

Gene expression changes associated with chemotherapy resistance in Ewing sarcoma cells

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Abstract. Ewing Sarcoma (ES) is a highly aggressive bone and soft tissue childhood cancer. The development of resistance to chemotherapy is common and remains the main cause of treatment failure. We herein evaluated the expression of genes associated with chemotherapy resistance in ES cell lines. A set of genes (CCAR1, TUBA1A, POLDIP2, SMARCA4 and SMARCB1) was data-mined for resistance against doxorubicin and vincristine, which are the standard drugs used in the treatment of patients with ES. The expression of each gene in SK-ES-1 ES cells was reported before and after exposure to a drug resistance-inducing protocol. There was a significant downregulation of CCAR1 and TUBA1A in doxorubicin-resistant cells, with low expression of TUBA1A in vincristine-resistant cells. By contrast, POLDIP2 was significantly upregulated in cells resistant to either drug, and the expression of the SMARCB1 and SMARCA4 genes was upregulated in doxorubicin-resistant cells. These findings indicate that resistance to specific chemotherapeutic agents was accompanied by differential changes in gene expression in ES tumors.

Introduction

Ewing Sarcoma (ES) is a bone and soft tissue tumor of possible neuroectodermal or mesenchymal origin that afflicts children and young adults. Approximately 20-25% of cases

are metastatic at diagnosis, and the survival rates are poor in the advanced setting (1,2). ES is characterized by a frequent characteristic cytogenetic translocation of the EWSR1 (22q12) and FLI-1 (11q24) genes. The resulting fusion protein EWS-FLI-1 is responsible for oncogene activation, inhibition of tumor suppression, chromatin remodeling and epigenomic reprogramming (3-5).

Standard multimodality treatment consists of induction chemotherapy followed by local control with surgery and/or radiotherapy and then consolidation chemotherapy with multiple drugs. Active chemotherapy agents in the first-line setting include doxorubicin, vincristine, cyclophosphamide, etoposide and ifosfamide. Treatment efficacy appears to reach a plateau after dose intensification and interval compression of all active agents (6,7), and the development of resistance to chemotherapy remains as the main cause of treatment failure (8,9). Cooperative groups have examined different chemotherapy combinations, dose-intensifying regimens with bone marrow transplantation, metronomic therapy and other synergistic mechanisms associated with the EWS fusion protein in ES. However, these approaches have failed to improve survival rates in clinical trials thus far (10,11). Elucidating the molecular mechanisms underlying the development of chemotherapy resistance in ES may help develop new agents with improved synergy.

Doxorubicin belongs to the class of anthracyclines and acts by topoisomerase II poisoning, creation of double-strand DNA breaks (DSBs), and impairment of DNA repair and supercoiling, leading to changes in epigenetic processes (12,13). Mechanisms involved in resistance to doxorubicin include drug efflux transporters, alterations in the ability of doxorubicin to form DSBs, and alterations in downstream apoptosis signaling triggered by DNA damage (14).

Vincristine, a natural alkaloid extracted from *Vinca rosea*, interferes with microtubule formation and stability through depolymerization, resulting in cell cycle arrest and apoptosis (15). In addition to affecting chromatin stability (16) by interfering with DNA binding and histone eviction,

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vincristine also affects topoisomerase IIa levels (17). Resistance to vinca alkaloids involves overexpression of ATP-binding cassette (ABC) transporters such as P-glycoprotein, alterations of β -tubulin (β II, β III and β IV) and multidrug-resistance proteins (18).

The aim of the present study was to investigate whether the expression of genes associated with resistance to chemotherapy in other tumor types is different in chemotherapy-resistant ES cells. The literature was first data-mined for genes associated with resistance to drugs used in ES treatment, focusing on doxorubicin and vincristine, and then SK-ES-1 cells resistant to these drugs were developed. Subsequently, the expression of the selected genes was evaluated with quantitative polymerase chain reaction (qPCR) analysis.

