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Research Article

Differential Expression of DDX3 and microRNAs in Response to Hormone and Cisplatin Against Cervical Cancer

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Abstract

Objectives: To discover micro ribonucleic acids (miRNAs) involved in the regulation of DDX3 expression using sexual hormones in combination with the well-known anticancer medication cisplatin.

Methods: SiHa cells were treated with estradiol, dihydrotestosterone, and cisplatin and evaluated the expression of ER beta, Ki67, and DDX3 via quantitative reverse transcription–polymerase chain reaction. We generated a chimeric fusion construct five untranslated region (UTR)–hLUC–3UTR in the pEZX-MT06 miRNA vector under the control of the SV40 promoter. Reporter activity is measured with/without hormones, and their activity is compared with 5'- and 3'-UTR respectively. Various reporter deletion constructs were generated to identify the minimal UTR region in regulating the expression of DDX3. We identified the potential miRNA binding sites on the DDX3 UTR region, and their expression is monitored in cancer patients and cisplatin-treated SiHa cells.

Results: Hormones increased the proliferation of SiHa cells and expression of DDX3. The 3'-UTR region 2135–4307bp contains miRNA sites that regulate DDX3 expression. miRNAs hsa-miR-671-5p, hsa-miR-361-5p, hsa-miR-140-5p, hsa-miR-564, and hsa-miR-769-5p downregulated in patient samples but upregulated in cisplatin-treated cells. miRNA hsa-miR-671-5p and hsa-miR-564 were associated with patient data and cisplatin-treated cancer cells.

Conclusion: We discovered that sexual hormones enhanced DDX3 expression in SiHa cells. MiR-671-5p and miR-564 are two potential therapeutic miRNAs that can be used to treat DDX3-related malignancies.

Keywords: Cancer patients, Cervical cancer, Cisplatin, DDX3, miRNA

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DDX3 is a member of the DEAD box family of ATP-dependent ribonucleic acid (RNA) helicases found on X (DDX3X) chromosomes.[1,2] For the first time, we reported the expression of DDX3 using a second-hand tobacco constituent and in the progression of breast and oral cancer.[3,4] Low or negative expression of DDX3 can be used as a prognostic marker for the diagnosis of nonsmoking patients with oral cancer.[5] DDX3 expression is positively associated with p21 expression. Conversely, low p21 expression was associated with poor relapse-free survival in early-stage lung cancer.[6-8] DDX3 loss via p53 inactivation has been shown to promote tumor malignancy via the MDM2/Slug/E-cadherin pathway and poor patient outcomes in nonsmall-cell lung cancer.[7] DDX3 expression is noted in colorectal cancer patient samples and treatment of patient-derived colorectal cancer spheroid cell lines with RK-33, a DDX3 inhibitor, displayed

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sensitivity. It suggests that DDX3 can be used as a biomarker for the diagnosis of colorectal cancer patient samples.[9] DDX3 was found to be expressed in both pediatric (55%) and adult (66%) medulloblastoma patients.[10] Conversely, analysis of 31 patient glioblastoma multiforme samples revealed a significant correlation between the levels of DDX3 and Snail.[11] Breast cancer metastases caused by hypoxia have been shown to correlate with DDX3 expression and have been shown to correlate with hypoxia.[12] Overall, it suggests that DDX3 is a multifunctional protein and its expression has been shown to correlate with ERα expression in hypoxic regions of breast cancer.[13] Under stress conditions (hypoxic, metabolic, and therapeutic), DDX3 has been shown to localize in cytoplasmic foci termed the stress granule and or processing body, where unwanted messenger RNA (mRNA) gets silenced or translation inhibition via miRNAs.[14,15] DDX3 was shown to mediate posttranscriptional regulation of the androgen receptor (AR), causing repression of AR protein translation. Hence, prostate cancer cells cannot be targeted by hormonal therapies, resulting in poor prognosis and AR-independent cell survival.[16] Recently, DDX3 overexpression was shown to be associated with the FIGO stage and also shown to associate with poor overall survival ($p<0.05$) in cervical cancer patients.[17]

microRNAs (miRNAs) are small RNAs that posttranscriptionally regulate gene expression.[18] More than 1000 functional miRNAs have been discovered so far, and they have been implicated in normal development and disease progression, including cancer.[19-21] The role of miRNAs in cancer was exemplified when miRNA signature patterns obtained from patient samples correctly predicted the phenotype of tumors.[22] miRNAs have been demonstrated to function as either tumor suppressors (let-7 family)[23] or oncogenic (miR17-92 [24]). Recent reports even suggest that the expression profiling of miRNAs may be a more accurate method of classifying cancer.[25,26] In this paper, we focused on understanding the hormone-mediated posttranscriptional regulation of DDX3 in cervical cancer cells using a Luciferase Reporter Assay. Our results showed that estradiol (E2) and dihydrotestosterone (DHT) hormones induced the expression of DDX3 mRNA in the SiHa cell line. Reporter assays revealed that the 3' untranslated region (UTR) is important for the posttranscriptional regulation of DDX3 and reporter deletion constructs allowed to select tumor suppressor miRNAs. Based on cell line and patient data, we conclude that miR-671-5p, 361-5p, and 769-5p can be used as potential biomarkers to diagnose cervical cancer.

