Original Paper

MiR-129-5p Sensitizes the Response of Her-2 Positive Breast Cancer to Trastuzumab by Reducing Rps6

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Key Words

Her-2-positive breast cancer • Trastuzumab resistance • MiR-129-5p • Rps6

Abstract

Background/Aims: Trastuzumab is an important treatment used for patients with Her-2-positive breast cancer, but an increasing incidence of trastuzumab resistance has been observed clinically during the past decade. Aberrant microRNA (miR) expression levels are correlated with prognosis and response to trastuzumab in breast cancer. MiR-129-5p is downregulated in trastuzumab-resistant human breast cancer cells (JIMT-1), but its potential function and underlying mechanism remain unclear. **Methods:** Quantitative RT-PCR (qRT-PCR) was used to determine the expression levels of miR-129-5p and its potential target genes. The effects of miR-129-5p on cell responses to trastuzumab were analyzed by CCK-8 and flow cytometry assays in Her-2-positive breast cancer cells (SKBR-3 and JIMT-1). Bio-informatics analyses were performed to predict target genes of miR-129-5p, and luciferase assays were carried out to confirm the binding of miR-129-5p and rpS6. **Results:** MiR-129-5p, which was downregulated and predicted to target rpS6 in trastuzumab-resistant breast cancer cells, enhanced the sensitivity of breast cancer cells to trastuzumab by reducing the expression of rpS6. Moreover, the overexpression of rpS6 reversed the sensitivity of cells to trastuzumab induced by miR-129-5p. **Conclusions:** MiR-129-5p sensitized Her-2-positive breast cancer to trastuzumab by downregulating rpS6. These findings provide novel insights into the common role of rpS6 and its related molecular mechanisms in mediating trastuzumab-resistance in Her-2-positive breast cancers.

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Published by S. Karger AG, Basel

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Introduction

ErbB-2/Her-2 is amplified in 20-30% of human invasive breast cancers and is associated with a poor prognosis and reduced disease-free survival rates [1, 2]. The humanized anti-Her-2 antibody trastuzumab (Herceptin) has significantly increased response rates in Her-2-positive breast cancer patients; however, most patients who respond to the initial treatment develop resistance within 1 year [3]. Furthermore, trastuzumab-resistant breast cancers have been identified to be more aggressive [4]. Hence, it is necessary to clarify the underlying mechanisms of trastuzumab resistance to develop new strategies for treating Her-2-positive breast cancer.

During the past decades, it has been confirmed that approximately 70% of Her-2-positive breast cancers receive few benefits from trastuzumab treatment [3], which indicates that Her-2 expression is not the only factor involved in the response of cancers to trastuzumab. A series of studies found that aberrant PI3K/Akt/mTOR/rpS6 signaling activity and loss of PTEN expression contributed greatly to trastuzumab resistance in breast cancers [5-7]. Recently, many miRNAs, such as miR-210, miR-375, and miR-200c, have been found play important roles not only in chemoresistance but also in trastuzumab resistance [8-10].

MiRNAs belong to a class of small non-coding RNA molecules that are 19-25 nucleotides in length; they regulate the expression of a variety of genes via sequence-specific binding to the 3'-untranslated region (3'-UTR) of target mRNAs, which results in the degradation of target mRNAs or inhibition of the mRNA-protein translation process [11, 12]. MiRNAs are phylogenetically conserved, and over 50% of miRNAs are located in cancer-associated genomic regions [13], which indicates that miRNAs may play important roles in a variety of biological processes [14], such as tumorigenesis. Additionally, miRNAs are also closely associated with drug resistance. Jung et al. found that miR-210, a hypoxia-induced miRNA in MCF-7 breast cancer cells, was significantly upregulated in trastuzumab-resistant BT474 cells [8]. Hypoxia is an important initiator of chemoresistance in cancers; therefore, miR-210 might mediate hypoxia-induced chemoresistance in breast cancer. Additionally, the authors confirmed that circulating miR-210 levels are associated with trastuzumab sensitivity. In another study, Bai et al. demonstrated that miR-200c, a well-known chemoresistance-related miRNA, could induce trastuzumab resistance by regulating epithelial-mesenchymal transition (EMT) in breast cancer cells [10] though chemoresistance-related miRNAs have been recognized as regulatory molecules in trastuzumab-resistant breast cancers, their underlying mechanisms remain unclear.

