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Mode of Action of Carboplatin Via Activating p53/miR-145 Axis in Head and Neck Cancers

Ahsen Kilic, BSc; Neslisah Barlak, MSc; Fatma Sanli, MSc; Abdulmelik Aytatli, BSc; Ozel Capik, MSc; Omer F. Karatas, PhD

**Objective**: In this study, we aimed at investigating the expressions of miR-145 and its well-characterized direct targets on carboplatin treatment.

**Study Design**: Laboratory study.

**Methods**: The effect of carboplatin and miR-145 on the proliferative capacity of head and neck squamous cell carcinoma cells was evaluated using Cell Viability Detection Kit-8. Expressions of miR-145 and its targets were evaluated using quantitative real-time polymerase chain reaction on carboplatin treatment and p53 inhibition. Western blot was used to measure the levels of p53 and its acetylated versions in cells treated with carboplatin and/or pifithrin-α.

**Results**: We demonstrated that carboplatin induced the expression of miR-145 in a dose-dependent manner and suppressed the expressions of miR-145 direct targets. In addition, we showed that inhibition of p53 by pifithrin-α in carboplatin-treated cells reduced miR-145 expression and reversed the suppression of miR-145 direct targets.

**Conclusions**: Considering all these findings together, one of the proposed mechanisms of carboplatin to kill cells might be the induction of miR-145 and deregulation of its targets in parallel, via p53 activation, which happens through carboplatin’s DNA-damaging property. To the best of our knowledge, these findings are the first to reveal the relationship between carboplatin and miR-145 in cancer cells.

**Key Words**: Head and neck cancer, carboplatin, p53, miR-145, microRNA.

**Level of Evidence**: NA

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**INTRODUCTION**

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancer types in the head and neck region.¹ With approximately 700,000 new cases diagnosed each year worldwide, it accounts for 95% of the head and neck cancers and 5% of all cancers.²³ Lifestyle, various genetic risk factors, smoking, alcohol consumption, poor oral hygiene, and human papillomavirus infection are known as major risk factors for HNSCC.⁴⁻⁵ Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignant tumors of the upper respiratory tract and is the second most common type of the respiratory cancers in the world.⁵⁻⁷ Because the larynx has important functions, such as breathing and swallowing, its treatment significantly affects quality of life.⁸ In addition, hypopharyngeal squamous cell carcinoma (HSCC) accounts for 3% to 5% of all head and neck cancers.⁹ It is the most aggressive head and neck cancer and has the worst 5-year survival rate of about 30% to 40%.¹⁰ Although various treatment approaches, such as surgery, radiotherapy, and chemotherapy are widely used, there has been no significant improvement in the mean survival rates of both LSCC and HSCC cancer patients. Therefore, new diagnostic and therapeutic approaches are needed to increase the survival rates and improve the quality of life of the patients.

Carboplatin, as a platinum-based alkylating agent, has been developed as an analogue of cisplatin and is used to treat several cancers including breast, ovarian, lung, and head and neck cancers.¹¹ Carboplatin forms cross-links on DNA by binding covalently to guanine or adenine bases and causes DNA damage, which inhibits cell proliferation by activating cell signaling pathways involved in cell cycle arrest and apoptosis.¹²⁻¹³ High-dose carboplatin in combination with fluorouracil was demonstrated to be effective against late-stage head and neck cancers with low toxicity potential.¹⁴ In the meantime, carboplatin has been shown to cause changes in the microRNA profile of tumor cells.¹⁵⁻¹⁶ However, there is limited study, especially in HNSCC, about the involvement of microRNAs in the cytotoxic affects of carboplatin while it kills the tumor cells, and further exploration is needed to reveal alternative modes of action for carboplatin.

MicroRNAs are single-stranded, 18 to 24 nucleotides-long, noncoding, endogenous short RNAs that have recently become one of the most popular topics in cancer research.¹⁷
MicroRNAs are thought to posttranscriptionally regulate the expression of more than 60% of human genes by targeting their 3’ untranslated regions (3’UTR). MiR-145, a well-known tumor suppressor microRNA, was discovered by identifying its homology to mice in 2002. This highly conserved microRNA transcription is controlled by p53, and its expression has been shown to be downregulated in several tumors, including head and neck cancers compared to normal tissues. As a tumor suppressor, miR-145 inhibits proliferation, metastasis, and invasion of tumor cells, and plays important roles in many processes during carcinogenesis such as sensitivity to chemotherapeutic drugs, differentiation, and angiogenesis in various cancer types.

