG ACT-3').

Western blot

Total cell extracts were prepared and subjected to spectrophotometric measurement of protein concentration. Forty micrograms of total cell proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). The membrane was blocked for 1 h at room temperature in PBS containing 0.3% Tween 20 and 5% skim milk and incubated overnight at 4°C with an anti-MDR1 antibody (dilution 1:1,000; Chemicon, USA) or anti-actin antibody (dilution 1:1,500; Chemicon). Antibody binding was revealed by incubation with horseradish peroxidase-coupled secondary antibody (dilution 1:5,000; Pierce, USA) for 1 h at room temperature. Chemiluminescence was detected using enhanced chemiluminescence reagents (Pierce). The relative level of the MDR1 protein to actin was determined by densitometric scanning.

Statistical analysis

Statistical analysis was performed using the SPSS 11.0 software package (SPSS Inc., USA). Data are reported as means ± SD. The means between two groups were compared using the Student t-test. The comparison of multiple means was performed using analysis of variance. Categorical data were compared using the chi-square test.

Results

Successful generation of a multidrug-resistant Hep-2 subline (Hep-2/v)

A multidrug-resistant variant of the human LC line Hep-2 was selected by adaptation to stepwise increasing concentrations of VCR and designated as Hep-2/v. The IC50 values for VCR, DDP, and 5-FU were significantly
higher in Hep-2/v cells than in Hep-2 cells (VCR = 0.04 ± 0.01 vs 1.8 ± 0.20; DDP = 0.59 ± 0.26 vs 1.97 ± 0.17; 5-FU = 60.92 ± 4.23 vs 330 ± 4.65; all P < 0.05), and the RI for VCR, DDP, and 5-FU were 45.00, 5.10, and 5.56, respectively.

Multidrug-resistant Hep-2/v cells exhibited altered biological characteristics compared to Hep-2 cells

Hep-2/v cells showed significant morphological changes compared to Hep-2 cells. Both Hep-2 and Hep-2/v cells grew in an adherent manner. Hep-2/v cells were larger and rounder, had more cytoplasmic granules, and aggregated into small clusters. The adherence ability of Hep-2/v cells was weaker than that of Hep-2 cells. Electron microscopy showed projected microvilli on the cell surface and abundant organelles in Hep-2 cells (see Supplementary Figure S1). In contrast, loss of surface microvilli, slight nuclear vacuolation, organelle swelling, and a marked reduction in the number of organelles were observed in Hep-2/v cells.

Hep-2/v cells grew slower than Hep-2 cells. Figure 1A shows the growth curves of Hep-2 and Hep-2/v cells. The doubling time of Hep-2/v cells was significantly longer than that of Hep-2 cells (42.33 ± 1.76 vs 28.75 ± 1.12 h, P < 0.05), indicating that the growth rate of multidrug-resistant Hep-2/v cells was slower than that of wild-type Hep-2 cells.

Hep-2/v cells in G0/G1 phase arrest compared to Hep-2 cells. Flow cytometric analysis showed that the percentage of cells in G0/G1 phase was significantly higher in Hep-2/v cells than in Hep-2 cells (80.98 ± 0.52 vs 69.14 ± 0.89, P < 0.05), whereas the percentage of cells in S phase was significantly lower in Hep-2/v cells than in Hep-2 cells (9.76 ± 0.88 vs 19.43 ± 2.04, P < 0.05). No significant difference was noted in the percentage of cells in G2/M phase in the two groups of cells (8.99 ± 0.48 vs 11.52 ± 1.20, respectively, P > 0.05; Figure 1B).

Hep-2/v cells had increased efflux of rhodamine 123 compared to Hep-2 cells. As shown in Figure 2A, the percentage of rhodamine 123-positive Hep-2 cells was significantly higher than that of rhodamine 123-positive Hep-2/v cells (95.97 ± 0.56 vs 12.40 ± 0.44%, respectively, P < 0.01), suggesting an increased efflux of rhodamine 123 in multidrug-resistant Hep-2/v cells.

MDR1 mRNA and protein expression is upregulated in Hep-2/v cells compared to Hep-2 cells. As shown in Figure 2B and C and Supplementary Figure S2, the relative expression levels of MDR1 mRNA and protein were 9.61- and 3.48-fold higher in Hep-2/v cells than in Hep-2 cells (both P < 0.01).

