miR-30a attenuates drug sensitivity to 5-FU by modulating cell proliferation possibly by downregulating cyclin E2 in oral squamous cell carcinoma

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\textbf{A B S T R A C T}

We aimed to determine the functional role of the miRNA, which affects drug sensitivity to 5-FU in oral squamous cell carcinoma (OSCC), using two types of 5-FU-resistant and parental OSCC cell lines. MiRNA microarray data showed that miR-30a was significantly upregulated in two resistant cell lines. Therefore, we investigated the effects and molecular mechanism of miR-30a on 5-FU sensitivity. Stable overexpression of miR-30a in parental OSCC cells decreased cell proliferation and attenuated drug sensitivity to 5-FU. Cell cycle analysis indicated that miR-30a overexpression increased the proportion of G1 phase cells and decreased the proportion of S phase cells. MiR-30a knockdown using siRNA reversed the effects of miR-30a overexpression. DNA microarray analysis using miR-30a-overexpressing cell lines and a TargetScan database search showed that cyclin E2 (CCNE2) is a target of miR-30a. A luciferase reporter assay confirmed that a miR-30a mimic interacted with the specific binding site in the 3’ UTR of CCNE2. CCNE2 knockdown with siRNA in OSCC cells yielded decreased drug sensitivity to 5-FU, similar to miR-30a overexpressing cells. These findings suggest that miR-30a in OSCC may be a novel biomarker of 5-FU-resistant tumors, as well as a therapeutic target for combating resistance.

\section{1. Introduction}

Five-fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents and a potent radiosensitizer [1]. Chemotherapy or chemoradiotherapy based on 5-FU treatments can improve the survival of patients with head and neck squamous cell carcinoma (HNSCC), including oral squamous cell carcinoma (OSCC) [2–4]. However, recurrent or metastatic HNSCC often shows resistance to 5-FU-based chemotherapy, resulting in poor outcomes [5]. Overcoming this resistance is important in improving prognoses for HNSCC patients.

Anticancer drug resistance is currently regarded as a multifactorial phenomenon, with genetics and epigenetic processes proposed as possible explanations for its development [6,7]. There is limited evidence to indicate genetic changes following chemotherapeutic treatment [8], but numerous studies have indicated the importance of epigenetic changes occurring through altered expression of short
incubation at 37 °C.

Evidence has accumulated for miRNA-mediated drug resistance, and attention is focused on targeting miRNAs as a novel strategy for therapeutic intervention [9]. There have been reports of miRNA-related chemoresistance in OSCC [16–20], but most have focused on cisplatin resistance, and only one has dealt with 5-FU [17]. Herein, we aimed to identify novel targets involved in 5-FU resistance, using specific miRNAs that are commonly altered in two 5-FU-resistant OSCC cell lines.

2. Materials and methods

2.1. Cell lines and cell culture

Human OSCC cell lines derived from the SAS tongue and Ca9-22 gingival tumors were obtained from RIKEN BioResource Center (Ibaraki, Japan). The HOC-313 cell line was kindly donated by the Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science. Cell lines were cultured with Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Gland Island, NY, USA) supplemented with 10% FBS and maintained under humidified 5% CO2 incubation at 37 °C.

2.2. Establishment of 5-FU-resistant OSCC cell lines

SAS and Ca9-22 cells were continuously exposed to increasing concentrations of 5-FU for two years. The surviving cells were cloned, and two 5-FU-resistant sublines (designated SAS/FR2 [21] and Ca9-22/FR2 [22]), which were able to survive exposure to 2.0 μg/ml of 5-FU, were used for subsequent study. To ensure continued resistance, these lines were maintained in DMEM containing 2.0 μg/ml 5-FU. To eliminate the effects of 5-FU from the experimental outcomes, the resistant cells were cultured in a drug-free medium for at least two weeks prior to all experiments.

2.3. Cell proliferation assay

To assess the degree of proliferation, viable cells treated without 5-FU were quantified every 24 h using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

2.4. Drug sensitivity assays

Cells were seeded onto 96-well plates at a density of 2 × 103/well and incubated in DMEM with 10% FBS at 37 °C. After 24 h, DMEM containing 5-FU at 0.05, 0.15, 0.31, 0.63, 1.25, 2.5, 5.0, or 10.0 μg/ml was added to each well, and cells were incubated at 37 °C for another 72 h. For the assay, WST-8 was added to each well and plates were incubated for an additional 2 h at 37 °C. Absorbance was measured at 450 nm using a microplate reader (Model 680, Bio-Rad). Eight wells were used for each drug concentration, and the experiment was performed in triplicate. The 50% inhibitory concentration (IC50) was calculated based on the survival curve.