Methods

Cell Culture and Real-Time Polymerase Chain Reaction (PCR)

The human cervical cancer cell line, SiHa, was purchased from the National Centre for Cell Science, Pune, India. SiHa cells were cultured on standard tissue culture plastic ware in high glucose Dulbecco's Modified Eagle Medium (DMEM) (HIMEDIA, AL241A) containing 10% fetal bovine serum and 1X antibiotic. The cells were cultured to a density of more than 90% at 37 °C in 5% CO^2. Then, the cells were starved for 5–6 days by culturing them in high glucose DMEM without phenol red, containing 10% charcoal-stripped medium. After starvation for several days, the cells were individually treated with 100 nm concentration of β-E2 (SIGMA, E2758-5G) and 5α-Androstan-17β-ol-3-one (SIGMA, 10300-5G-F) hormones.[27,28] Following incubation, total RNA was isolated at six different time points (30 min, 1, 6, 12, 18, and 24 h) using the standard operating protocols (QIAGEN). RNA was quantified using Nanodrop and approximately 1 µg of RNA was reverse transcribed using the Prime Script complementary deoxyribonucleic acid (cDNA) synthesis kit (Takara, #6110A). Primer sequences used for the quantitative relative expression of DDX3X, Erα, Er β, AR, and Ki67 genes were listed in Table 1.

Table 1. List of primers used for the amplification of selected genes

S.No	Gene	Direction	Primer Sequence
1	GAPDH	Forward	TGACATCAAGAAGGTGGTGA
		Reverse	TCCACCACCCTGTTGCTGTA
2	Oestrogen receptor Alpha	Forward	CCACCAACCAGTGCACCATT
		Reverse	GGTCTTTTCGTATCCCACCTTTC
3	Oestrogen receptor Beta	Forward	AGAGTCCCTGGTGTGAAGCAAG
		Reverse	GACAGCGCAGAAGTGAGCATC
4	Ki67	Forward	TCCTTTGGTGGGCACCTAAGACCTG
		Reverse	TGATGGTTGAGGTCGTTCCTTGATG
5	Androgen receptor	Forward	CGGAAGCTGAAGAAACTTGG
		Reverse	ATGGCTTCCAGGACATTCAG
6	DDX3X	Forward	GGAGGAAGTACAGC CAGCAAAG
		Reverse	CTGCCAATGCCATCG TAATCACTC

Cloning 5' and 3'UTR of the Human DDX3X

The entire 3'UTRs of DDX3X (NM_001356.5) (2080–4635 bp) are PCR-amplified using forward: 5'-GATCCGCGAGATCCTGAT-3' and reverse: 5' CCTATTGGCGTTACTATG-3' primers. The amplified product is cloned downstream of the Luciferase reporter into the pEZX-MT06 miRNA target cloning vector (GeneCopoeia). Recombinant clones were confirmed using AsiS1 and XhoI restriction enzymes (BM1). Simultaneously, we amplified the 5'UTR of DDX3X using forward: 5'-GAGCTCCGCTAGCCATATGCGCT-3' and reverse 5'-GATATCCCCTGAAGAGTACCGAGAACTC-3' primers. The products were gel-purified and cloned into the PUC vector. Recombinant clones were confirmed using Kpn1 and HindIII flanking enzymes and DNA sequence analysis. Next, 5'UTR fragments were transferred upstream of the luciferase and downstream of the SV40 promoter into pEZX-MT06 (BM2). Following confirmation of the clone, we finally generated a fusion construct (5UTR–hLUC–3UTR) to mimic the endogenous cassette of DDX3. DNA sequence analysis confirms the recombinant clone. Following confirmation of the entire clone, we prepared three truncated 3'UTR constructs based on the availability of the restriction enzymes EcoRI (BM4 and BM5) and PciI (BM6). BM1ΔPciL (BM7) is used as a control to measure BM6 activity. Standard molecular biology kits and protocols were followed for the generation of all the constructs.