Materials and methods

Data mining and refining. To select drug resistance genes, the literature was searched for studies investigating resistance to doxorubicin and vincristine in all cancer types. Approximately 270 genes were identified, but only 23 genes appropriately validated by experimental methodologies (mutational and knockout gene analysis) were selected. This database was further enriched with information from online tools, such as DrugBank, Gene Ontology and UniProt. Finally, a set of five genes (CCAR1, TUBA1A, POLDIP2, SMARCA4 and SMARCB1) was selected based on pathways that are important for ES development.

Cell culture. The standard Ewing sarcoma cell line SK-ES-1 (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI-1640 medium (Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% bovine serum, 4 mg/ml gentamicin (Nova Farma, São Paulo, Brazil) and amphotericin B (Fungizone®, Invitrogen; Thermo Fisher Scientific, Inc.).

Chemoresistance induction. For chemotherapy resistance induction, the cells underwent treatment for 72 h, with increasing drug concentration every 2 weeks, for a period of ≥ 10 weeks. The concentrations were 10, 20, 30, 40 and 50 nM for doxorubicin (Libbs, São Paulo, Brazil) and 0.5, 1, 2, 3 and 4 nM for vincristine (Pfizer, Inc., New York, NY, USA). Untreated cells served as control (9).

Cell proliferation. SK-ES-1 cells were seeded at a density of 2.104 cells per well in 48-well plates. After 24 h, the cells were treated with vincristine and doxorubicin individually, including control. The concentrations for this treatment were 10, 30 and 50 nM for doxorubicin and 1, 3 and 5 nM for vincristine. The medium was removed after 72 h of treatment; the cells were washed with phosphate-buffered saline, detached with 0.25% trypsin solution (no EDTA; Invitrogen; Thermo Fisher Scientific) and then counted with the trypan blue exclusion method in a hemocytometer, as previously described (19). The mean of three experiments for each dose was utilized for calculations.

RNA extraction and cDNA synthesis. RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA was synthesized using the reverse transcription (RT)-PCR kit SuperScript™ III

Table I. Quantitative polymerase chain reaction primer sequences.

Gene	Primer sequence	Product
SMARCB1	F: 5'-TCCGTATGTTCCGAGTTCT-3' R: 5'-CTGGTGGCTAGAGTCGTGA-3'	154
SMARCA4	F: 5'-GCTCCGAGGTCTGATAGTGA-3' R: 5'-CGCTGTCTGGATCTGGAATC-3'	133
CCAR1	F: 5'-AGAGTTCGACGTGTTGTTCC-3' R: 5'-GCGCCTTAGTTCATCATGT-3'	90
TUBA1A	F: 5'-TTGTTCACTGGTACGTTGGG-3' R: 5'-AATCCACACCAACCTCCTCA-3'	105
POLDIP2	F: 5'-TTCCAGTATAGCAGCCACGT-3' R: 5'-GAACATCAAAGTGGGAGCCA-3'	97
GAPDH	F: 5'-CAAGATCATCAGCAATGCCTCC-3' R: 5'-GACTGTGGTCATGAGTCCTTCC-3'	103

F, forward; R, reverse.

First-Strand Synthesis SuperMix (Invitrogen; Thermo Fisher Scientific).

qPCR. qPCR was performed using the AB 7500 system (Thermo Fisher Scientific Inc.), with positive and negative controls. Reactions were prepared with KiCqStart® qPCR Ready mix™ (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA), using a 0.5- μ l sample cDNA. Expression levels were evaluated using the 2- $\Delta\Delta$ Cq method, with GAPDH used as the housekeeping gene. The primers used for CCAR1, TUBA1A, POLDIP2, SMARCA4 and SMARCB1 are listed in Table I.

Statistical analysis. Statistical differences were analyzed using one-way analysis of variance, with the Sidak correction method for multiple comparison tests. Experiments were conducted three times and in triplicates. All statistical analyses were performed using SPSS 16.0 for Windows. The differences were considered statistically significant when P-values were < 0.05 .

