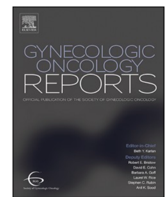
Gynecologic Oncology Reports 45 (2023) 101138

Contents lists available at ScienceDirect

Gynecologic Oncology Reports

journal homepage: www.elsevier.com/locate/gynor



Research Report

Hypoxia-induced paclitaxel resistance in cervical cancer modulated by miR-100 targeting of USP15

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ARTICLE INFO

Keywords:
Hypoxia
miRNA
Chemotherapy
Apoptosis
Paclitaxel resistance
Cervical cancer

ABSTRACT

Objective: Hypoxia, which occurs during the development of cervical cancer, confers chemotherapy resistance. MicroRNA expression is regulated by hypoxia and is associated with the onset and progression of certain types of cancer. MicroRNA-100 (miR-100) is a microRNA, associated with nasopharyngeal and oral squamous cell carcinomas, whose expression is decreased in cervical cancer. This study aims to ascertain the effect of hypoxia on expression levels of both miR-100 and its target genes, as well as exploring the sensitivity to paclitaxel under hypoxic conditions.
Methods: We investigated the effect of hypoxia on miR-100 expression. We also explored the regulators of paclitaxel response under hypoxic conditions of cervical cancer.
Results: Using RT-qPCR, we found that expression of miR-100 in cervical cancer cell lines SiHa and HeLa is significantly higher under hypoxic conditions (1% O_2). We also confirmed that human ubiquitin-specific protease 15 (USP15) is the one of the target proteins of miR-100. Hypoxia and overexpression of miR-100 both reduced the activity of the luciferase reporter containing the 3′-untranslated region of USP15, which contains the miR-100 binding site. Furthermore, a western blot analysis showed that hypoxia suppresses the expression of the USP15 protein, while RT-qPCR showed hypoxia-induced downregulation of USP15 mRNA levels. We also discovered that overexpression of miR-100 induces paclitaxel resistance, thereby reducing the drug's therapeutic effect on cell death.
Conclusions: Our results are consistent with the hypothesis that cervical cancer cells overexpress miR-100 in response to hypoxia and that miR-100 is a facilitator of USP15 downregulation and inactivation.

1. Introduction

Cervical cancer is the leading cause of cancer-related death among women in developing countries (Siegel, et al., 2011, Arbyn, et al., 2020). Approximately 90 % of new cases and deaths from cervical cancer worldwide in 2020 occurred in low- and middle-income nations (Sung, et al., 2021). In its advanced stages, cervical cancer is more likely to exhibit treatment resistance, contributing to poor prognoses, despite recent advancements in cancer treatment, highlighting the need for more innovative and effective chemotherapy treatments. Paclitaxel is one of the most effective anticancer agents for ovarian, gastrointestinal, lung, breast, and cervical cancers (Seino, et al., 2016, Soetekouw, et al., 2013, Al-Batran, et al., 2016, Fader and Rose, 2009, Musaev, et al., 2015), though its efficacy is limited, with many patients exhibiting resistance to the drug. To treat these cancers more effectively, resistance is an issue that needs to be addressed.

We have previously shown that ubiquitin-specific peptidase 15 (USP15) is a key protein affecting paclitaxel sensitivity (Xu, et al., 2009), as it enhances rates of paclitaxel-induced apoptosis. We have also found that USP15 expression is regulated by oxygen levels. USP15 consists of 952 amino acids, with a predicted molecular mass of 109.2 kDa (Baker, et al., 1999), and it contains highly conserved Cys and His boxes, which

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https://doi.org/10.1016/j.gore.2023.101138
Received 21 November 2022; Received in revised form 6 January 2023; Accepted 17 January 2023
Available online 21 January 2023
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are present in all UBP family members.

Research indicates that hypoxia has a significant impact on carcinogenesis and cancer development. Furthermore, tumor hypoxia serves as an independent prognostic marker for several cancers, such as cervical, head, neck, and soft tissue cancers (Sasaki, et al., 2014, Brizel, et al., 1996, Brizel, et al., 1997). Hypoxic stress has also been shown to underlie many of the biologically important processes responsible for chemotherapy resistance among cancer cells (Vaupel, et al., 2001).

