MicroRNAs contribute to the chemoresistance of cisplatin in tongue squamous cell carcinoma lines

Zhi-wei Yu, Lai-ping Zhong *, Tong Ji, Ping Zhang, Wan-tao Chen, Chen-ping Zhang *

Department of Oral and Maxillofacial Surgery, Ninth People's Hospital, School of Stomatology, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology and Shanghai Research Institute of Stomatology, Shanghai 200011, China

MicroRNAs (miRNAs) are small non-coding RNAs that function as negative regulators of gene expression. They are strongly implicated in human cancers, including oral squamous cell carcinoma (OSCC). Evidence for the involvement of miRNAs as important regulators of chemosensitivity and chemoresistance in OSCC is not well understood. In this study, miRNA microarray was firstly used to compare the differential miRNAs levels between the cisplatin-sensitive tongue squamous cell carcinoma line (Tca8113) and its cisplatin-resistant subline (Tca/cisplatin). Three miRNAs of miR-21, -214, and -23a were validated by miRNAs real-time PCR, and intervened by anti-miRNA oligonucleotides (miR-214 and -23a) and pre-miRNA plasmid transfection (miR-21). Further relationship between miR-23a and DNA topoisomerase II beta (TOP2B) on the chemoresistance against cisplatin was studied. There were 19 out of 480 differential miRNAs between the Tca8113 and Tca/cisplatin cells. miR-214 and -23a were found increased as with chemoresistance against cisplatin in the Tca/cisplatin cells while miR-21 was found decreased as with chemosensitivity for cisplatin in the Tca/cisplatin cells. Intervention of these three miRNAs could decrease the chemoresistance against cisplatin in Tca/cisplatin cells. Transfection of anti-miR-23a into the Tca/cisplatin cells could increase the TOP2B protein expression. Our results suggest the existence of differential miRNAs with chemosensitivity and chemoresistance between the cisplatin-sensitive and resistant tongue squamous cell carcinoma lines. miR-21 serves as a chemosensitive miRNA, while miR-214 and -23a serve as chemoresistant miRNAs in the tongue squamous cell carcinoma lines. miR-23a is an up-stream regulator of TOP2B to realize the chemoresistance of cisplatin.

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Contents lists available at ScienceDirect

Oral Oncology 46 (2010) 317–322

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tant roles in the regulation of normal gene expression for developmental timing, cell proliferation, and apoptosis. In addition, aberrant miRNA expression is strongly implicated in carcinogenesis and cancer progression. About 50% of known miRNAs are located in the fragile position of genes related to tumors. Some biological processes of miRNAs may also be relevant to cancer chemosensitivity and chemoresistance.

Chemotherapy has been widely accepted as one of the three major therapies (surgery, chemotherapy and radiotherapy) treating advanced squamous cell carcinoma in the head and neck region. Some drugs such as cisplatin, 5-Fu, taxanes, and EGFR-inhibitors are currently used. Cisplatin is one of the main anti-neoplastic drugs against oral squamous cell carcinoma. Resistance to anticancer drugs is present in the majority of human tumors and is associated with failure of chemotherapy. For the clinicians, it would be very useful to have tools that could predict the sensitivity of certain drugs, in particular with regard to cisplatin, whose toxicity profile is wide and severe and, moreover, enhances the radiation-induced toxicity. Selecting patients in advance that will not benefit from the addition of cisplatin is of major importance for further treatment optimization. Existing knowledge indicates that tumors develop chemical resistance through multiple cellular response pathways that alter gene and protein expression. Although the roles of gene signaling cascades and proteins as regulatory factors in chemoresistance are becoming well established, the evidence on the involvement of regulatory miRNA is still unclear.

Topoisomerase II is involved in DNA replication, transcription and chromosome segregation, and the beta form of DNA topoisomerase II beta (TOP2B) functions as the target for several anticancer agents and a variety of mutations in this gene have been associated with the development of drug resistance. Herzog et al. have reported that down-regulation of miRNA and protein levels of TOP2B could cause chemoresistance of ansamycin in human leukemia cell line, which suggests the TOP2B involved in the chemosensitivity. TOP2B is also involved in chemosensitivity of etoposide in melanoma. However, there are few reports on the relationship between the TOP2B expression and cisplatin/cisplatin-resistance in malignancies, especially in TSCC.

In the present study, in order to investigate the potential roles of miRNAs in cancer chemotherapy, we measured the expression levels of most known miRNAs in the cisplatin-sensitive TSCC line (Tca8113) and its cisplatin-resistant subline (Tca/cisplatin) which we had previously established, and further intervened three miRNAs of miR-21, miR-214, and miR-23a, which were associated with chemosensitivity and chemoresistance. Then, we explored the potential relationship between the miR-23a and TOP2B expression. We found the potential value of miRNAs playing important role on the chemosensitivity in TSCC.