In this study, we investigated the expression of miR-145 and its well-characterized direct targets upon carboplatin treatment, which causes DNA damage and thereby initiates p53 expression in HNSCC cell lines to reveal a potential mechanism of action for carboplatin while it exerts its function against cancer cells.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**

The human LSCC Hep-2 cell line was obtained from SAP Institute (Ankara, Turkey; Ministry of Food Agriculture and Livestock, Turkey), and the human HSCC FaDu cell line was kindly provided by Dr. Fatma Sogutlu from Ege University (Izmir, Turkey). Hep-2 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). FaDu cells were cultured in Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). Cells were maintained in a 37°C and 5% CO2 incubator.

**Preparation of Carboplatin and Pifithrin-α Stock Solution**

Five milligrams of commercially available carboplatin (MediChemExpress, Monmouth Junction, NJ) was dissolved in distilled water to have a 10 mM stock solution, which was aliquoted into 2-mL intermediate stocks and stored at −80° C. Pifithrin-α purchased from Cayman Chemical (Ann Arbor, MI) was dissolved in dimethyl sulfoxide to have a 2 mM stock solution, which was diluted to 20 μM final concentration when applied to cells.

**MicroRNA Transfection**

Hep-2 and FaDu cells were seeded in six-well plates at a concentration of 2 x 10⁵ and 1.8 x 10⁵ cells/well, respectively. Cells were transfected with mir-145 mimic and nontargeting negative control (NC) mimic (Invitrogen, San Diego, CA) using Lipofectamine 3000 Transfection Reagent kit (Invitrogen) according to the manufacturer’s protocol. Cells were collected 24 hours or 48 hours after transfection according to the protocol of the experiments.

**Cell Proliferation Assay**

The viability of cells treated with carboplatin and/or miR-145 was measured using Cell Proliferation Assay Kit-8 (CVDK-8; EcoTech Biotechnology, Erzurum, Turkey) following the manufacturer’s protocol. Hep-2 and FaDu cells were seeded in 96-well plates at a density of 3 x 10⁴ cells/well in six replicates and incubated at 37°C overnight. Then, cells were treated with carboplatin at varying concentrations for 24 hours and 48 hours (0.5, 1, 2, 4 μg/mL) to assess the effects of carboplatin on its own. In the meantime, cells were treated with carboplatin (4 μg/mL) and mir-145 mimic or NC mimic for 24 hours and 48 hours to evaluate their effects in combination.

Then, CVDK-8 reagent was added to each well as diluted in 1/10 in RPMI medium, plates were incubated for 3 hours protected from light, and optical densities were measured at 450 nm with an Epoch 2 Microplate Spectrophotometer (BioTek, Winoo-ski, VT) to assess the viability of cells.

**Total RNA Isolation**

Total RNA was extracted from Hep-2 and FaDu cells using EcoPURE Total RNA Kit (EcoTech Biotechnology, Erzurum, Turkey) according to the manufacturer’s protocol and were stored at −80°C until their use. RNA concentrations and purities were measured with Epoch 2 Microplate Spectrophotometer (BioTek).

**cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction**

For microRNA quantification, total RNA samples diluted to 15 ng/μL were reverse transcribed to complementary DNA (cDNA) using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and specific primers (Applied Biosystems). Expression analysis was then performed using TaqMan Universal Master Mix II, with UNG (Applied Biosystems) and microRNA-specific probes (Applied Biosystems). RNU43 was used as internal control.

To detect changes in the mRNA level, equal amounts of total RNA samples were initially converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Then, 5x HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and primers listed in Table I were used for relative mRNA quantification. Glyceraldehyde 3-phosphate dehydrogenase was utilized as internal control.