Recent research shows that microRNA (miRNA) expression is regulated by hypoxia (Gervin, et al., 2020, Tapeh, et al., 2021). These single-stranded RNA molecules are non-coding and range in length from 20 to 22 nucleotides. They typically act as negative post-transcriptional regulators of gene expression by binding to the 3′-untranslated region (UTR) of their target genes (Bartel, 2004, Bartel, 2009). Such direct binding either inhibits translation or causes mRNA degradation (Bartel, 2004, Bartel, 2009, Pillai, et al., 2007). Moreover, miRNAs play pivotal roles in many biological and pathological processes among all eukaryotes, including in cancer development (Bandres, et al., 2007). Their varied expression in cancer cells is connected to the initiation and progression of various cancer types (Tsuchida, et al., 2011, Yamaguchi, et al., 2012, Tadokoro, et al., 2013, Kobayashi, et al., 2014, Kanekura, et al., 2016, Deng, et al., 2021). For example, Li et al. previously showed that miR-100 expression is downregulated in cervical cancer (Li, et al., 2011). Given this finding, we sought to elucidate the specific role of miR-100′s altered expression in the progression of this cancer type. Additionally, we focused on examining: 1) the effect of hypoxia on regulation of miR-100 expression, 2) characterizing the relationship between miR-100 and its target gene USP15, and 3) investigating downstream sensitivity to paclitaxel.

2. Materials and Methods

2.1. Cell culture

The human cervical cancer cell lines, HeLa and SiHa, were cultured in modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum and incubated in a water-jacketed CO_2 incubator (NAPCO, Winchester, VA, USA) at 37 °C in a humidified atmosphere with 5 % CO_2. For the hypoxic exposure condition, the HeLa and SiHa cells were incubated in a 1 % O_2 atmosphere.

2.2. RT-qPCR analysis

To quantify the levels of miR-100 and USP15 mRNA expression, we utilized RT-qPCR analysis. Isogen reagent (Nippon Gene, Tokyo, Japan) was used to isolate total RNA from the HeLa and SiHa cells, which had been seeded at densities of 1.0×10^7 cells/100 mm dish in 10 mL of growth medium and incubated under normoxia or hypoxia conditions for 24 h. We quantified total RNA with A260/A280 measurement using the Ultraspec 3000 (Amersham Biosciences, Piscataway, NJ, USA). Next, miRNA quantification was performed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) and miRNA-specific stem-loop primers (has-miR-100; Applied Biosystems, Waltham, MA, USA). Then, miR-100 RNA was reverse-transcribed (RT) into cDNA according to the manufacturer's protocol using an Applied Biosystems 9800 Fast Thermal Cycler. Quantitative real-time polymerase chain reaction (qPCR) was performed on miR-100 cDNA with an miR100-specific primer along with Universal Master Mix and carried out using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). For the qPCR protocol, the reaction mixture was first incubated at 95 ℃ for 2 min. After the initial incubation, the following thermal protocol was applied: 50 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The cycle thresholds (Ct) were calculated and normalized to miR-16, which is the most widely used endogenous control miRNA for RT-qPCR (Ohyashiki, et al., 2011). We then calculated the miR-100 expression levels using the comparative cycle threshold (C_T) method, wherein the average C_T value of the control miR-16 was subtracted from the C_T value of the mature miR-100 reaction, thereby producing the ΔC_T value. The -ΔΔCt value was then calculated by subtracting the -ΔCt value of samples under the normoxia condition from the respective -ΔCt values of samples under the hypoxia condition. Finally, miR-100 expression was normalized using the $2^{-\Delta\Delta Ct}$ method (Schrauder, et al., 2012).

RT-qPCR was performed to gain a quantitative estimate of USP15 mRNA levels. Total RNA was reverse-transcribed into cDNA using a cDNA reverse transcription kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's protocol. Then, PCR mixtures (20 μL) were prepared with final concentrations of 5 mM $MgCl_2$, 5 μL cDNA (1:10 diluted), 0.3 μM each of the forward and reverse primers, and 2 μL SYBR green master mix (Roche Applied Science, Mannheim, Germany). The primer sequences were as follows:

USP15: 5′-TGGACTTC TCAAAGATGGTGATGC-3′ and 5′-GGTGCTGTCTGGTTTATTCAGTGG-3′.

β-actin: 5′-GGGAAATCGTGCGTGACATTAAG-3′ and 5′-TGTGTTGGCGTACAGGTCTTTG-3′.