Results

Protein network integration. Protein interaction networks are crucial for understanding the biological cellular processes. We designed a protein network to visualize the interactions between the selected genes and molecular pathways associated with ES. A set of genes associated with resistance to doxorubicin and vincristine was manually curated. The open-source software programs Cytoscape version 3.6.0 and String version 10.5 were used to build the network. The interactions between the investigated genes (CCAR1, TUBA1A, POLDIP2, SMARCA4 and SMARCB1) and DNA-topoisomerase II (TOP2A), a target of both doxorubicin and vincristine, and genes directly binding to EWS-FLI1 fusion protein, including those associated with spliceosomal activity that is crucial to ES pathogenesis, are shown in Fig. 1.

Induction of chemotherapy resistance. Chemotherapy resistance was successfully induced for both doxorubicin and vincristine in SK-ES-1 cells. Doxorubicin resistance was

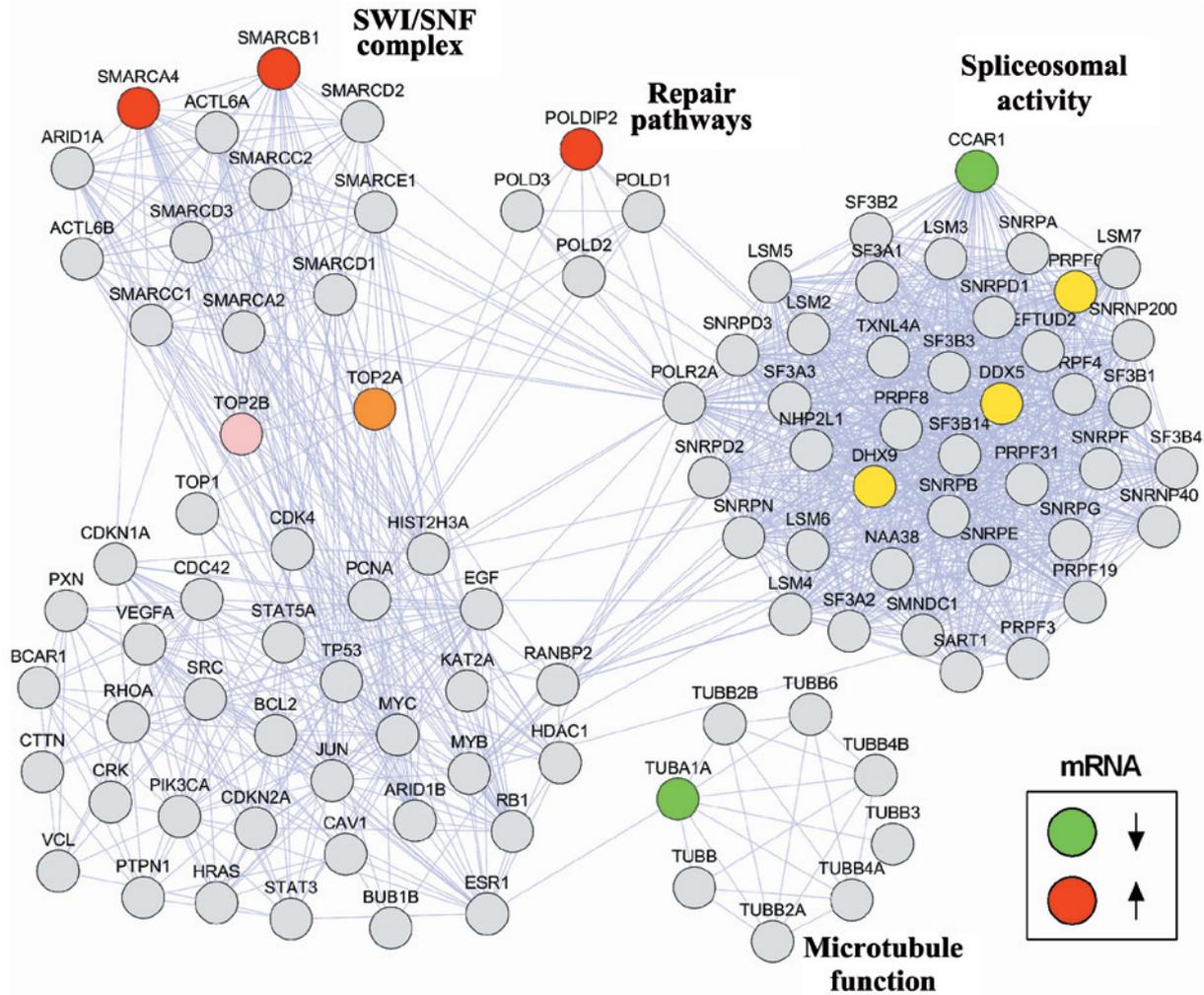


