Cloning and functional characterization of TCRP1, a novel gene mediating resistance to cisplatin in an oral squamous cell carcinoma cell line

Yixue Gu a,d, Shasha Fan a, Yan Xiong b, Bo Peng a, Guopei Zheng a, Yanhui Yu a, Yongmei Ouyang a, Zhimin He a,c,⇑

a Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha 410078, Hunan, PR China
b Department of Pharmacology, Guangzhou Medical University, Guangzhou 510182, Guangdong, PR China
c Cancer Research Institute and Cancer Hospital, Guangzhou Medical University, Guangzhou 510182, Guangdong, PR China
d Medical School, University of South China, Hengyang 421001, Hunan, PR China

Abstract

To explore the mechanisms of chemotherapy resistance, we previously established a multi-drug resistant cell line, Tca8113/Pingyangmycin (Tca8113/PYM) and identified differential expression in known genes and ESTs using microarray analysis. From among those ESTs we have now identified a novel gene producing an mRNA of 1834 nucleotides translated into a protein having 235 amino acids. This gene was denominated as tongue cancer resistance-associated protein 1 gene (TCRP1, accession number: EF363480). We further determined its functional characteristics. The results demonstrate that TCRP1 mediates a specific resistance to cisplatin in Tca8113 cells by reducing the cisplatin-induced apoptosis. This suggests that TCRP1 might be a novel molecular target to develop agents to reverse cisplatin-induced chemoresistance.

1. Introduction

The squamous cell carcinoma of head and neck (SCCHN) is the fifth most common cancer worldwide and is a significant source of cancer morbidity and mortality. Oral squamous cell carcinoma (OSCC) is one of the most common types of the SCCHN [1,2]. Currently, chemotherapy is the first line of treatment, especially for some advanced OSCC patients. In the past decades, cis-diaminedichloroplatinum (cisplatin, CDP), pingyangmycin (PYM, also known as bleomycin A5) and 5-fluoro-2,4(1H,3H) pyrimidinedione (5-FU) were the most potent and indispensable chemotherapeutic agents used in the treatment of OSCC due to their wide antitumor spectrum and synergistic cytotoxic effects [3–6]. However, parallel to high efficacy in the chemotherapy of the tumors, the resistance to chemotherapeutic agents developed during sequential treatments and became a major obstacle for their clinical application [7–9]. The molecular genetic basis of resistance to chemotherapy is extraordinarily complex, involving multiple processes such as drug transport, drug metabolism, DNA repair, apoptosis regulation, etc [10]. Currently the mechanisms involved in chemoresistance still remain to be fully elucidated. Thus, exploring the mechanisms responsible for the development of resistance to chemotherapeutic agents is still an urgent need.

To understand the chemotherapy resistance mechanisms in OSCC, we recently established a new OSCC multiple drug resistance (MDR) cell line (Tca8113/PYM) by continuously exposing the Tca8113 OSCC cells to a stepwise escalating concentration of PYM [11]. The resistance index (RI) to PYM in this cell line was upregulated 18 fold compared to the parent Tca8113 cells. Simultaneously, it was revealed to be cross-resistant to a subset of clinically relevant antitumor agents such as cisplatin, paclitaxol, adriamycin (ADM), tetrahydropyrane (THP) and mitomycin C (MMC), but not to etoposide (VP-16) and 5-FU. A cDNA microarray analysis identified 41 known genes and 4 ESTs whose expressions were upregulated, and 48 genes and 9 ESTs that were downregulated in the Tca8113 cells. Among these candidates, one of the upregulated ESTs (GenBank ID: AL707095; UniGene Cluster ID: HS475334) attracted our interest as the most strongly upregulated gene in Tca8113 cells [11].

In this study, we cloned the complete AL707095 cDNA, and identified a tongue cancer resistance-associated protein 1 gene (TCRP1), a new member of the chemoresistance-related gene family. We further investigated how it exerts its role in chemoresistance. Our finding provided new insight to elucidate chemoresistance...
mechanisms in OSCC, and a new target to develop agents to reverse cisplatin-induced chemoresistance.