Materials and methods

Cell cultures

Human tongue squamous cell carcinoma cell line (Tca8113) and its cisplatin-resistant subline (Tca/cisplatin) were obtained from Oral Oncology Laboratory of Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine. Cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% FBS (Invitrogen, USA) in a cell culture incubator under humidified atmosphere of 5% CO₂ at 37 °C. They were routinely passaged at 2–3 days intervals.

miRNA microarray

Total RNA isolation was performed with Trizol reagent according to the instructions of manufacturer (Invitrogen, USA). About 5 μg of total RNA was used for hybridization of miRNA microarray chip containing 480 human miRNAs (Shanghai Biochip Co Ltd., China). Oligonucleotide arrays were printed with trimer oligonucleotide probes (antisense to miRNAs) specific for the 480 miRNAs on GeneScreen Plus membranes (NEN Life Science Products, USA). Hybridization signals were detected by biotin-containing transcripts. To ensure accuracy of the hybridizations, each labeled RNA sample was hybridized with three separate membranes. Processed slides were scanned by using a microarray scanner (Axon, USA). Raw data was normalized and analyzed with the use of GeneSpring software (Agilent, USA). The expression level larger than 2.0-fold was set as a threshold indicating significant change, and the multiple testing correction of Bonferroni correction was used as a confidence filter of differential miRNAs.

Real-time PCR for miRNA and mRNA

For miRNAs, total RNA was isolated with Trizol reagent and the miRNAs were reverse transcribed into cDNAs. The reaction mixture containing 5 × RT buffer, DTT (0.1 M), dNTP (10 μl), MgCl₂ (25 mM), miRNA-RT primers (1 μM), Rnasin (40 U/μl), M-MLV reverse transcriptase, and nuclease-free water was mixed with 20 ng of each total RNA. The mixture was incubated for 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C. Real-time PCR was performed using a standard protocol of by Hairpin-it™ miRNAs qPCR Quantification Kit Reagents (GenePharma, Shanghai, China). For miR-23a, miR-21 and miR-214, the PCR master mix of a total reaction volume of 20 μl for each reaction containing 2 × real-time PCR PCR master mix, miRNA TaqMan probe, Taq DNA polymerase, miRNA primers for each target, nuclease-free water and cDNA template was prepared. The PCR condition was as following: 94 °C for 5 min, followed by 50 cycles of 94 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s. Expression of target mRNA gene was also validated by real-time PCR. Total RNA was extracted and reverse transcribed to cDNA. PCR was carried out according to standard protocol of SYBR Premix Ex Taq using real-time PCR equipment (Stratagen, USA). To quantify changes in gene expression, the ΔΔCt method was used to calculate the relative miRNA and mRNA expression.

Antisense inhibition of miRNA expression

Anti-miRNA oligonucleotides were prepared using conventional phosphorodiester chemistry and DNA synthesis equipment. The inhibitors had the following sequences: anti-miR-23a: 5’-GGAA AUCCCUUGCAUGUGAU-3’; anti-miR-214: 5’-ACUGCC GUCCUGCUGU-3’. Tca8113 cells were transfected using Lipofectamine™ 2000 transfection reagent (Invitrogen, USA) 24 h after plating. Transfection complexes were prepared according to the manufacturer’s instructions and added to the cells. Transfection medium was replaced 6 h later. Forty-eight hours after transfection, the cells were collected and prepared for further studies.

Pre-miR-21 plasmid construction and transfection

Vector pGenesil-1.1 was utilized to construct pre-miR-21 plasmid. PCR amplification was used to create expression plasmid of pre-miR-21 using the primers as following: pre-miR-21, 5’-TGAATCTTCAAGCAACACCATGCGTTCATGGTACATT TTGGACCCAGAATTC-3’ (sense) and 5’-CCATGAGATCCTGAGGAGCCAATT-3’ (antisense). The reaction mixture contained 10 × LA buffer 2 μl, dNTP (10 mM) 2 μl, pre-miR-21-sense (10 μM) 1 μl, pre-miR-21-antisense (10 μM) 1 μl, pGenesil-1.1 0.5 μl, LA Taq 0.5 μl. The PCR condition was as follows: 30 cycles of 55 °C for 30 s
Cisplatin was added in a dilution series of 0.3, 0.4, 0.5, 0.6, 0.7, oligomers or pre-miRNA plasmid, and incubated at 37 °C for 5 min. The PCR products were confirmed by DNA sequencing. The plasmid was then transfected into the Tca/cisplatin cells using Lipofectamine™ 2000 transfection reagent (Invitrogen, USA).