2.5. Total RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Life Technology) according to manufacturer’s instructions. RNA quantity, purity, and integrity were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 1.0 μg of total RNA using the miScript II RT Kit (Qiagen, Valencia, CA, USA). For quantitative PCR, cDNA was amplified using PCR with specific primers for cyclin E2 (CCNE2) (forward: 5′- TCAAGACGAATGTGCGTCTAC-3′; reverse: 5′- TGCACATCTGGTTAAAGTCTC-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-GGTGGCCATCAATGAGCCCTTT-3′; reverse: 5′-CTCAGCAGTACTCAGGCG-3′), where GAPDH was used as an internal control. Expression of miR-30a (MS00007350) was analyzed using miScript SYBR Green PCR Kits (Qiagen, Valencia, CA, USA) and normalized to RNU6B (MS00029204). All reactions were performed in triplicate.

2.6. Establishment of OSCC cells stably expressing miR-30a

Cells were seeded into 6-cm dishes (1 × 106 cells per well) and stably transfected at 80% confluence with pmR-ZsGreen-miR-30a transfectant using Lipofectamine™ LTX Reagent (Invitrogen), as described by the manufacturer. After 3 days, fresh medium containing the selection reagent G418 (500 μg/ml; Gibco) was added to the cells. Selection continued for 14 days, with the medium refreshed every two days. Transfected cells were identified by the coexpression of ZsGreen1, a human codon-optimized variant of the reef coral Zoanthus sp. green fluorescent protein (ZsGreen) engineered for brighter fluorescence with respective excitation and emission maxima of 493 and 505 nm. Coexpression of pmR-ZsGreen1-miR-30a, SAS/st-30a, and Ca9-22/st-30a allows easy monitoring and/or selection of miR-30a overexpressed cells using fluorescence microscopy, flow cytometry, or qRT-PCR. SAS/st-cont and Ca9-22/st-cont clones were used as controls were created using transfection in a similar fashion as pmR-ZsGreen1-miR-control.

2.7. Cell cycle analysis and apoptosis detection using flow cytometry

Cells were harvested 48 h after transfection, washed with ice-cold PBS, and stained using CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson). DNA content was evaluated using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) with ModFit LT software (Verity Software House) for histogram analysis. For apoptosis detection, cell lines were incubated with 5-FU at 2.0 μg/ml or culture medium alone. After 72 h, apoptosis was quantified using Annexin-V Allophycocyanin (APC) (Becton Dickinson). Untreated cells served as a negative control for double staining. Cells were analyzed immediately after staining using a FACS Calibur flow cytometer and Cell Quest Pro software.

2.8. miR-30a knockdown

SAS/st-30a cells and Ca9-22/st-30a cells were transfected with 40 nM of miR-30a inhibitor (Stem loop ID: MI0000088; Life Technologies) or miR-control inhibitor (life technology) in the 24-well plates (1 × 105 cells per well) by Lipofectamine RNAi MAX Reagent (Invitrogen) according to manufacturer’s instructions. (Life Technology) for each drug concentration, and the experiment was performed in triplicate. Expression of miR-30a was measured using miScript SYBR Green PCR Kits (Qiagen, Valencia, CA, USA) and normalized to RNU6B (MS00029204). All reactions were performed in triplicate.

2.9. Small interfering RNA (siRNA) transfection

Twenty-four h before siRNA transfection was performed, SAS and Ca9-22 cells were diluted in fresh medium without antibiotics and transferred to 24-well dishes (Nunc, Waltham, MA), where they were cultured and transfected with CCNE2 specific siRNA and Stealth™ RNAi Universal negative control (Shealth siRNA, 40 nM, Invitrogen) using Lipofectamine RNAi MAX (Invitrogen) per manufacturer’s instructions. The sequences for siRNA were sense 5′-CGU CUA ACA AUC UCC UGG CUA A-A-3′ and antisense 5′-UUA GCC AGG AGA UUG UUA UAG G-3′ for CCNE2. Cells were harvested 48 h after transfection and total RNA
was extracted for subsequent experiments.