MiR-129-5p, which is downregulated in breast cancer, has been related to EMT-mediated chemoresistance and poor prognosis in breast cancer [15]. Interestingly, rpS6, a post-treatment indicator of Her-2 signaling-induced drug resistance, is a downstream target of not only mTOR [16, 17] but also miR-129-5p. Hence, we propose that miR-129-5p might inhibit trastuzumab resistance through downregulating rpS6 in Her-2-positive breast cancer cells. In this study, we aimed to identify the effect of miR-129-5p on trastuzumab sensitivity and elucidate the underlying mechanism.

Materials and Methods

Patients and samples

Patients were identified through electronic records obtained from Jiangyin Peoples' Hospital. The study was approved by the Ethics Committee of the Jiangyin Peoples' Hospital. Blood samples were collected from 32 women with Her-2 amplified breast cancer after obtaining informed consent between 2012 and 2014. The status of Her-2 was detected by immunohistochemistry. The specimens with 2+ immunostaining scores were identified as HER2 amplification if the Her-2 (red arrow); CEP17 (green arrow) ratio was greater than or equal to 2 by fluorescence in situ hybridization (Fig. 1). All the patients were treated with trastuzumab during the adjuvant and/or advanced treatment. Trastuzumab primary resistance was defined as progression at first radiological reassessment at 8-12 weeks or within 3 months after first-line

trastuzumab in the metastatic setting or new recurrences diagnosed during or within 12 months after adjuvant trastuzumab [18]. Whole blood samples were collected in EDTA-containing lavender top tubes (Becton Dickinson, NJ) before the treatment of trastuzumab and chemotherapy. Then the samples were centrifuged at 3,000 g for 10 minutes, and the serum supernatant was transferred to new tubes for subsequently experiments.

Cell culture

Human breast cancer cell lines SKBR3 (trastuzumab-sensitive) and JIMT-1 (trastuzumab-resistant) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). SKBR3 cells were cultured in RPMI-1640 (Multicell, USA), and JIMT-1 cells were cultured in DMEM (Gibco, UK) with 10% Fetal Bovine Serum (FBS, Gibco), containing 100 U/ml penicillin and 100 µg/ml streptomycin (Multicell, USA). Cells were cultured at humidified 37°C in 5% CO₂ and the cell medium was replaced every 48 h.

Methyl thiazolyl tetrazolium (MTT) assay

SKBR3 and JIMT-1 cells were seeded in 96-well plates at a density of 4000 cells per well and incubated for 24 h. After incubation with different doses of trastuzumab (0, 2, 20, 200, 2000 µg/ml) for 24 h, 48 h and 96 h at 37°C, 20 µL of 3-(4, 5-di methylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT, Sigma Aldrich) solution (5 mg/mL in phosphate buffered saline) was added to each well, and the incubation was continued for an additional 4 h at 37°C. Then, the medium was rapidly removed, and the MTT crystals were solubilized in 150 µL DMSO. The absorbance of each well was read in a plate reader at 490 nm [19]. The absorbance readings were subtracted from the value of the blank wells; the reduction in cell growth was calculated as percentage of the control absorbance in the absence of any drug. The data represent the mean ± SD of at least three independent experiments.

Target prediction and functional annotations of miR-129-5p

The potential targets of miRNAs were predicted using the Target-Scan, PicTar, and microRNA.org programs with the default parameters [20]. Intersecting elements identified in each miRNA target prediction program were manually selected as miRNA targets. To investigate the possible biological processes regulated by the miRNAs, the MAS3 program (<http://www.bioinfo.capitalbio.com/mas3/>) was used to determine the putative functions of the potential miRNA target genes by annotation using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

Quantification Real-time RT-PCR (qRT-PCR)