All experiments were performed in triplicates following the manufacturers’ instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a Rotor-Gene qRT-PCR (Qiagen, Dusseldorf, Germany) device using standard parameters. Differential expression of miR-145 and its target genes were measured using the 2−ΔΔCT method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tr>
<td>SOX2-F</td>
<td>5’-CTCCGGACGACATGATCAGC-3’</td>
</tr>
<tr>
<td>SOX2-R</td>
<td>5’-GGTATGCTGGACATGATG-3’</td>
</tr>
<tr>
<td>KLF4-F</td>
<td>5’-CCCCATGACCCTGCTCC-3’</td>
</tr>
<tr>
<td>KLF4-R</td>
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</tr>
<tr>
<td>OCT4-F</td>
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</tr>
<tr>
<td>OCT4-R</td>
<td>5’-CAAAATTGCTGAAGCTTCATG-3’</td>
</tr>
<tr>
<td>ABCG2-F</td>
<td>5’-AGCAGCAGTCTGACAGTG-3’</td>
</tr>
<tr>
<td>ABCG2-R</td>
<td>5’-GATCGATCCTGCTTACC-3’</td>
</tr>
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<td>GAPDH-F</td>
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</tr>
<tr>
<td>GAPDH-R</td>
<td>5’-GGCATTGCTGATCTGAGG-3’</td>
</tr>
</tbody>
</table>

2819

Laryngoscope 130: December 2020

Kilic et al.: Carboplatin Induces p53/miR-145 Axis in HNSCC
**Western Blot**

Hep-2 and FaDu cells seeded in six-well plates at a concentration of $2 \times 10^5$ and $1.8 \times 10^5$ cells, respectively, were treated with carboplatin, miR-145, and/or pifithrin-α for 48 hours. Cells were lysed within radioimmunoprecipitation assay lysis buffer (EcoTech Biotechnology, Erzurum, Turkey) supplemented with phenylmethanesulfonfyl fluoride (Roche, Basel, Switzerland), protease inhibitor cocktail (Santa Cruz Biotechnology, Dallas, TX), Cell lysates diluted in 10x Laemmli Sample Buffer (EcoTech Biotechnology, Erzurum, Turkey) were boiled for 5 minutes at 100 °C. Subsequently, equal amounts of protein samples were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred onto a nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA). Membranes were initially blocked with 5% nonfat milk powder for 1 hour and then incubated overnight at 4 °C with appropriate commercially available primary antibodies: β-Actin (1:200; Santa Cruz Biotechnology), p53 (1:1000; Cell Signaling Technology, Danvers, MA), anti p53 acetyl K120 (1/1000; Abcam, Cambridge, United Kingdom), and anti-acetyl p53 Lys 373&382 (Upstate Biotechnology, Lake Placid, NY). Membranes were then incubated with appropriate secondary antibodies for 1 hour at room temperature. Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Scientific) was used to detect signals. β-actin was used as internal control.

**Statistical Analysis**

Experimental data were presented as mean ± standard error of the mean and tested with the Student t test. A $P$ value ≤.05 was accepted as statistically significant.

**RESULTS**

**Carboplatin Reduces Proliferation and Induces miR-145 Expression**

To evaluate the effects of carboplatin on the proliferation of Hep-2 and FaDu cells, they were exposed to varying concentrations of carboplatin for 24 hours and 48 hours. Although only the highest dose of carboplatin reduced the viability of cells after 24 hours compared to controls, doses from 1 μg/mL to 4 μg/mL effectively killed the cells after 48 hours of carboplatin administration (Fig. 1A,B). To look
for potential mechanisms carboplatin uses while it kills the cells, we evaluated the expression of a well-characterized tumor suppressor microRNA in various cancers including HNSCCs, miR-145, and found that increased doses of carboplatin could effectively and in a dose-dependent manner induce the expression of miR-145 in both Hep-2 and FaDu cells compared to corresponding controls (Fig. 1C,D). This finding points to miR-145 as an important potential endogenous effector of carboplatin. To further analyze the involvement of miR-145 during carboplatin administration, we utilized further in vitro tests, where we used 4 μg/mL carboplatin dose as the optimum dose, because it effectively inhibits the proliferation and induces miR-145 expression in both cell lines.