2. Materials and methods

2.1. Cell culture

Tca8113 is a human oral squamous cell carcinoma (OSCC) cell line and was obtained from China Type Culture Collection Center (Wuhan, China). The Tca8113/PYM cell line was previously established in our laboratory [11]. Tca8113 cells were cultured in RPMI-1640 (Gibco St. Louis, MO, USA) containing 10% fetal calf serum (Gibco St. Louis, MO, USA) at 37 °C, 5% CO2. Tca8113/PYM cells were routinely cultured under the same conditions with addition-ally 100 ng/ml PM (Harbin Bolai Pharmaceutical Co., China). However, Tca8113/PYM cells were maintained in PM-free medium for at least two weeks before experiments.

2.2. In silico cloning and full-length cDNA amplification

To clone the EST AL707095-representing gene, we performed BLAST analysis against the public human EST database using AL707095 as a query sequence. The matched ESTs were assembled into a contig from overlapping ESTs through CAP3. To amplify the sequence, we designed primers according to the contig, and RT-PCR was performed using PimeSTARTM HS DNA Polymerase. The coding sequence (CDS) was amplified using forward primer: 5’-CACATGAAACCTGGTTTACAG-3’ and reverse primer: 5’-TCAGGTAGGAGGAAGG-3’. The full-length cDNA was amplified by using forward primer: 5’-CCGCTACTGACAGGATCTCA-3’ and reverse primer: 5’-TCAGTTCAGTACGTCAGTTAC-3’. The PCR products were then inserted into pEGM-T Easy vectors and sequenced using an ABI PRISM 377 Sequencer.

2.3. Indirect immunofluorescence microscopy

To locate the TCRP1 protein in cells, cells were grown on cover slips placed in six-well tissue culture plates. Indirect immunofluorescence was carried out as previously described [12] using an Olympus immunofluorescence microscope.

2.4. RT-PCR assay of TCRP1 mRNA

Total RNA was isolated using Trizol reagent (Invitrogen) and reverse transcription was performed using Reverse Transcription System (Promega). PCR was conducted in triplicate for each sample. TCRP1 was amplified using forward primer: 5’-ACT CCGGGGCAACATACAGG-3’ and reverse primer: 5’-GGGCATACAGATTTCTGCGGGG-3’. The β-actin was used as an internal standard and was amplified using forward primer: 5’-ACCTGGAGAAGAGCTGAG-3’ and reverse primer: 5’-GACACCTGGACACAGGAGCAG-3’.

2.5. Construction of pcDNA3.1/TCRP-myc expression and TCRP1 shRNA silencing plasmids

The ORF of TCRP1 was amplified from pGEM-T/KIAA0280, a human genome plasmid was cloned using the same strategy (5’-CCAAACGCTGCCACCTAGAACCCTGGTTACACGCC-3’ and reverse primer: 5’-CCGATCCATTTATCCGAGGAGCGAGCTT-3’). The resulting plasmids were pGEM-TCRP1 and pcDNA3.1-TCRP1, respectively.

To design the small interfering RNA-encoding minigenes for TCRP1, we used a siRNA designer available at the website of Ambion Inc. (Austin, TX). Two complementary oligonucleotide DNA sequences (5’-TGACACACAGATCCGCTTACATCAAGAGG-3’ and 5’-CTGCAAGAACAGTCTTTTTTTG-3’) were synthesized with Sal I and Xba I-compatible overhangs (gift from Professor David Engelke, University of Michigan). The resulting plasmid was pAU-siTCRP1. The control plasmid encoding a non-sense minigene with no homology to any known sequences in the human genome plasmid was cloned using the same strategy (5’-TGACAGCTTACATGAGGACAGTCTTGTTGA-3’).

2.6. Transfection of Tca8113 and Tca8113/PYM cell lines

Tca8113 and Tca8113/PYM cell lines were transfected with pcDNA3.1-TCRP1 or pAU-siTCRP1 and their controls using Lipofectamine 2000 according to the manufacturer’s instructions. G418 was used for colony selection and the resulting cell lines were Tca8113/TCRP1, Tca8113/pDNA3.1, Tca8113/PYM-siRNA and Tca8113/PYM-con.

2.7. Western blot analysis

The whole cell lysates were prepared and immunoblotting performed as described previously [13]. Antibodies against caspase 3 and PARP were obtained from Cell Signaling Technology (Beverly, MA), and the α-tubulin monoclonal antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA) and was used as a loading control. To detect the protein expression of TCRP1 in Tca8113 cells, we prepared rabbit polyclonal antibody against TCRP1. Briefly, pGEM-TCRP1 plasmid was transformed into BL21 (DE3) E. coli. The GST-fused TCRP1 protein was produced and purified using an established protocol [14]. The purified TCRP1 protein was injected into rabbit and sequentially, rabbit polyclonal antibody against TCRP1 was prepared as previously described [14].