Identification of 7 putative MDR-associated miRNAs in Hep-2/v cells

A total of 7 differentially expressed miRNAs were identified when Hep-2 and Hep-2/v cells were compared by microarray analysis (Table 1 and Figure 3A). Two miRNAs (has-miR-210 and has-miR-923) were significantly up-regulated and five miRNAs (has-miR-93, has-miR-93-star, has-miR-424-star, has-miR-25-star, and has-miR-494) were significantly down-regulated in Hep-2/v cells. To verify the reliability of the microarray results, real-time RT-PCR was performed to measure the expression of has-miR-210, has-miR-923, and has-miR-93. As shown in Figure 4A, RT-PCR results showed that has-miR-210 and has-miR-923 expression had a 4.78- and 1.53-fold up-regulation, and has-miR-93 expression showed a 1.46-fold down-regulation in Hep-2/v cells compared with Hep-2 cells. These findings were well matched with the microarray results.
Identification of 605 putative MDR-associated mRNAs in Hep-2/v cells

A total of 605 differentially expressed mRNAs were identified when Hep-2 and Hep-2/v cells were compared by microarray analysis (Figure 3B, Supplementary Figure S3, and Supplementary Table S1). Of these mRNAs, 270 were significantly up-regulated and 335 were significantly down-regulated in Hep-2/v cells. These mRNAs have been implicated in carcinogenesis, signal transduction, cytoskeletal organization and cell motility, protein translation, DNA synthesis, and repair and metabolism, as well as other cellular functions. Table 2 shows representative mRNAs that were differentially expressed between Hep-2 and Hep-2/v cells. Criteria for choosing these representative mRNAs were: i) showing significantly differential expression, ii) having either a direct or indirect relationship with the identified differentially expressed miRNAs (revealed by searching miRNA target prediction databases), and iii) genes of our interest for further study.

HTRA1 and NUPR1 expression was down-regulated and RGS10 expression was up-regulated in Hep-2/v cells compared to Hep-2 cells

To identify putative target genes of differentially expressed miRNAs, we used the miRanda software to deduce target genes. NUPR1 was identified as the putative target of has-miR-210 and has-miR-923, whereas HTRA1 and RGS10 were identified as putative targets of has-miR-210 and has-miR-93, respectively. Real-time RT-PCR analysis confirmed that the relative expression levels of HTRA1 and NUPR1 mRNA were significantly lower (HTRA1: 2.12- and 5.08-fold relative to beta-actin and GAPDH, respectively; NUPR1: 2.81- and 4.06-fold relative to beta-actin and GAPDH, respectively), whereas the RGS10 mRNA expression level was significantly higher.

Table 1. Differential expression of miRNAs by Hep-2 and Hep-2/v cells.

| miRNA     | Fold-change | Regulation | q-value |
|-----------|-------------|------------|---------|
| has-miR-210 | 4.69        | up         | 4.06    |
| has-miR-923 | 2.30        | up         | 4.06    |
| has-miR-93  | 0.44        | down       | 0.00    |
| has-miR-93-star | 0.44   | down       | 0.00    |
| has-miR-424-star | 0.45  | down       | 0.00    |
| has-miR-25-star | 0.42   | down       | 0.00    |
| has-miR-494  | 0.31        | down       | 0.00    |

miRNA = microRNA.
significantly higher (2.35- and 2.71-fold relative to beta-actin and GAPDH, respectively) in Hep-2/v cells (Figure 4B).

**Discussion**

MDR is a major obstacle to the successful chemotherapeutic treatment of LC, and therefore the elucidation of the mechanisms responsible for MDR in LC represents an important step toward overcoming this problem. The establishment of multidrug-resistant tumor cell lines provides an important tool for the study of MDR in cancer (24). In the present study, we generated a multidrug-resistant human LC subline, which was designated Hep-2/v, by exposing Hep-2 cells to stepwise increasing concentrations of VCR. In comparison to Hep-2 cells, Hep-2/v cells showed approximately 45-fold resistance to VCR, 5.1-fold resistance to DDP, and 5.6-fold resistance to 5-FU. Thus, the Hep-2/v subline may represent a promising cell model for the study of MDR and screening of anti-MDR agents in LC.

The doubling time of Hep-2/v cells was 13.58 h longer than that of Hep-2 cells, suggesting that the growth rate of Hep-2/v cells decreased remarkably. Because chemotherapy mainly affects rapidly dividing cells, tumor cells with a long doubling time are generally insensitive to chemotherapy (25). The slower growth rate of Hep-2/v cells could be explained by the observation that the percentage of cells in G0/G1 phase significantly increased and the percentage of cells in S phase significantly decreased in Hep-2/v cells compared to Hep-2 cells. This may be because VCR is able to bind to tubulin dimers, prevent the formation of spindles, and induce mitotic arrest in metaphase.