The reactions were performed in LightCycler 96 System (Roche Applied Science, Basel, Switzerland). The following thermal protocol was used: one denaturation cycle at 95 ℃ for 10 s, 45 cycles off (temperature transition of 20 ℃/s) 95 ℃ for 10 s, 62 ℃ for 10 s, and 72 ℃ for 6 s. Melting curve analysis was then performed using continuous fluorescence readings taken at 72 ℃. For each sample, each gene was quantified through correlation with a standard curve generated from measurements taken during the exponential phase of amplification. All samples were analyzed in duplicate.

2.3. Western blot analysis

To quantify the level of USP15 protein expression under both normoxic and hypoxic conditions, we utilized western blot analysis. HeLa and SiHa cells were plated at a density of 2.5×10^7 cells per 150-mm dish, and cultured at 37 °C, 20 % O_2. Subsequently, the passaged cells were exposed to 1 % O_2 for 24 h to mimic hypoxic conditions. Harvested cells were lysed on ice for 30 min in lysis buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 400 mM NaCl, 10 % glycerol, 0.5 % NP40, 5 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) along with protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). The lysate was then centrifuged at 13,000 × g for 15 min to collect the soluble fractions. The protein concentrations of the soluble fractions were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA); 40 μg of each protein lysate was then loaded onto a 4 %–12 % SDS-polyacrylamide gel and subjected to electrophoresis at 200 V for 50 min before being transferred onto a polyvinylidene difluoride (PVDF) membrane. After stripping the PVDF membrane with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, IL, USA), each protein was probed with an anti-USP15 antibody (M01, clone 1C10, Abnova Corporation, Taipei, Taiwan) and again with an anti-actin antibody (C4; Boehringer Mannheim, Indianapolis, IN, USA). Each protein band was visualized using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence western blotting detection reagent (Amersham Biosciences, Amersham, UK). The intensity of each USP15 band was then normalized to that of the corresponding actin band.

2.4. Transfection and luciferase reporter assays

To determine if miR-100 suppresses USP15 expression, we utilized a luciferase reporter assay. In experiments using transfected cells, transfection was performed prior to hypoxia exposure. The luciferase reporter construct pLightSwitch_3′UTR-USP15, containing the 3′-UTR of USP15, was prepared by ligating the region into the pLightSwitch_3′UTR vector. Luciferase assays were performed using a standard protocol (Aldred, et al., 2011). Each cell line was seeded at a density of 5.0×10^5 cells per

35-mm dish and incubated overnight at 37 °C and 5 % CO_2. For each transfection, 100 ng of pLightSwitch_3′UTR-USP15 and 0 to 25 nM of either miR-100 miCENTURY OX siMature mimic (Exiqon A/S, Vedbaek, Denmark) or negative control were mixed with Lipofectamine (Invitrogen, Waltham, MA, USA) in Opti-MEM (Invitrogen) containing. Cells were washed with Opti-MEM, and then the transfection mixtures were applied to the cells. The cells were then incubated for 24 h under normoxic or hypoxic conditions. Luciferase activity was measured 24 h after transfection using the LightSwitch Luciferase Assay System (Switch Gear Genomics, Menlo Park, CA, USA). All luciferase assays were performed in triplicate.

2.5. Infection with retrovirus

To see how miR-100 expression impacts paclitaxel sensitivity, we employed retroviral infection. First, miR-100 was expressed in HeLa cells through retroviral infection with Pri-miR-100 in pBABE-puro and using platinum-GP cells (Cell Biolabs, Inc. San Diego, CA, USA) as packaging cells. The cells were infected with either empty pBABE as a control vector or pBABE miR-100 and then selected with puromycin. Paclitaxel resistance was then evaluated in both the empty pBABE and pBABE miR-100 infected HeLa cells. After 24 h in culture, different concentrations of paclitaxel (10, 30, and 50 nM) were added to the cells, which had been plated in 12-well culture plates ($n = 6$). The cells were incubated with paclitaxel for 48 h, before being collected and stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA). The number of live cells (represented by the unstained cells) were then counted using a hemocytometer.

2.6. Statistical analysis

All values are presented as the mean ± SD of at least three independent experiments. Statistical analyses were performed using the paired *t*-test, with $P < 0.05$ being considered statistically significant.