2.8. Cytotoxicity assay and plate colony assay

Cytotoxicity of cisplatin, 5-FU, ADM (Sigma–Aldrich) and PYM (Shanghai Pharmacy Co., China) was determined by an improved MTT assay as previously described [15]. The absorbance was measured at 570 nm using a microplate reader (Bio-Tech, USA). The results represented the absorbance ratio between the treated and untreated cells at indicated time points. Each data point represented the mean and SD of three assays.

For plate colony assay, Tca8113 cells were treated with 20 μM cisplatin for 30 h, and then trypsinized and reseeded in 6-well plates (5 x 104/well), and cultured at 37 °C for 2 weeks. Cells were fixed with cold methanol at −20 °C for 30 min and then stained with crystal violet (0.1%). Colonies containing more than 50 cells were counted using an imaging system (Syngene) and three independent experiments were analyzed.

2.9. Comet assay and DNA ladder

The alkaline (pH >13.0) single cell gel electrophoresis assay was carried out as previously described [16,17], with modifications. After exposing to 20 μM of cisplatin for 30 h, the Tca8113 cells were washed twice with ice-cold phosphate buffered saline (PBS) and suspended in low melting agarose (LMA) at 1 x 104 cells/ml at 37 °C.
Eighty milliliter of 0.75% LMA/cell suspension was pipetted onto frosted glass microscope slide pre-coated with a 100 μl of 0.75% normal melting agarose (NMA). Coverslips were placed gently to allow even spreading of gel. The slides were kept on ice for 10 min to allow the gel to solidify. The coverslips were then removed and the slides were immersed into freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10.0) to dissolve the cell proteins and to unfold DNA. After incubating at 4 °C for at least 1 h in the dark, the slides were covered with fresh buffer (1 mM Na2 EDTA, 300 mM NaOH, pH >13.0) in a horizontal electrophoresis unit. The slides were rinsed in this fresh buffer for 20 min to continuously unfold DNA before subjecting to electrophoresis at 20 V, 300 mA for 30 min at 4 °C. After electrophoresis, the slides were washed gently with freshly prepared 0.4 M Tris–HCl, pH 7.5 for three times and then stained with 50 μl ethidium bromide (20 μg/ml) for 20 min. All steps described above were conducted under yellow light or in the dark to prevent additional DNA damage. Images of 50 cells per treatment sample (25 cells/slide) were taken individually under a fluorescence microscope (Olympus, BX51) with digital camera (Olympus, DP50) at 200× magnification and the test was carried out three times. The Olive tail moment (Tail DNA% × [Tail Mean-Head Mean]) was analyzed using Comet Assay Software Project (CASP).

DNA ladder assay were performed as previously described [18]. Briefly, cells were lysed and treated with lysis buffer (10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100), followed by 1 h incubation with RNase A. The cell lysates were precipitated for proteins and spun at 15,000 rpm for 20 min. After centrifugation, the low molecular weight DNA was purified with phenol/chloroform and precipitated with ethanol. Ten micrograms of DNA from each sample were electrophoresed on a 1.8% agarose gel.

2.10. Apoptosis assay

Apoptotic cell death was assessed by Annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining to discriminate apoptotic cells from live cells and necrotic cells. Briefly, 30 h after the treatments, 2 × 10^5 trypsinized cells from each group were stained according to the instruction of the Annexin-V FITC Apoptosis Detection Kit (R&D systems, Abingdon, United Kingdom), and analyzed by a Flow Cytometer (BD FACSAriaTM). The data are presented as dot plots showing fluorescence intensity of Annexin-V FITC and propidium iodide.

2.11. Statistical analysis

Data was analyzed using the statistical package for the Social Sciences Version 13.0 (SPSS 13.0) and statistical analysis was done with student t-test. P < 0.05 was considered statistically significant. All data are presented as mean ± standard error of the mean.