**Carboplatin Treatment Reduces the Expressions of miR-145 Targets**

Because carboplatin treatment induces miR-145 expression, we wanted to see whether carboplatin treatment and ectopic miR-145 expression causes similar changes in the phenotypes of the cancer cells. To validate the overexpression of miR-145 after transfection, we initially measured miR-145 level in cells treated with miR-145 mimic, and found that miR-145 was significantly upregulated in both Hep-2 and FaDu cells transected with miR-145 compared to corresponding control cells (Fig. 2A,B). To also validate the tumor suppressor potential of miR-145, we demonstrated that its ectopic overexpression inhibited the viability of cells (Fig. 2C,D). To see whether the expressions of direct targets of miR-145 are deregulated upon carboplatin treatment, we evaluated the expression of OCT4, SOX2, KLF4, and ABCG2, which are well-characterized direct targets of miR-145. Initially, we confirmed downregulation of those genes’ expressions upon miR-145 overexpression (Fig. 2E,F). Then, we measured their expressions in cells treated with carboplatin and found a significant decrease in the expression levels of OCT4, SOX2, KLF4, and ABCG2 when compared to controls (Fig. 2E,F). These results suggest that carboplatin treatment–induced alteration of the gene expression profile might partially stem from induction of miR-145.

![Fig. 2. Relative expression level of miR-145 in Hep-2 and FaDu cells treated with either carboplatin or miR-145 (A, B). Proliferation of Hep-2 and FaDu cells treated either with carboplatin or miR-145 (C, D). Relative expression levels of miR-145 target genes in Hep-2 and FaDu cells treated either with carboplatin or miR-145 (E, F). Mean ± standard error of the mean is shown. *P < .05, t test. Carbo = carboplatin. NC = non-targeting negative control mimic.](image-url)
Carboplatin Induces miR-145 Expression Through p53 Activation

Carboplatin exerts its function through its DNA damaging property, which is thought to be the main cause of its cytotoxicity.\textsuperscript{29} We demonstrated that carboplatin treatment resulted in an increase in expression of p53, which is activated upon DNA damage, and its acetylated versions K120ac and K373ac (acetylated at lysine 120 or 373) associated with activation of proapoptotic genes, in Hep-2 and FaDu cells (Fig. 3A,B). We also demonstrated that miR-145 itself has the potential for a proapoptotic effect, which is exerted through p53 activation (Fig. 3A,B).

Since p53 has been demonstrated to transcriptionally activate the expression of miR-145 through interaction with a potential p53 response element in the \textit{miR-145} promoter,\textsuperscript{30} we inhibited p53 using pifithrin-\(\alpha\), which is an inactivator of p53 that blocks p-53–dependent transcriptional activation (Fig. 3C,D), and demonstrated that inhibition of p53 resulted in suppression of miR-145 expression in both Hep-2 and FaDu cells (Fig. 3E,F). Reduction of p53 and miR-145 expressions upon pifithrin-\(\alpha\) alone and in combination with carboplatin was observed, indicating that p53 is a key mediator of miR-145 expression in response to carboplatin treatment.

**Fig. 3.** Relative protein levels of p53 and its acetylated versions in Hep-2 and FaDu cells treated with carboplatin or transfected with miR-145 (A, B). Relative protein levels of p53 and its acetylated versions in Hep-2 and FaDu cells treated with carboplatin and/or pifithrin-\(\alpha\) (C, D). (E, F) Relative miR-145 expression level in Hep-2 and FaDu cells treated with carboplatin and/or pifithrin-\(\alpha\). \(\beta\)-actin was used as internal control in Western blot analysis. Mean \(\pm\) standard error of the mean is shown. *\(P < .05\), t test. Carbo = carboplatin. NC = non-targeting negative control mimic.

**Fig. 4.** Relative expression levels of miR-145 target genes in Hep-2 and FaDu cells treated with carboplatin and/or pifithrin-\(\alpha\) (A, B). Mean \(\pm\) standard error of the mean is shown. *\(P < .05\), t test. Carbo = carboplatin.

**Fig. 5.** Schematic representation of the mode of action of carboplatin via activating p53/miR-145 axis in head and neck cancers.
Carboplatin is a chemotherapeutic agent commonly used in the treatment of head and neck cancers, which induce DNA damages and thereby activate the p53-signaling pathway. We showed for the first time in the literature that carboplatin induces miR-145 expression via activation of p53 in a dose-dependent manner. Moreover, miR-145 overexpression was accompanied by reduced expressions of miR-145 direct targets. These results showed that one of the mechanisms carboplatin
uses when it kills HNSCC cells might be exerted via induction of miR-145 expression, which is an important tumor suppressor microRNA.

BIBLIOGRAPHY