2.10. Western blot analysis

Whole cell proteins were separated using 10% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies against CCNE2 (1:1,000; Cell Signaling Technology), and β-actin (1:5,000; Sigma). After overnight incubation, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Dako). Finally, membranes were washed and visualized using the ECL Plus detection kit (GE Healthcare).

2.11. Statistical analysis

Differences in the mean values between the two groups were statistically analyzed using Student’s t-test. All P-values were based on two-tailed statistical analyses, with P < 0.05 were considered to indicate statistical significance. All statistical analyses were completed using the JMP 9 software program (SAS Institute Inc, Cary, NC).

The supplementary methods describe details on the following: RNA isolation, gene expression, and miRNA microarray analysis; luciferase reporter assay; Western blot analysis; clinical specimens of OSCC.

3. Results

3.1. Cell proliferation and drug resistance of 5-FU-resistant OSCC cell lines

There were no significant differences between cell proliferation of the parental (SAS, Ca9-22) and resistant (SAS/FR2, Ca9-22/FR2) cell lines (Supplementary Figs. S1A and B), suggesting that 5-FU resistance is not due to increased cell proliferation. We next examined the cytotoxic effects of 5-FU in the parental and resistant cells (Fig. 1A and B). After 72 h incubation with 2.5 μg/ml of 5-FU, apoptotic changes (shrinkage and rounding of the cells) were observed more frequently in SAS and Ca9-22 cells compared to resistant cells under phase-contrast microscopy (Supplementary Figs. S2A and B). IC₅₀ values for 5-FU of the SAS and SAS/FR2 cells were 0.28 and 1.79 μg/ml, and 0.47 and 4.47 μg/ml for Ca9-22 and Ca9-22/FR2, respectively (P < 0.01). Therefore, SAS/FR2 5-FU showed a 6.4-fold greater resistance to 5-FU than SAS cells, and Ca9-22/FR2 showed a 9.5-fold greater resistance to 5-FU than Ca9-22 cells.

3.2. MicroRNA microarray analysis and upregulation of miR-30a

According to the criteria for screening resistance-related miRNA,
microRNA analysis using 5-FU-sensitive and -resistant cell lines yielded 5 candidate miRNAs (Supplementary Table S1). Among those 5 miRNAs, miR-30a was the only, about 2-fold upregulated miRNA in both resistant cell lines. Therefore, we focused on the analysis of miR-30a in this study.

RT-qPCR quantitation of differences in miR-30a expression between SAS and SAS/FR2 and Ca9-22 and Ca9-22/FR2 showed that resistant cells expressed about 2-fold more miR-30a (Fig. 1C).

Overexpression of miR-30a affects cell proliferation, cell cycle distribution, and drug sensitivity to 5-FU.

Expression of miR-30a in the SAS/st-30a and Ca9-22/st-30a lines respectively increased 2.7-fold and 3.9-fold compared to controls (Fig. 2A). The functional significance of miR-30a was evaluated using a gain-of-function assay using these miR-30a-overexpressing lines. Cell proliferation of these lines was significantly inhibited compared with each control cells after 96 h (SAS/st-30a: 67 % and Ca9-22/st-30a: 38 %, respectively, P < 0.05; Supplementary Figs. S3A and B).

Flow cytometry showed that the percentage of G1 phase fraction in miR-30a-overexpressing cells were elevated (SAS/st-30a, 43.8 % versus...
SAS/st-cont, 32.1 %; P < 0.05, and Ca9-22/st-30a, 39.2 %; P < 0.05; Fig. 2B). These results suggest that miR-30a overexpression may suppress cell cycle progression from moving past the G1 phase. IC\(_{50}\) values for SAS/st-cont and SAS/st-30a cells against 5-FU were 0.29 and 0.49 μg/ml, and 0.18 and 0.41 μg/ml for Ca9-22/st-cont and Ca9-22/st-30a, respectively (P < 0.05; Fig. 2C). That is, miR-30a overexpressing cells SAS/st-30a and Ca9-22/st-30a showed 1.7-fold and 2.3-fold greater resistance to 5-FU than control cells. SAS/st-30a and Ca9-22/st-30a cells showed 1.5 % and 2.1 % decreases in apoptosis rates compared with their controls (P < 0.05; Fig. 2D).