3. Results

3.1. Cloning of the full-length cDNA of TCRP1 and bioinformatic analysis

To assemble the complete sequence of the gene represented by the EST of interest, AL707095, we performed BLAST analysis using the AL707095 sequence as a query to align with sequences in the human EST database. The matched ESTs were assembled into a contig, and an 1834 bp sequence was obtained. This sequence was previously characterized as “Homo sapiens family with sequence similarity 168, member A” (FAM168A) or KIAA0280 (Genbank accession number NM_015159.1). We suggest the novel names tongue carcinoma chemotherapy resistance-associated protein 1 (TCRP1, Genbank accession number: EF363480). The putative open reading frame extends from position 280–987, which would encode a putative protein of 235 amino acids with molecular weight of 25 kDa, and theoretical pl of 9.18. The ORF and a full-length cDNA were cloned via RT-PCR as described in Section 2.2.

The TCRP1 gene is located on human chromosome 11q13.4. Some transcription factor binding sites, such as for c-Myc and SP-1, were found in the 5′-flanking region.
3.2. Subcellular localization of TCRP1

To determine the subcellular localization of TCRP1, we performed an immunofluorescence assay of TCRP1 protein. As shown in Fig. 1, green fluorescence was mainly detected in the cytoplasm, but was rarely seen at the cell membrane and in nucleus.

3.3. TCRP1 expression is correlated with sensitivity to cisplatin in Tca8113 cell lines

To investigate the roles of TCRP1 on multi-drug resistance in Tca8113 cells, firstly TCRP1 was overexpressed in Tca8113 cells to examine whether TCRP1 expression affected cellular sensitivity.
to cisplatin, PYM, 5-Fu and ADM. The mRNA and protein expression of TCRP1 in Tca8113, Tca8113/PYM, Tca8113/pcDNA3.1 and Tca8113/TCRP1 cell lines was analyzed. (Fig. 2a and b). As shown in Fig. 2c, overexpression of TCRP1 resulted in increased cellular resistance to cisplatin, but not to PYM, 5-Fu, or ADM. In addition, TCRP1 expression was knocked down using vectors expressing either TCRP-specific or control shRNA, and then treated with aforementioned agents. As shown in Fig. 2d–f, the sensitivity to cisplatin was significantly increased in TCRP1-silenced cells (Tca8113/PYM-i) compared with control cells (Tca8113/PYM-c), but such effect was not observed with PYM, 5-Fu, or ADM. Similar results were obtained with a second siRNA construct for silencing (not shown).

3.4. Effect of TCRP1 expression on cell growth

To determine whether TCRP1 has an effect on long-term cell survival, we performed colony formation assays. Tca8113 cells were incubated with 20 μM of cisplatin for 30 hrs, and then cultured at 37 °C, 5% CO₂. After 2 weeks, cells were fixed and stained to visualize the colonies. As shown in Fig. 3a and b, the cells with high expression of TCRP1 had a significantly higher number of colonies, and silencing of TCRP1 expression significantly decreased the colony formation. This result suggests that TCRP1 plays a crucial role in cell survival after treating with cisplatin.

3.5. TCRP1 protects Tca8113 cells against cisplatin-induced DNA damage

To further investigate how TCRP1 increased the cell survival, we conducted comet assays to test the role of TCRP1 in cisplatin-induced DNA damage in Tca8113 cells. Overexpression of TCRP1 significantly decreased the Olive tail moment in cisplatin-treated cells (Fig. 4a and b). As shown in Fig. 4c, Tca8113 cells treated with cisplatin.
cisplatin showed apparent DNA ladders, while this was blocked by overexpression of TCRP1.

3.6. TCRP1 expression is correlated with apoptosis in Tca8113 cell lines

To determine whether TCRP1 is involved in apoptosis, the percentage of apoptotic nucleus in Tca8113 cell was examined by Annexin-V FITC and PI double staining 30 h after treatment with 20 μM cisplatin. Apoptosis was significantly decreased in cells with overexpression of TCRP1 compared to the control, whereas apoptosis was significantly increased in TCRP1-silenced cells (Fig. 5a and b). These results indicate that TCRP1 is an apoptosis resistance gene. The activated caspses are critical initiators of apoptosis in many biological systems [12]. To address whether the anti-apoptotic effect of TCRP1 was coincident with reduced caspase activity, the activities of caspases-3 and PARP were measured in cells treated with cisplatin for 30 h. TCRP1 obviously lowered the cleaved caspases-3 and PARP levels (Fig. 5c). These results suggest that TCRP1 could block cisplatin-induced apoptosis in OSCC tumor cells.