Total RNA of samples was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol [21]. The purified total RNA (1 µg) was then reverse transcribed using the PrimeScript RT reagent kit (Takara, Japan), and qPCR was performed on an ABI 7900 PCR System (Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (2X, Applied Biosystems). The primers are: miR-129-5p (F) 5'-GGGGGCTTTTTCGGTCTGG-3', miR-129-5p (R) 5'-AGT GCGTGTCGTGGAGTC-3'; U6 (F) 5'-CTCGCTTCGGCAGCAC-3, U6 (R) 5'- AACGCTTCACGAATTTGCGT-3'; rpS6 (R) 5'-GTCATCCAGCATGGGTGCTG-3' and rpS6 (F) 5'-GGCTGATACCTTTTGGGACAG-3'; GAPDH (F) 5'-CGCGCCCGG TTTCTA-3', GAPDH (R) 5'-[22]GGCTCGGCTGGCGAC-3'. Each reaction was performed in triplicate. The relative expression levels were determined using the comparative threshold cycle 2^{-ΔΔCt} analysis method.

Fig. 1. Identification of Her-2-positive breast cancer samples by FISH. The specimens with 2+ immunostaining scores were identified as Her-2-positive if the Her-2 (red arrow): CEP17 (green arrow) ratio was greater than or equal to 2 according to fluorescence in situ hybridization.

Western blotting

After the cell protein components were collected, a standard western blotting analysis was used to investigate the rpS6 expression levels. Briefly, Protein lysates were boiled in SDS-sample buffer for 5 min and then subjected to 8% SDS polyacrylamide gel electrophoresis and transferred to PVDF (polyvinylidene fluoride) membranes (Bio-Rad). Membranes were then blocked for 2 h in 5% milk-Tris-Buffered Saline Tween-20 (TBST) at room temperature, and incubated overnight at 4 °C either with the monoclonal antibodies (Cell Signaling Technology, USA) of anti-GAPDH, or anti-rpS6. Membranes were washed four times in TBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Blots were visualized by enhanced chemiluminescence (Thermo, USA) and analyzed using a scanning densitometer with the molecular analysis software FluorChem M system (Protein Simple, USA).

Luciferase Reporter Assays

The 3'-UTR of the wild-type rpS6 and a variant containing mutations in the putative miR-129-5p binding site (Fig. 6A) were inserted downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, USA). Trastuzumab-resistant JIMT-1 cells were co-transfected with reporter constructs, an internal control vector (pGL4.73), and synthetic miR-129-5p mimics. 48 h after transfection, cells were rinsed with phosphate buffered saline (PBS), and then luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, USA) and a luminometer [23]. The luciferase activity of each lysate was normalized to the activity of Renilla luciferase driven by the constitutively expressing promoter in the phRL vector. Basal promoter activity was measured as the fold change relative to the activity observed with the basic pGL3 vector alone.

Statistical analysis

The differences between the treated and the control cells about protein expressions were by applying one way ANOVA (analysis of variance) method followed by standard student t-test analysis. A p-value of less than 0.05 was considered statistically significant, each experiment was performed at least triple times. Computer-based calculations were conducted using SPSS version 20.0 (SPSS Inc. Chicago, Illinois, USA).

Results

Resistance index of SKBR-3 and JIMT-1 cells for trastuzumab

To assess the resistance index of SKBR-3 and JIMT-1 cells for the anti-Her-2 agent trastuzumab, cell viability was determined by MTT assays. SKBR-3 and JIMT-1 cells were treated with different concentrations of trastuzumab (0, 2, 20, 200, or 2000 µg/ml). The results showed that the IC₅₀ for SKBR-3 cells was 63.25 µg/ml trastuzumab. However, the IC₅₀ for JIMT-1 cells was 169.6 µg/ml trastuzumab; this IC₅₀ was increased 2.68-fold compared with the IC₅₀ of SKBR-3 cells (Fig. 2). These results are consistent with those of a previous report indicating that JIMT-1 cells are resistant to trastuzumab [24].

Fig. 2. Resistance index of SKBR-3 and JIMT-1 cells for trastuzumab. The data show that the resistance index of JIMT-1 cells was 2.68-fold higher than that of SKBR-3 cells.