3. Results

3.1. Hypoxia induces miR-100 expression but also downregulates USP15 expression

We investigated at how hypoxia affected the expression of miR-100 and USP15 in the cervical cancer cell lines HeLa and SiHa in order to determine whether there is a link between them. Total RNA lysates were extracted from both the normoxic cells (which had been exposed to 5 % O_2 for 24 h) and hypoxic cells (which had been exposed to 1 % O_2 for 24 h), and differences in both miR-100 and USP15 expression levels were analyzed using RT-qPCR. As shown in Fig. 1, miR-100 expression increased significantly under hypoxic conditions. This result indicates that hypoxia induces endogenous expression of miR-100. However, USP15 mRNA expression significantly decreased under hypoxic conditions (Fig. 2A). To quantify USP15 protein expression, whole cell extracts from both normoxic cells and hypoxic cells were subjected to western blot analysis. Results showed that USP15 protein expression is significantly decreased under hypoxic conditions (Fig. 2B). These results indicate that hypoxia downregulates the endogenous expression of USP15.

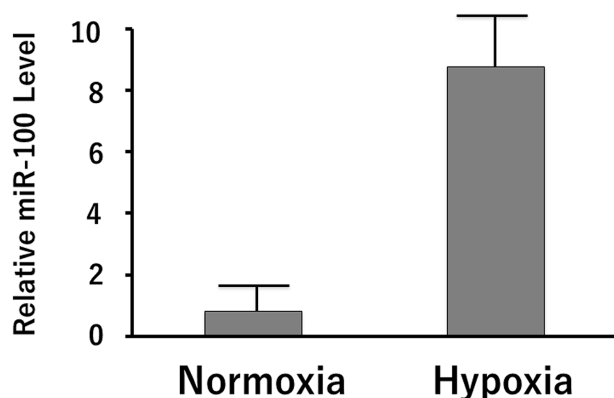

Fig. 1. RT-qPCR data show hypoxia is associated with an increase in miR-100 mRNA expression. Relative levels were quantified using the comparative cycle threshold (C_T) method. Error bars indicate standard deviation among the triplicate assays.

3.2. Hypoxia downregulates USP15 translation

To determine whether the reduction of USP15 protein expression by hypoxia is mediated by miR-100, HeLa and SiHa cells were transfected with pLightSwitch_3′UTR-USP15 and then exposed to the hypoxic condition of 1 % O_2 for 24 h. This hypoxic condition resulted in a 1.7- to 2.4-fold range of downregulation of luciferase activity between (Fig. 3A). Additional co-transfection experiments were performed using either pLightSwitch_3′UTR-USP15, miR-100, or control shRNA. The concentrations of the mentioned constructs were varied to include 5 μM, 10 μM, and 25 μM. Subsequent measurements showed that miR-100 overexpression downregulated luciferase activity by 1.7- to 3.7-fold depending on the miR-100 concentration (Fig. 3B).

3.3. Overexpression of miR-100 induces paclitaxel resistance

Next, we sought to find whether miR-100 overexpression increases paclitaxel resistance. We constructed an miR-100 expression vector, called pBABE miR100, using the pBABE-puro retrovirus vector. We generated HeLa cells stably expressing miR-100 and treated them with either 10, 30, or 50 nM of paclitaxel. The viability of miR-100-overexpressing HeLa cells decreased slower than that of the control

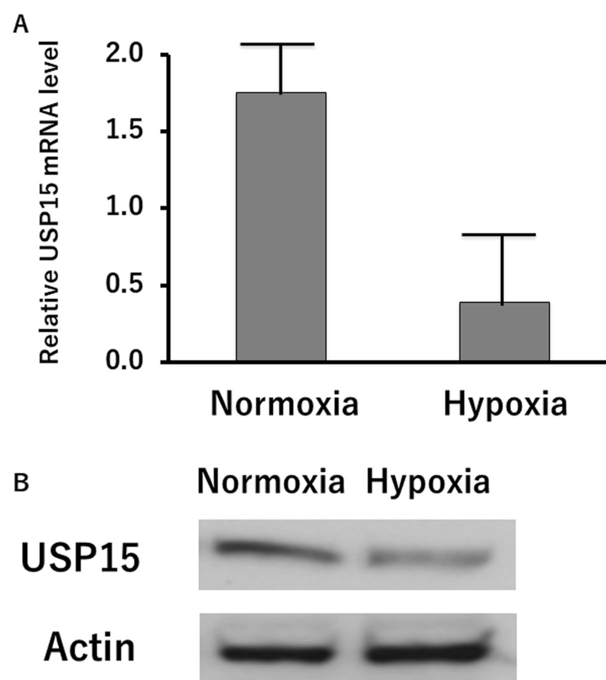

Fig. 2. Hypoxia drives lower USP15 expression. a. RT-qPCR data show hypoxia is associated with a decrease in USP15 mRNA expression. Relative levels were quantified using the comparative cycle threshold (C_T) method. Error bars indicate standard deviation among the triplicate assays. b. Western blot results show that hypoxia results in decreased USP15 protein expression. Total protein was extracted from cells under both the normoxic and hypoxic conditions and then western blotted for USP15. Actin bands served as a loading control.