Effects of miR-30a knockdown on proliferation, cell cycling, and drug sensitivity to 5-FU.

The optimal concentration of anti-miR-30a for efficient down-regulation of miR-30a was found to be 50 nmol/ml, which yielded 71 % and 76 % reductions in miR-30a expression of SAS/st-30a and Ca9-22/st-30a, respectively (Fig. 3A). As expected, downregulation of miR-30a led to a significant increase in cell proliferation (Fig. 3B) and a significant decrease in the percentage of the G1 phase fraction (Fig. 3C) in both lines. Drug sensitivity assay 24 h after transfection with anti-miR-30a inhibitor or anti-miR-control showed that miR-30a targeting significantly enhanced sensitivity to 1 μg/ml 5-FU treatment compared with the controls, resulting in 15 % more cell death in the SAS/st-30a line and 9 % more in the Ca9-22/st-30a line (Fig. 3D). These results suggest that inhibition of miR-30a may chemosensitize OSCC cells to 5-FU by reducing the number of cells arrested in the G1 phase and increasing cell proliferation.

3.3. CCNE2 is a direct target of miR-30a

DNA microarray analysis using SAS/st-30a and Ca9-22/st-30a and their control cell lines yielded 10 candidate genes according to the TargetScan database. Among these, cyclin E2 is the only one related to the cell cycle (Supplementary Table S2), in regulating G1/S transition. Therefore, this gene may be a target of miR-30a.

We then examined whether CCNE2 was actually regulated by miR-30a using the luciferase reporter vector containing the total sequence
of the CCNE2 3'UTR, including the predicted miR-30a target sites (position 213–220 and 476–483; Supplementary Fig. S4A). Luminescence intensity in the OSCC cells was significantly reduced by cotransfection of the miR-30a mimic compared with the non-targeting control (Supplementary Fig. S4B). Furthermore, downregulated CCNE2 protein in miR-30a-overexpressing cells was significantly increased by miR-30a knockdown. (Supplementary Fig. S4C).

Effects of CCNE2 knockdown on proliferation, cell cycle, and drug sensitivity to 5-FU.

A loss-of-function assay using siRNA analysis was performed to assess the oncogene function of CCNE2 that is directly targeted by miR-30a. CCNE2 knockdown using si-CCNE2-1 and si-CCNE2-2 significantly inhibited CCNE2 expressions in OSCC cells at both the mRNA and protein levels (Fig. 4A). The optical concentration for efficient downregulation of CCNE2 was 10 nmol/ml si-CCNE2-1, which yielded an 80–90% reduction in mRNA expression in both cell lines (Fig. 4A). Since the data obtained from all experiments using CCNE2 si-RNA-1 were similar to those obtained using CCNE2 si-RNA-2, we present the only data from CCNE2 si-RNA-1 for the subsequent experiments. The cell proliferation assay indicated significant inhibition (60% of control for SAS and 75% of control for Ca9-22) in the si-CCNE2 transfectant (Fig. 4B) through increased G1-arrested cells (Fig. 4C). Drug sensitivity assay showed that CCNE2 targeting significantly enhanced 5-FU resistance of the cells compared to controls (Fig. 4D).

3.4. miR-30a expression in OSCC clinical specimens

In the analysis of clinical samples (Supplementary Table S3), the expression of miR-30a in OSCC tissues was significantly reduced compared with adjacent normal tissues (Supplementary Fig. S5). In addition, we examined the relationships among clinical characteristics such as gender, age, stage and miR-30a expression status (Supplementary Fig. S5). Although a tendency for reduced expression of miR-30a in OSCC tissues compared with normal tissues was seen in all the groups classified by clinical characteristics, we couldn’t obtain remarkable findings.
4. Discussion

Emerging evidence indicates that miRNAs are aberrantly expressed in several types of human cancers [23]. Therefore, characterizing the biological processes affected by miRNA dysregulation may help develop novel miRNA-based cancer treatments. Herein, we identified miR-30a as a candidate for 5-FU resistance-related miRNA in OSCC. Consistent with our data obtained using clinical samples, miR-30a has been reported to be downregulated in a variety of solid tumors, including nasopharyngeal [24], colon [25], lung [26], liver [27], and prostate cancers [28]. These results suggest that miR-30a fundamentally functions as a tumor-suppressive miRNA. However, the effects of miR-30a on drug resistance in various human cancers are not well understood.