Fig. 3. Expression pattern of miR-129-5p in trastuzumab-resistant breast cancers. miR-129-5p levels were significantly decreased in trastuzumab-resistant serum (A) and cell (B) samples.

Expression pattern of miR-129-5p in trastuzumab-resistant breast cancers

It has been reported that low levels of miR-129-5p are correlated with breast cancer chemoresistance. In this study, the expression levels of miR-129-5p were measured in the serum of Her-2-positive breast cancer patients. The data showed that miR-129-5p levels were significantly lower in trastuzumab-resistant samples than trastuzumab-sensitive samples (Fig. 3A). Similar to the blood sample results, miR-129-5p was also downregulated in trastuzumab-resistant JIMT-1 cells compared to trastuzumab-sensitive SKBR-3 cells (Fig. 3B).

MiR-129-5p increased trastuzumab sensitivity in Her-2-positive breast cancer cells

To identify the effects of miR-129-5p on the sensitivity of breast cancer cells to trastuzumab, JIMT-1 cells in which miR-129-5p mimics were used to overexpress miR-129-5p were used. After transfection, miR-129-5p levels were increased approximately 3.21-fold compared to those of the scrambled mimic control group (Fig. 4A). Moreover, cell viability was low in the mimic group treated with trastuzumab (Fig. 4B), which indicated that miR-129-5p overexpression increased trastuzumab sensitivity in JIMT-1 cells. In contrast, SKBR-3 cells in which miR-129-5p was overexpressed were transfected with the miR-129-5p sponge. After transfection, miR-129-5p levels were decreased 2.17-fold compared to those in the scrambled mimic control group (Fig. 4C). Cell viability was high in the miR-129-5p sponge group treated with trastuzumab (Fig. 4D), which indicated that downregulating miR-129-5p decreased trastuzumab sensitivity in SKBR-3 cells.

GO and pathway analyses of miR-129-5p target genes

We predicted the potential targets and function of miR-129-5p by using bio-informatics tools. GO analysis results showed that genes in the biological processes of the positive regulation of RNA polymerase II promoter, negative regulation of RNA polymerase II promoter transcription, DNA-templated transcription from the RNA polymerase II promoter, positive regulation of transcription, DNA-templated regulation of transcription, DNA-templated negative regulation of transcription, DNA-templated axon guidance, positive regulation of the nuclear-transcribed mRNA catabolic process, and adult behavior were enriched (Fig. 5A). The molecular functions of these genes include protein binding, DNA binding, transcription factor activity, and sequence-specific DNA binding (Fig. 5B). These genes are primarily located in the nucleus, nucleoplasm, cytoplasm, and intracellular ribonucleoprotein cytoplasm (Fig. 5C). Then, we used pathway mapping tools to identify the biological pathways of these genes; 10 categories were found, including the calcium signaling pathway, stem cell pluripotency regulation, the oxytocin signaling pathway, adrenergic signaling in cardiomyocytes, the TGF-beta signaling pathway, and the cAMP signaling pathway (Fig. 5D).

Fig. 4. MiR-129-5p increased trastuzumab sensitivity in Her-2-positive breast cancer cells. MiR-129-5p overexpression increased the sensitivity of JIMT-1 cells to trastuzumab (A & B). In contrast, miR-129-5p knockdown reduced the sensitivity of SKBR-3 cells to trastuzumab (C & D).

MiR-129-5p reduced the expression levels of the rpS6 gene by targeting the 3'-UTR region of rpS6 mRNA

To confirm the miR-129-5p binding sites within the 3'-UTR region of the rpS6 gene, we constructed a wild type (wt) and mutant (mut) miR-129-5p binding sites in the rpS6 3'-UTR (Fig. 6A). Then, we evaluated the effects of the mutant and wild type constructs in miR-129-5p-overexpressing JIMT-1 cells (mimic group) using a dual-luciferase analysis system. The results showed that the luciferase activity was significantly lower in the mimic group co-transfected with the wild type 3'-UTR construct than the mimic group co-transfected with the mutant construct (Fig. 6B), suggesting that miR-129-5p suppressed rpS6 gene expression by binding to the 3'-UTR of rpS6 mRNA. Consistent with the luciferase analysis data, miR-129-5p overexpression decreased the mRNA and protein expression levels of rpS6 compared to those in the scrambled mimic groups, whereas miR-129-5p inhibition increased the mRNA and protein expression levels of rpS6 compared to those in the scrambled mimic groups (Fig. 6C & 6D). These results indicate that miR-129-5p reduced the expression levels of the rpS6 gene by targeting the 3'-UTR region of rpS6 mRNA in breast cancer cells.