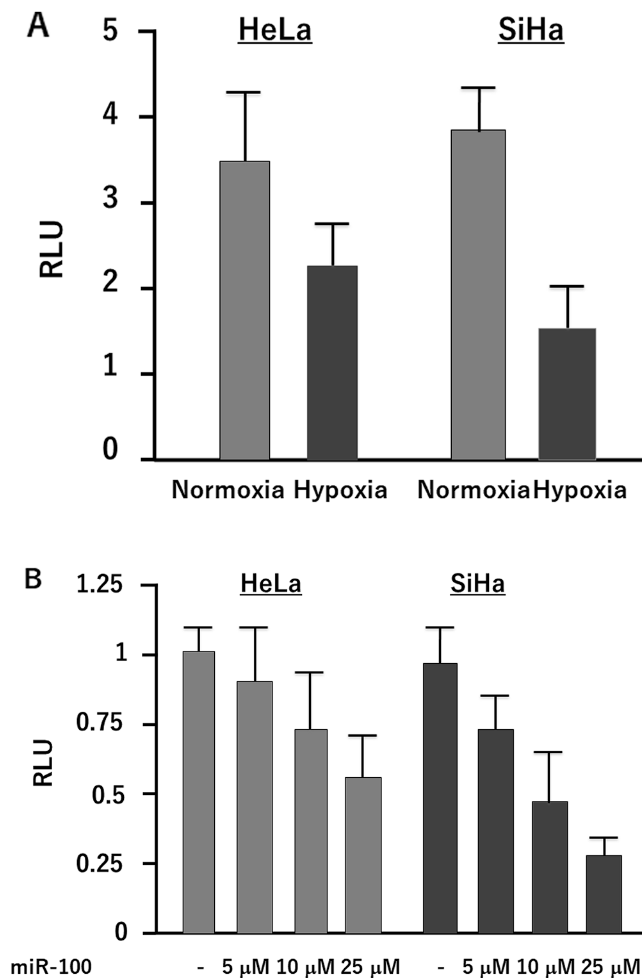

Fig. 3. Luciferase activity of pLightSwitch_3′UTR-USP15 construct containing the 3′-UTR of USP15 gene under normoxia and hypoxia conditions. a. HeLa and SiHa cells were transfected with the pLightSwitch_3′UTR-USP15 and incubated under hypoxic conditions. Measured luciferase activity is reported here as relative luminescence units (RLU). Error bars indicate standard deviation among the triplicate assays. b. HeLa and SiHa cells were co-transfected with both pLightSwitch_3′UTR-USP15 (100 ng) and either the miR-100 expression vector or an empty vector. Luminescence activity is reported in RLU. The amounts of miR-100 constructs used varied to include 5, 10, and 25 μM. Error bars indicate standard deviation among the triplicate assays.

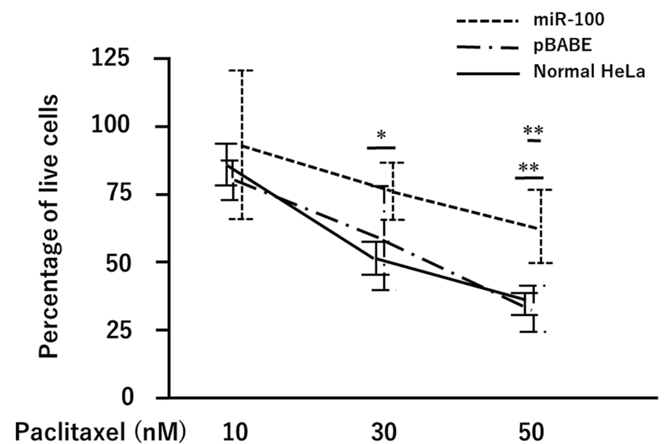

Fig. 4. Overexpression of miR-100 exerts an effect on paclitaxel sensitivity. Following paclitaxel treatment, the miR-100 expression vector-transfected HeLa cells showed a smaller decrease in the percentage of surviving cells compared with that of the control vector, pBABE-transfected cells, or wild type HeLa cells. This phenomenon was observed across varying concentrations of paclitaxel. *$p < 0.05$; **$p < 0.01$. Error bars indicate standard deviation among assays.

vector-expressing HeLa cells or the wild-type HeLa cells (Fig. 4). These results suggest that the expression level of miR-100 is related to paclitaxel resistance.