To the best of our knowledge, this is the first study to demonstrate the association between altered miR-30a expression and 5-FU resistance in head and neck cancer. This agent is considered to be a purely S phase-active chemotherapeutic agent, with no activity when cells are in G0 or G1 phases [29]. We found that miR-30a-overexpressing OSCC cells had a reduced proliferation rate, an increased proportion of G1 phase cells, and a decreased proportion of S phase cells, which may indicate that miR-30a-induced G1 arrest can protect OSCC cells against 5-FU. In addition, this effect implies that delayed S phase entry and/or reduced S phase transit may provide resistant cells with sufficient time to repair 5-FU-induced damage. Many miR-30a targets are shared with other miR-30 family members through a common seed sequence [30]. This supports our finding and suggests that the miR-30 family members induce growth inhibition through an increase of G1 arrested cells in colorectal [31] and breast cancer [32].

A luciferase reporter assay demonstrated that miR-30a interacts directly with binding sites in the 3’UTR of CCNE2. The encoded protein, cyclin E, is required for mammalian cells to transition from G1 to S phase, by activating cyclin-dependent kinase 2 (CDK2) to form a CCNE2-CDK complex [33] and initiate DNA synthesis. Therefore, the down-regulation of CCNE2 by miR-30a could induce G1 arrest, leading to 5-FU resistance. This association between cancer cell growth suppression and 5-FU resistance is supported by previous reports showing that a reduced proliferation rate is inversely correlated with 5-FU cytotoxicity and the therapeutic response [34-36].

Cells overexpressing miR-30a showed markedly decreased cellular growth activity (Supplementary Figs. S3A and B), whereas 5-FU-resistant cells showed no significant decrease in growth compared with parent cells (Supplementary Figs. S1A and B). This discrepancy may be attributed to difference in the mechanisms of acquired resistance to 5-FU. The proliferation of cells overexpressing miR-30a was considerably suppressed through miR-30a-mediated downregulation of CCNE2 and increased G1 arrest, thus leading to 5-FU resistance. Meanwhile, drug resistance to 5-FU is exerted as a combination of multiple mechanisms, including altered drug metabolism [10], increased drug efflux [10], enhanced apoptosis evasion [21], cell-extracellular matrix adhesion-mediated survival enhancement [22], and increased miR-30a-mediated growth inhibition. Under drug selective pressure, the emergence of cell subpopulations for resistance can be induced by tumor heterogeneity. Therefore, drug resistance of 5-FU-resistant OSCC cells is possibly based on the synthetic effects of diverse molecular mechanisms that heterogeneous tumor cells acquired. Five-FU-mediated miR-30a upregulation seems to be one of such mechanisms to attenuate drug sensitivity to 5-FU.

One major limitation of this study was that the underlying mechanisms of miR-30a upregulation in 5-FU-resistant OSCC cells are not known. Analyses of miR-30a expression regulation are needed at the pre-transcriptional, transcriptional, and post-transcriptional levels. Another limitation is that we focused only on CCNE2 as a key functional target of miR-30a in 5-FU-resistant OSCC cells, whereas a single miRNA can concurrently target multiple effectors of pathways involved in cancer biology. Therefore, further analyses are required to determine the influence of the other targets of miR-30a on 5-FU resistance in OSCC.

Another limitation is that the novel findings in our study are largely based on in vitro data. We hope to be able to conduct in vivo studies that will help us to assess the significance of miR-30a in OSCC in the near future.

Notably, miR-30a is reportedly involved in numerous biological processes including proliferation, autophagy, epithelial-mesenchymal transition, and cell cycle regulation [37], and plays versatile roles as an oncogene or a tumor suppressor in different types of human cancers [38]. Collectively, its functional roles appear flexible and context-dependent according to tissue- or tumor-specific environments. Further studies are warranted to determine its effects on oral cancer biology, including autophagy and epithelial-mesenchymal transition. This information would contribute to a better understanding and control of 5-FU sensitization in OSCC.

In conclusion, we demonstrate, for the first time, that miR-30a attenuates drug sensitivity to 5-FU by modulating cell proliferation in OSCC. Our data indicate that miR-30a could be a predictor of 5-FU sensitivity, and that miR-30a targeting therapy might be an effective treatment option in patients with 5-FU-resistant OSCC.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1152/jb.brep.2021.101114.

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