MiR-129-5p regulated trastuzumab sensitivity in Her-2-positive breast cancer cells through rpS6

A rescue strategy was adopted to confirm the function of rpS6 in miR-129-5p induced trastuzumab-sensitivity. SKBR-3 cells were co-transfected with the miR-129-5p mimic/rpS6 vector or mimic/empty vector (pcDNA-3.1-rpS6 or pcDNA-3.1); JIMT-1 cells were co-transfected with the miR-129-5p sponge/rpS6-siRNA or sponge/scrambled control. The expression levels of rpS6 were determined by qRT-PCR and western blotting (Fig. 7A & 7B). Moreover, after rpS6-siRNA treatment, the trastuzumab resistance induced by miR-129-5p

Fig. 5. GO and pathway analyses of miR-129-5p target genes. GO analysis results showed that genes involved in the biological processes of the positive regulation of RNA polymerase II promoter, negative regulation of RNA polymerase II promoter transcription, and DNA-templated transcription from the RNA polymerase II promoter were enriched (A). The molecular functions of these genes included protein binding and DNA binding (B), and these genes are located primarily in the nucleus, nucleoplasm, and cytoplasm (C). KEGG pathway analyses indicated that these genes are involved in the calcium signaling pathway, stem cells pluripotency regulation, the oxytocin signaling pathway, adrenergic signaling in cardiomyocytes, the TGF-beta signaling pathway, and the cAMP signaling pathway (D).

inhibition was significantly reduced in SKBR-3 cells; in contrast, the trastuzumab sensitivity induced by miR-129-5p overexpression was reversed by rpS6 overexpression in JIMT-1 cells (Fig. 7C & 7D). These results demonstrated that miR-129-5p could induce trastuzumab sensitivity in Her-2-positive breast cancer cells through regulating rpS6 expression levels.

Fig. 6. MiR-129-5p reduced the expression levels of the rpS6 gene by targeting the 3'-UTR region of rpS6 mRNA. We constructed wild type (wt) and mutant (mut) miR-129-5p binding sites in the rpS6 3'-UTR (A). The luciferase activity was significantly lower in the mimic group co-transfected with the wild type 3'-UTR construct than the mimic group co-transfected with the mutant construct (B). MiR-129-5p overexpression decreased the mRNA and protein expression levels of rpS6 compared to those of the scrambled groups, whereas miR-129-5p inhibition increased the mRNA (C) and protein expression (D) levels of rpS6 compared to those of the scrambled groups.

Discussion

Over the past decade, trastuzumab has been used successfully for the clinical treatment of patients with Her-2-positive breast cancers. However, trastuzumab resistance has become the major obstacle to achieving positive clinical outcomes. Her-2 gene amplification is necessary, but not sufficient, for responsiveness to trastuzumab. It has been confirmed that aberrant PI3K/Akt/mTOR/rpS6 signaling activity contributes to acquired resistance to trastuzumab in breast cancers [6]. Recently, many chemoresistance-related miRNAs have been found to regulate the sensitivity of breast cancer cells to trastuzumab. MiR-129-5p has been correlated with EMT-mediated chemoresistance and poor prognosis in breast cancer. Interestingly, rpS6, a downstream target of the mTOR signaling pathway, was predicted to be a target gene of miR-129-5p. In this study, we proposed that downregulating miR-129-5p contributed to trastuzumab resistance in Her-2-positive breast cancer cells through increased rpS6 expression.