Figure 1. Protein interaction network of genes involved in resistance to chemotherapy. Genes selected for this study are represented in red or green according to higher or lower relative mRNA expression, respectively, between control and chemoresistant Ewing sarcoma cells. DNA-topoisomerase (TOP)2A and TOP2B (pink/orange) were included due to their role as doxorubicin targets. Genes involved in spliceosomal activity and EWS/FLI1 activity are shown in yellow. The network was constructed using the STRING database, version 10.5.

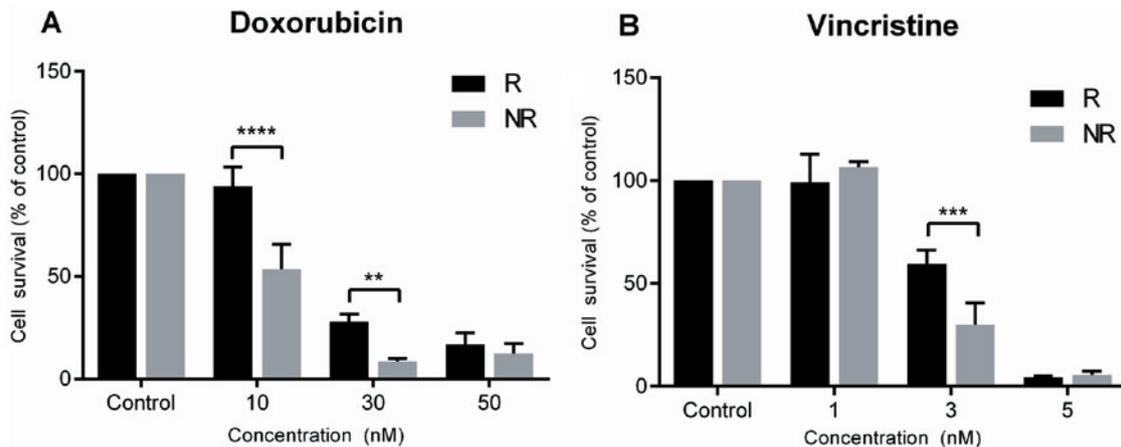


Figure 2. Chemotherapy resistance in SK-ES-1 Ewing sarcoma cells. Evidence of resistance was observed at concentrations of 10 and 30 nM for doxorubicin and 3 nM for vincristine. Values are presented as percentage of survival of resistant (R) vs. non-resistant (NR) SK-ES-1 cells, for three independent experiments; **P<0.005; ***P<0.0005; and ****P<0.0001 compared with controls cells.

evidenced at concentrations of 10 nM (P<0.0001 compared with controls) and 30 nM (P<0.005 compared with controls),

and vincristine resistance at a concentration of 3 nM (P<0.0005 compared with controls; Fig. 2).