**Growth inhibition assay**

Anticancer drug (cisplatin) was used to test cell growth and drug potency with MTT assay. Ten thousand cells per well were seeded in 96 well plates after transfection with antisense miRNA oligomers or pre-miRNA plasmid, and incubated at 37 °C for 24 h. Cisplatin was added in a dilution series of 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 μg/ml, six replicate plate columns per concentration. Seventy-two hours after treatment, 20 μl MTT per well was added and the plates were incubated for 4 h subsequently. The plates were then centrifuged at 1000 rpm for 5 min and supernatant fluid was discarded. The 200 μl DMSO per well was added to the plates and mixed vibrationally. Absorbance was read in a microplate reader at 450 nm. To determine IC50 values (the concentration causing 50% cell growth inhibition), the absorbance of control cells was set at 1. Dose–response curves were plotted using Prism software (GraphPad Software, USA). Each experiment was done independently at least thrice.

**Statistical analysis**

Statistical analysis was performed with non-parametric tests by using the software package of SPSS11.0 for Windows (SPSS Inc., USA). P values less than 0.05 were considered statistically significant.

**Results**

**Differential miRNAs between the Tca8113 and Tca/cisplatin cells**

Nineteen out of the 480 miRNAs were found differentially expressed between the Tca8113 and Tca/cisplatin cells (P < 0.05). The increase levels of the following miRNAs were found in the Tca/cisplatin cells comparing with the Tca8113 cells: let-7c, -7d, -7e, -7g, miR-20b, -23a, -30d, -181d, -188, -181d, -214, -373*, -432, -498, -518c*, -584, -608, and -628. Conversely, the decrease levels of miR-23a, -214 increased and miR-21 decreased in Tca/cisplatin comparing with Tca8113 (Fig. 1). Among these miRNAs, there were a few miRNAs have been previously reported with anticancer drug potency. Here, three miRNAs, miR-23a, miR-214, and miR-21 were validated by miRNAs real-time PCR to confirm the findings of miRNA microarray. And the results showed the relative levels of miR-23a, -214 increased and miR-21 decreased in Tca/cisplatin comparing with Tca8113 (Fig. 1).
Effects of miRNA silencing on drug potency

The loss-of-function assay was used to confirm the function of miR-23a and miR-214, anti-miR-23a and -214 were synthesized and transfected into the Tca/cisplatin cells. Conversely, expression level of miR-21 was elevated by a pre-miR-21 plasmid construction and transfection into the Tca/cisplatin cells. Decreased levels of miR-23a and -214 was found after transfection of anti-miR-21.

Figure 2 (A) Plasmid construction of pGenesil-1.1/pre-miR-21. (B) Relative expression levels of the miR-23a, -214 and -21 in the Tca/cisplatin cells transfected with miRNA inhibitor or plasmid were determined by miRNAs real-time PCR. The miR-23a and -214 levels decreased significantly after transfection with anti-miR-23a and anti-miR-214, and the miR-21 level was increased significantly after transfection with pre-miR-21 plasmid. (C) Cells inhibition by cisplatin following pre-treatment with anti-miR-23a, anti-miR-214 and pre-miR-21 plasmid was determined using the MTT assay. All of the three miRNAs displayed dose-dependent growth inhibition. The cells after transfection showed a decreased ratio of surviving cells ($P < 0.05$). (D) IC$_{50}$ values of the Tca/cisplatin cells to cisplatin with transfection of anti-miR-23a, anti-miR-214 and pre-miR-21 were measured. The resistance cisplatin concentration of the Tca/cisplatin cells after transfection was much lower than the cells without transfection ($P < 0.01$).
and -214 by miRNAs real-time PCR, and increased level of miR-21 was found after transfection of pre-miR-21 plasmid also by miRNAs real-time PCR (Fig. 2A).

The Tca/cisplatin cells transfected with anti-miRNA or pre-miRNA plasmid was then challenged with serious concentrations of cisplatin, and the surviving cells were determined using the MTT assay. Paired t-test was performed to determine the inhibition ratio for the cells transfected with miRNA inhibitor comparing with those without transfection. The Tca/cisplatin cells transfected with anti-miR-23a, anti-miR-214 and pre-miR-21 plasmid all showed an evident decreasing ratio of surviving cells compared to those without transfection (P < 0.05) (Fig. 2B). The IC50 values for cisplatin over 72 h treatment: 4.10 ± 0.29 μg/ml with 95% confidence interval [3.39, 4.88] for anti-miR-23a group, 3.50 ± 0.27 μg/ml with 95% confidence interval [2.77, 4.17] for anti-miR-214 group, and 2.93 ± 0.24 μg/ml with 95% confidence interval [2.32, 3.57] for pre-miR-21 group, respectively. For the group of Tca/cisplatin cells without transfection, the IC50 was 6.24 ± 0.33 μg/ml with 95% confidence interval [5.38, 7.10]. The resistance of Tca/cisplatin cells with transfection was much lower than that without transfection (Fig. 2C).