4. Discussion

USP15 has previously been implicated in the regulation of caspase-3, β-catenin stability, and the NF-κβ and TGF-β signaling pathways (Schweitzer, et al., 2007, Huang, et al., 2009, Eichhorn, et al., 2012). USP15 is also an HPV16 E6-associated protein (Vos, et al., 2009, Yaginuma, et al., 2018, Li, et al., 2019). HPV16 E6 protein levels are elevated by overexpressing USP15, while E6 protein levels are lowered by USP15 knock down with small interfering RNA (Vos et al., 2009). These findings imply that USP15 controls the stability of the E6 protein. The HPV16 E6 protein is a key regulator in cervical cancer development (Dokianakis, et al., 1998); thus, miR-100′s suppression of USP15 may have a role in the pathophysiology and progression of cervical cancer.

Although paclitaxel confers anti-tumor activity against a variety of solid cancers, the development of clinical drug resistance severely restricts this effect (Han, et al., 2021, Alharbi, et al., 2018). Our data show that hypoxia mediates the induction of miR-100 expression and that miR-100 directly downregulates USP15 expression. In turn, this reduction in USP15 leads to paclitaxel resistance among cervical cancer cells (Xu et al., 2009). Through RT-qPCR and western blot experiments, we determined that the level of both USP15 mRNA and protein expression decreases under hypoxic conditions. These data show that tumor hypoxia is one of the most critical conditions for the development of paclitaxel resistance (Das, et al., 2015). This concept may be important for future therapeutics, because acquiring resistance to chemotherapy is one of the most challenging clinical obstacles in treating cervical cancer.

In a previous study, we used HeLa cells to test a siRNA library and discovered that suppressing USP15 expression led to paclitaxel resistance (Xu et al., 2009). Paclitaxel is a potent chemotherapeutic agent, which exerts its effects by targeting the microtubule cytoskeleton, leading to blockage of mitosis and ultimately cell death (Wang, et al., 2000, Jordan and Wilson, 2004). Earlier research has shown that paclitaxel induces caspase-dependent apoptosis (Perkins, et al., 2000, Fawcett, et al., 2005). Active caspase-8 and 10 catalyze this process by cleaving and activating downstream effector proteases such as caspase-3 and 7, which induce apoptosis (Chandra, et al., 2004, Tang, et al., 2000). We have previously found that USP15 overexpressing HeLa cells rapidly and significantly increased the activity of both procaspase-3 and cleaved caspase-3 when compared to that of the control cells (Xu et al., 2009). This indicates that expression level of USP15 is related to paclitaxel sensitivity.

The coordinated inactivation of USP15 by miR-100 suggests that miR-100 inhibitors may have therapeutic utility as paclitaxel sensitizers. Thus, miR-100 inhibitory compounds may be evaluated as anti-cancer therapeutic agents. Additionally, miR-100 and USP15 expression levels could be considered molecular biomarkers predicting response to miR-100 inhibitors in treating paclitaxel resistance. The primary limitation of this study is that it is an in vitro experiment, therefore it has not been confirmed whether the exact same phenomenon can be seen in vivo. Appropriate use of miR-100 and USP15 could help tailor treatment for cervical cancer patients in the future. Our findings show that hypoxia induces miR-100 production in cervical cancer cells and that miR-100 is a mediator of USP15 downregulation and inactivation. This study raised the hypothesis that miR-100 has a role in resistance to chemotherapy in cervical cancer, a promising topic for future research.

5. Authors' contributions

The contribution of authorship is as follows: HN designed the study. MO, SO, ZY, and TS performed experiments. KO, JHO, and MK analyzed the data. HN and MO wrote the manuscript. HN, MO, SO, ZY, TS, KO, JHO, and MK confirmed the authenticity of all raw data. All authors have read and approved the final manuscript.

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.

Acknowledgements

This study was supported by funding from the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (grant nos. 20K09654 and 22K09556) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) - Supported Program for the Strategic Research Foundation at Private Universities (grant no. S1311016).

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