The identification of patients who might benefit from chemotherapy is important because the individualized selection of therapeutic agents could maximize the benefits and minimize the side-effects of ineffective therapies [25]. MiRNA expression analyses are effective tools for predicting therapeutic responses to clinical treatments, especially when used in conjunction with well-established gene expression array-based profiling technology. MiRNAs can play differing roles in regulating cancer occurrence and drug resistance by targeting oncogenes or tumor suppressors. Dysregulated expression levels of miRNAs are correlated with trastuzumab resistance in breast cancers [8-10]. In this study, we found

Fig. 7. miR-129-5p regulated trastuzumab sensitivity in Her-2-positive breast cancer cells through rpS6. SKBR-3 cells were co-transfected with the miR-129-5p mimic/rpS6 vector or mimic/empty vector; JIMT-1 cells were co-transfected with the miR-129-5p sponge/rpS6-siRNA or sponge/scrambled control. The expression levels of rpS6 were determined by qRT-PCR (A) and western blotting (B). After rpS6-siRNA treatment, the miR-129-5p-induced inhibition of trastuzumab resistance was significantly suppressed in SKBR-3 cells (C); in contrast, trastuzumab sensitivity induced by miR-129-5p overexpression was reversed by rpS6 overexpression in JIMT-1 cells (D).

that miR-129-5p was downregulated in trastuzumab-resistant serum samples and cells (Fig. 3), which is consistent with the expression patterns found in chemoresistant samples. Furthermore, the upregulation of miR-129-5p increased the sensitivity of Her-2-positive breast cancer cells to trastuzumab (Fig. 4). However, the underlying mechanism is not clear.

RpS6 was predicted to be a target of miR-129-5p by bio-informatics analysis tools. Previous evidence suggests that rpS6 is downstream of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling pathway and is dysregulated in a variety of cancers. Interestingly, rpS6 always participates in regulating translation initiation and thus protein synthesis [26], in accordance with GO term analysis (Fig. 5A); on the other hand, rpS6 phosphorylation level regulates several protein signaling pathways involving TGF- β [27, 28] accordance with Pathway analysis (Fig. 5D), which suggested that rpS6 probably mediated miR-129-5p induced trastuzumab sensitivity. mTOR exists as 2 distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), and rpS6 is one of best mTORC2 substrates [29]. Both genetic and biochemical data suggest that the activation of the PI3K/Akt/mTOR/rpS6 pathway contributes to the overexpression of ER, PR, and Her-2 in breast cancer [30]. In contrast, Her-2-positive tumors can escape the effects of trastuzumab through the PI3K/Akt/mTOR/rpS6 pathway; combination therapy with mTOR inhibitors could reverse trastuzumab resistance [31, 32]. The data in Fig. 6 show that the altered expression levels of miR-129-5p in Her-2-positive breast cancer cells led to opposite changes in rpS6 expression. Moreover, the rpS6 mRNA 3'-UTR has a miR-129-5p binding site; after miR-129-5p transfection, the expression levels of rpS6 mRNA and protein were both suppressed. However, there were no obvious effects of miR-129-5p on the mutant rpS6 3'-UTR, which highlighted the negative regulation of rpS6 by miR-129-5p.

To further analyze whether rpS6 functions in miR-129-5p-mediated breast cancer sensitivity to trastuzumab, we performed rescue assays. We found that miR-129-5p increased trastuzumab sensitivity through targeting rpS6 in Her-2-positive breast cancer cells (Fig. 7) because restoring the expression of rpS6 abolished miR-129-5p-mediated breast cancer sensitivity to trastuzumab. In conclusion, we identified that miR-129-5p is suppressed in trastuzumab-resistant breast cancer cells and serum samples. Restoring cellular miR-129-5p levels increased the sensitivity of Her-2-positive breast cancer to trastuzumab by directly targeting rpS6, which is downstream of the PI3K/Akt/mTOR pathway. Our study indicates the therapeutic potential of miR-129-5p in combination with trastuzumab for treating Her-2-positive breast cancers.

Acknowledgements

This study was financially supported by the Natural Science Foundation of Jiangsu (BL2013012, BE2016656), and Health and Family Planning Commission Foundation of Wuxi (Q201630, Q201601).

Disclosure Statement

We declare that there is no conflict of interests.

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