### Relationship between miR-23a and TOP2B

Based on our miRNA microarray results and three miRNA databases of miRBase, TargetScanS and PicTar, possible targets of the miRNA mir-23a target site for TOP2B. In addition to 8mer sites, TargetScanS predicts 7mer sites of two types: 7mer-m8: an exact match to positions 2–8 of the mature miRNA (the seed + position 8); 7mer-1A: an exact match to positions 2–7 of the mature miRNA (the seed) followed by an ‘A’. (B) Complementarity between mir-23a and the TOP2B 3’-UTR as predicted by PICTAR. (C) TOP2B protein and mRNA expressions in the Tca8113 and Tca/cisplatin cells. TOP2B protein and mRNA levels in the Tca/cisplatin cells were lower than those in the Tca8113 cells, and the change of TOP2B protein expression was significant (P < 0.05). (D) TOP2B protein and mRNA levels in the Tca/cisplatin cells transfected with anti-miR-23a increased after transfection with anti-miR-23a, and the change of TOP2B protein expression was significant (P < 0.05).
miRNAs in Table 1 were searched in the three miRNA databases. Among the candidates targeted, the TOP2B was found matching to the seed sequence of miR-23a, which was involved in chemosensitivity in several malignant tumors containing a putative region that matches to the seed sequence of miR-23a.

TargetScans predicted 7mer sites of miR-23a to TOP2B with an exact match to positions 2–8 of the mature miRNA (Fig. 3A). The degree of incomplete pairing between miR-23a and the human TOP2B 3′-UTR was showed in Fig. 3B.

To investigate this putative interaction, the TOP2B mRNA and protein levels in Tca8113 and Tca/cisplatin cells were detected by real-time PCR and Western blotting. The TOP2B protein level in the Tca/cisplatin cells was significantly lower than that in the Tca8113 cells. Although the TOP2B mRNA level in the Tca/cisplatin was lower than that in the Tca8113 cells, the difference was not significant (Fig. 3C). After the Tca/cisplatin cells transfected with anti-miR-23a, the TOP2B protein expression level increased (Fig. 3D).

Discussion

Using the cisplatin-sensitive TSCC line (Tca8113) and its cisplatin-resistant subline (Tca/cisplatin), 19 miRNAs are screened and identified by miRNA microarray method. The three miRNAs of miR-21, miR-214, and miR-23a associated with chemosensitivity and chemoresistance are then validated by miRNAs real-time PCR and confirmed to the miRNA microarray results. The miR-21 is down-regulated in the Tca/cisplatin cells comparing to the Tca8113 cells, while the miR-214 and miR-23a are up-regulated in the Tca/cisplatin cells.

The miR-21 has been reported to affect the chemosensitivity in some tumors. For example, it has been reported that suppression of miR-21 expression in a cholangiocarcinoma cell line increases the some tumors. For example, it has been reported that suppression of miR-21 expression in a cholangiocarcinoma cell line increases the chemosensitivity and chemoresistance. Although the TOP2B mRNA level in the Tca/cisplatin cells was lower than that in the Tca8113 cells, the difference was not significant (Fig. 3C). After the Tca/cisplatin cells transfected with anti-miR-23a, the TOP2B protein expression level increased (Fig. 3D).

Among the candidates targeted, the TOP2B was found matching to the seed sequence of miR-23a, which was involved in chemoresistance against cisplatin. Using the bioinformatic searching method based on three miRNA databases, we find the potential relationship between the miR-23a and TOP2B, which has the chemosensitivity for malignancies. Here, we find the decreased TOP2B mRNA and protein expression levels in the Tca/cisplatin cells comparing to the Tca8113 cells. This confirms the chemosensitivity ability of TOP2B. After the Tca/cisplatin cells are transfected with anti-miR-23a, the TOP2B protein expression level increases. So, the TOP2B protein expression could be regulated by miR-23a; that is to say that the TOP2B gene is one of the target genes of miR-23a. However, further studies are needed to make sure whether cleavage or translational repression is the primary mechanism of down-regulation of the TOP2B gene by miR-23a.

Conflict of interest statement

None declared.

Acknowledgements

These works were supported by research Grant Nos. 30973344 and 30700953 from National Natural Science Foundation of China; by research Grant 2007BA11B03 from National Key Technology R&D Program of China, and by research Grant 08QA14056 and 08XD14024 from Science and Technology Commission of Shanghai Municipality.

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