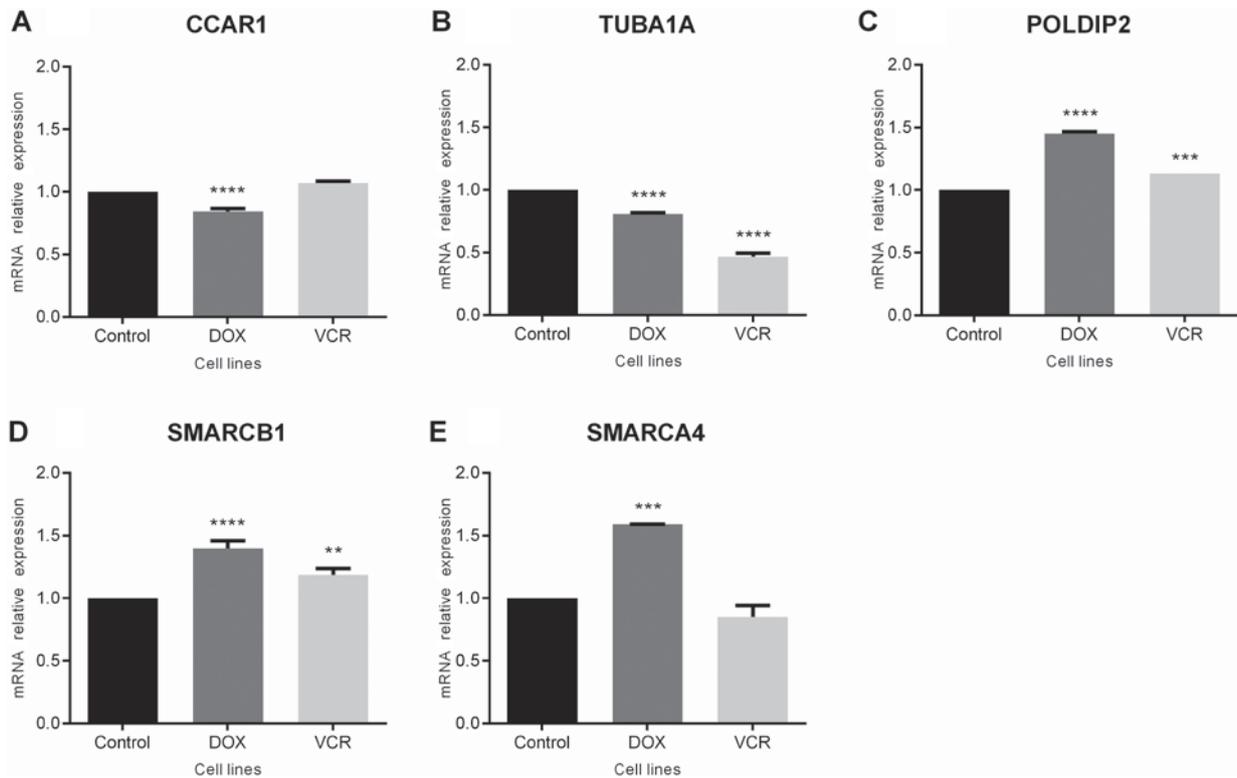


Figure 3. Gene expression analysis of (A) CCAR1, (B) TUBA1A, (C) POLDIP2, (D) SMARCB1 and (E) SMARCA4 in chemoresistant and non-resistant SK-ES-1 Ewing sarcoma cells. Values represent the relative expression of genes in doxorubicin (DOX)-resistant, vincristine (VCR)-resistant, and control SK-ES-1 cells (cell lines); ** $P < 0.005$; *** $P < 0.0005$; and **** $P < 0.0001$ compared with control cells.

Gene expression. Changes in expression for the selected genes in chemoresistant ES cells were analyzed. The results revealed a modest, but statistically significant downregulation of CCAR1 ($P < 0.0001$ compared with control cells; Fig. 3A) and TUBA1A ($P < 0.0001$ compared with controls) in doxorubicin-resistant cells, with decreased expression of TUBA1A also observed in vincristine-resistant cells ($P < 0.0001$ compared with controls; Fig. 3B). By contrast, POLDIP2 was upregulated in cells resistant to either drug ($P < 0.0001$ for doxorubicin-resistant and $P < 0.0005$ for vincristine-resistant cells; Fig. 3C). For SMARCB1 and SMARCA4, gene expression was upregulated in cells resistant to doxorubicin ($P < 0.0001$ and $P < 0.0005$, respectively), whereas changes were observed in vincristine-resistant cells for SMARCB1 only ($P < 0.0005$; Fig. 3D and E).

Discussion

Resistance to chemotherapy remains the main reason for treatment failure in patients with ES. In the present study, a group of genes was selected (TUBA1A, POLDIP2, SMARCA4, SMARCB1 and CCAR1), known in other tumors to be associated with resistance to drugs commonly used in ES, and the gene expression in chemotherapy-resistant ES cells was evaluated. It was demonstrated that the TUBA1A expression levels were lower in cells resistant to either doxorubicin or vincristine, when compared with the non-resistant cells. The TUBA1A gene encodes the α -tubulin protein, which belongs to the tubulin family of proteins that form and organize microtubules that are required for cell division and movement. Given that vincristine is a microtubule-depolymerizing agent,

resistance to this agent may develop from changes in tubulin levels (20). Doxorubicin may interfere in TUBA1A levels, with lower gene expression observed in resistant MCF-7 breast cancer cells (21).