The development of MDR is a multifactorial process mediated by multiple mechanisms (3-9). Increased drug efflux resulting from the up-regulated expression of
efflux drug transporters is a well-established cause of MDR (5). In this study, rhodamine 123 retention was significantly reduced and the expression of both MDR1 mRNA and protein was significantly up-regulated in Hep-2/v cells, suggesting that drug efflux mediated by MDR1 overexpression is an important mechanism that allows Hep-2/v cells to acquire MDR.

A strong association between miRNAs and the acquisition of MDR by tumor cells has been established in previous studies (15-20,26). In the present study, seven miRNAs were differentially expressed when Hep-2 cells were compared to Hep-2/v cells (Table 1). Of these identified miRNAs, has-miR-210 has been implicated in tumor hypoxia and drug resistance in head and neck squamous cell carcinoma (27,28), has-miR-923 has been found to be up-regulated in taxol-resistant cancer cells (29), and has-miR-93 has been shown to be down-regulated in adriamycin-resistant breast cancer MCF-7 cells (30). These findings, together with our observation, highlight the possible role of these miRNAs in MDR in LC.

MiRNAs exert their functions by modulating their target genes (10,11). For this reason, we performed a cDNA microarray analysis to identify mRNAs that are differentially expressed between Hep-2 and Hep-2/v cells in order to predict putative target genes for has-miR-210, has-miR-923, and has-miR-93. Of the 605 differentially expressed genes identified, three (HTRA1, NUPR1, and RGS10) were putative target genes for these miRNAs. Interestingly, Chien et al. (31) demonstrated that up-regulation of HTRA1 expression attenuates DDP- and paclitaxel-induced cytotoxicity, and, conversely, the forced expression of HTRA1 enhances DDP- and paclitaxel-induced cytotoxicity. This result is consistent with our finding that HTRA1 down-regulation that is mediated by has-miR-210 may be involved in the acquisition of MDR by Hep-2/v cells.

NUPR1 was down-regulated in Hep-2/v cells and may be regulated by both has-miR-210 and has-miR-923. Some studies have demonstrated that NUPR1 can protect some cancer cells from apoptosis and confer resistance to some chemotherapeutic drugs (32,33). In contrast, other studies have shown an inverse relationship between NUPR1 overexpression and apoptosis in different types of cancer (34,35). These findings suggest a dual role of NUPR1 in regulating apoptosis and chemoresistance of cancer cells. RGS10 was up-regulated in Hep-2/v cells and may represent a novel MDR-associated protein, since, to date, no reports have shown a link between RGS10 expression and the chemoresistance of cancer cells.

In conclusion, we have developed a multidrug-resistant human LC subline designated Hep-2/v by exposure of Hep-2 cells to stepwise increasing concentrations of VCR. Using this cell model, we performed microarray assays to compare the miRNA and mRNA expression profiles of Hep-2 and Hep-2/v cells and identified seven differentially expressed miRNAs and 605 differentially expressed mRNAs. Additionally, we explored the association between these differentially expressed miRNAs and mRNAs. These findings may open a new avenue for the clarification of mechanisms responsible for MDR in LC.
Table 2. Representative mRNAs that are differentially expressed between Hep-2 and Hep-2/v cells.

| Genbank accession No. | Full gene name                      | Gene symbol | Fold-change | q-value |
|-----------------------|-------------------------------------|-------------|-------------|---------|
| Up-regulated          |                                     |             |             |         |
| NM_016109             | Angiopoietin-related protein 4      | ANGPTL4     | 15.04       | 0.00    |
| NM_002228             | Transcription factor AP-1           | JUN         | 7.92        | 0.00    |
| NM_000927             | Multidrug resistance protein 1      | ABCB1       | 5.87        | 0.14    |
| NM_139317             | Baculoviral IAP repeat-containing protein 7 | BIRC7   | 4.29        | 0.14    |
| NM_002925             | Regulator of G-protein signaling 10 | RGS10       | 2.61        | 0.14    |
| NM_006545             | Tumor suppressor candidate 4        | TUSC4       | 2.57        | 0.00    |
| Down-regulated        |                                     |             |             |         |
| NM_006754             | Synaptophysin-like protein 1        | SYPL1       | 0.48        | 0.50    |
| NM_002775             | High-temperature requirement protein A1 | HTRA1     | 0.47        | 0.62    |
| NM_133377             | Cell cycle checkpoint protein 1     | RAD1        | 0.46        | 0.24    |
| NM_012385             | Nuclear protein 1                   | NUPR1       | 0.43        | 0.62    |
| NM_001259             | Cell division protein kinase 6      | CDK6        | 0.18        | 0.00    |
| NM_021073             | Bone morphogenetic protein 5        | BMP5        | 0.06        | 0.00    |

Supplementary Material

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Acknowledgments

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