4. Discussion

In present study, we first cloned the novel tongue cancer resistance-associated protein1 gene (TCRP1), which is located on human chromosome 11q13.4, one of the most frequently amplified regions in OSCC [19,20]. The promoter region was predicted to contain binding sites for transcription factors including c-Myc and SP-1. The oncogenes c-Myc and SP-1 are thought to be closely associated with cellular apoptosis and resistance to chemotherapy [21–23]. We therefore hypothesized that transcription of TCRP1 activated by c-Myc and SP-1 might play a crucial role in cellular proliferation, apoptosis, survival and chemoresistance. Indeed, our study proved that exogenous overexpression of TCRP1 increased the resistance to cisplatin, while TCRP1 knockdown sensitized the multi-drug resistant Tca8113/PYM cell to cisplatin-induced cell apoptosis and decreased cellular proliferation and long-term survival. These results suggest that TCRP1 exerts a very important role in resistance to cisplatin in Tca8113 cells through increasing cell proliferation and survival, as well as decreasing cell apoptosis.

Interestingly, we found that manipulating TCRP1 expression levels did not significantly alter the sensitivity to pingyangmycin (bleomycin A5) in Tca8113 cells (Fig. 2). The main reasons might be due to the molecular mechanism of resistance to pingyangmycin involves multiple factors such as activity of a bleomycin hydrolase [24], DNA repair genes [25] and metallothionein (MT) [26]. Coincidently, MT1 and MT2 were also found to be overexpressed in Tca8113/PYM cell [11]. We also found that TCRP1 was highly expressed in the other cisplatin-resistant cancer cells, such as non-small-cell lung cancer (NSCLC) resistance-cell line (A549/cDDP) compared with its parent cell line A549 (data not shown). This result suggests that TCRP1-mediated chemoresistance is not tumor cell-type specific, but is rather a cisplatin-specific resistance.

The platinum compound cisplatin is one of the important chemotherapeutic agents widely used to treat a variety of cancers, such as ovarian, testes, and the head and neck tumors. The cytotoxicity of platinum compounds is mediated by the formation of intra-strand cross-links in the DNA [27]. The adducts in DNA lead to G2 arrest and apoptosis, which is believed to be responsible for the antitumor activity of platinum [28]. However, tumors frequently developed resistance to cisplatin, which significantly decreased its application in the clinic. It is known that cellular resistance to
platinum can arise through multiple mechanisms such as GSH-based detoxification, metallothionein (MT) induction, and DNA repair capacity. Although involvement of transporters in MDR has also been implicated in cisplatin resistance in certain cases, platinum compounds are not a typical part of MDR phenotype [29]. Thus, it is not surprising that we did not find overexpression of the genes of the transporter superfamily (including MRPI, MDR1 and BCRP) in the Tca8113/PYM cell line [11]. Furthermore, genes functioning to increase cell survival and to decrease apoptosis, such as p53, XIAP, Bcl-2, AKT, Bcl-XL, FAS-L, and NF-kB, have also been revealed to affect cisplatin resistance in various cells [30–33]. In the present study, overexpression of TCRP1 was capable of blocking cisplatin-induced DNA damage and counteracting cisplatin–induced cell death (Figs. 4 and 5). Meanwhile, alterations in cisplatin uptake or expression of copper transporters have been demonstrated to contribute to cisplatin resistance [34]. However, changes in cisplatin uptake and copper transporter expression were not observed in Tca8113/PYM cell (data not shown). We therefore hypothesized that TCRP1-mediated resistance to cisplatin might be associated with alterations in the signaling pathways affecting apoptosis, GSH/Mt-based detoxification, or DNA repair capacity. In a cDNA microarray analysis, we found that the expression of NF-kB and Akt were reduced in the Tca8113/PYM–siRNA cell line comparing with Tca8113/PYM cell (data not shown). NF-kB and Akt play important roles in the control of cell growth, differentiation, apoptosis and many other physiologic processes [31,35,36]. Therefore, we considered that TCRP1 might mediate resistance to cisplatin partially through alteration of the expression or activation of NF-kB and Akt, but this will be a subject for further study.

In summary, our study provided strong evidence for the linkage between up-regulated TCRP1 expression and cisplatin resistance in tongue cancer cell. We therefore concluded that TCRP1 is a candidate gene involved in chemotherapeutic resistance and a potential molecular target to reverse resistance to cisplatin.

Acknowledgment

This study was supported by the National Natural Science Foundation of China (30873088).

References