Upregulation of POLDIP2 in the chemotherapy-resistant ES cells was also observed. POLDIP2 encodes a protein implicated in the activity of translesional polymerases. The translesion synthesis polymerase and primer extension activities of PrimPol play a role in DNA damage tolerance (22); its involvement in repair processes may explain the increases in cell sensitivity to oxidative stress when POLDIP2 is silenced (23). In addition, POLDIP2 plays an important role in DNA replication/repair and regulation of reactive oxygen species and participates in cytoskeletal reorganization as well as key pathways in cancer, including those involved in autophagy and cell cycle regulation (24). POLDIP2 is also involved in vascular integrity, smooth cell migration and adhesion (25). These processes are important for tumor development and survival, and its upregulation in drug-resistant cells may contribute to increased tumor aggressiveness.

Both SMARCB1 and SMARCA4 are part of the SWI/SNF chromatin-remodeling complex, which recruits TOP2A to DNA and leads to the formation of DSBs and cell death. Loss of the SWI/SNF complex results in drug resistance, including DSBs and repair pathways (26). Knockdown of SMARCA4 and SMARCB1 leads to increased chemotherapy resistance (27). Our results revealed higher expression levels of SMARCA4 and SMARCB1 in doxorubicin-resistant ES cells, suggesting a different landscape in ES. The oncogenic EWS-FLI1 fusion induces chromatin-remodeling patterns, stimulating or

repressing enhancers and establishing a modified oncogenic regulatory and interaction network that may explain divergences in drug resistance mechanisms among different tumors (4).

The results of the present study are consistent with previous evidence indicating that loss of SMARCB1 is found in a small percentage of ES patients, and this may interfere favorably with outcome. It is possible that the combination of EWSR1 translocation and SMARCB1 loss increases susceptibility of tumor cells to treatment (28).

CCAR1 is a biphasic regulator of cell growth and apoptosis, and plays an important role in tumorigenesis in gastric cancer (29) and hepatocellular carcinoma (30). CCAR1 can target gene activation by estrogen and glucocorticoid receptors in breast cancer cells (31), and androgen receptors in prostate cancer (32). EWS-FLI1 alters mRNA splicing in ES cells, giving rise to several protein isoforms implicated in oncogenesis (33), and CCAR1 is associated with spliceosomal activity. An unexpected decrease in CCAR1 expression levels was observed in doxorubicin-resistant cells. Further experiments are required to elucidate how this gene is associated with chemotherapy resistance and alternative splicing in ES. Although the genes selected in the present study have different functions and are involved in diverse pathways, our integration network reveals a possible connection among these different mechanisms. Therefore, repair pathways, SWI/SNF chromatin remodeling, microtubule rearrangements and spliceosomal activity may be interacting to maintain chemoresistance mechanisms in ES.

In summary, the present findings provide early evidence revealing novel changes in gene expression associated with chemotherapy resistance in ES cells. Gene knockout assays, characterization of resistance pathways and the use of tumor samples from patients are among the next steps required to confirm and extend these findings, and validate this set of genes as possible targets to counteract therapy resistance in ES.

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Availability of data and materials

The materials included in the manuscript will be made freely available to any researchers who wish to use them for non-commercial purposes, while preserving any necessary confidentiality and anonymity.

Authors' contributions

LH, MS, CAS, DBO and CBF made substantial contributions to the design of the present study; LH, MS, CAS and DBO performed the experiments; LH, MS, and CBF analysed the

data; LH, MS, CAS, DBO, LJJ, ALB, ATB, RR and CBF critically revised the manuscript for important intellectual content, wrote and reviewed the manuscript; LH, MS, ATB, RR and CBF wrote the manuscript. All authors critically revised the manuscript and all authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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