Transfection and Dual-Luciferase Reporter Assay

One day before the transfection, 1×10^5 serum-deprived cells were seeded into 24 well plates. One (1 µg) of the recombinant vectors was transfected into cells using Lipofectamine Stem Reagent (Invitrogen, STEM0003). Four replicates were performed for each transfection group. After 12 h, the transfected cells were individually treated with 100 nm of E2 and 5α DHT (Sigma Chemicals, St Louis, MO, USA) for 24 h. After 36 h of transfection, the luciferase activity was assayed using the Luciferase Reporter Assay kit (Promega, E1910, WI, USA) and the Berthold Sirius luminometer (Sirius, Oak Ridge, TN) according to the manufacturer's instructions. All experiments were performed in quadruplicate.

miRNA Prediction

Based on luciferase reporter activity and bioinformatics analysis, we searched miRNA targets for our sequence using miRDB, an online database server, and also confirmed against the target gene using miRTarBase.[29,30]

Patient Sample Collection. A total of 13 tissue samples, 10 biopsies from cervical cancer patients (squamous cell carcinoma), and three from normal subjects were collected from the Mehdi Nawaz Jung (MNJ) Cancer Hospital and the CC Shroff Hospital, Hyderabad respectively. Normal cervical tissues (noncancerous samples) were obtained from women who had a hysterectomy for other gynecological issues. The institutional ethical committee review board of Osmania University and MNJ Cancer Hospital has approved the study.

Validation of miRNAs by Quantitative Reverse Transcription-PCR (qRT-PCR)

Small RNA from patient tissue samples was isolated using the Nucleospin miRNA kit (Macherey–Nagel, cat. no-740971.250) and quantified using Nanodrop. Using the Mir-X miRNA first-Strand Synthesis kit (Takara, cat. no-638315), 1 µg of small RNA was reverse transcribed into cDNA. For miRNA quantification, real-time PCR was performed using TB green Advantage qPCR premix (Takara cat. no-639676). The relative expression of each miRNA was normalized to the internal reference gene U6. The miR-primer software was used to design primers, and the primers used in these studies are depicted in Table 2.

Table 2. List of miRNA primers used in this study

S.No	miRNA	Name	Primer sequence
1	hsa-miR-671-5p	Forward	GCCCTGGAGGGGCT
		Reverse	GGTCCAGTTTTTTTTTTTTTTTCTC
2	hsa-miR- 361-5p	Forward	CGCAGTTATCAGAATCTCCAG
		Reverse	GTCCAGTTTTTTTTTTTTTTTGTACC
3	hsa-miR- 140-5p	Forward	CAGCAGTGGTTTTACCCTATG
		Reverse	GGTCCAGTTTTTTTTTTTTTTTCTAC
4	hsa-miR-564	Forward	CAGAGGCACGGTGTCA
		Reverse	AGTTTTTTTTTTTTTTTGCCTGCT
5	hsa-miR-769-5p	Forward	GAGACCTCTGGGTTCTGA
		Reverse	GGTCCAGTTTTTTTTTTTTTTTAGC
6	U6	Forward	GCTTCGGCAGCACATATACTAAAAT
		Reverse	CGCTTCACGAATTTGCGTGTCAT

Receiver Operating Characteristic (ROC) Curves

The ROC curve depicts the marker's discriminatory accuracy in distinguishing between two groups. Each sample's Δcycle threshold (CT) values were used to create ROC curves to estimate the possible threshold values of all the identified markers. For different cut-off points of a parameter, the sensitivity-true positive rate is plotted as a function of 1-specificity-the false positive rate in a ROC curve. The area under the curve determines how well a parameter distinguishes between diseased and normal samples. The ROC curve was used to calculate the highest Youden index and is allied with the optimal threshold cut-off point (COP) for the identified markers. The COP values indicate whether the expression levels of identified markers are higher or lower in cancer and normal samples.

Assessment of Cytotoxicity by MTT Assay

The cytotoxic efficiency of cisplatin was determined by the MTT assay. Approximately 5 x 10^3 cells/ well were seeded in a 96-well plate. After 24 hrs, cells were treated with different concentrations of cisplatin (25, 50, 100,150, 200, and 250µM) for 24 hrs. Following treatment, the cells were washed with 1X PBS and 100µl of fresh culture media was added to the cells. 20µl of MTT solution (5mg/ml) was added to the cells, and the plate was incubated for 3–4 hrs at 37ºC in a CO_2 incubator. The purple color obtained by dissolving the formazan crystals in 100µl of DMSO was estimated by measuring the absorbance at 540nm in a Perkin-Elmer plate reader (multimode plate reader Enspire).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 3.03. An unpaired t-test is performed to evaluate the significance of data sets. P-values less than 0.05 were considered statistically significant and * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$.

Results

Estradiol and Dihydrotestosterone Induce the Expression of DDX3

To investigate whether the expression of DDX3 is regulated by hormones, we compared the mRNA levels of ERα, ERβ, and DDX3 in cells cultured in the presence of E2 and DHT hormones at different time intervals (30 min, 18, and 24hrs). A time course experiment in SiHa cells demonstrated that the ERβ mRNA expression was transient (Fig. 1a) and maximum at 30 min, but after 12 and 24 hrs, ER beta levels returned to or fell below normal. DHT is unable to induce ERβ. Next, we measured the expression of Ki67, a cell proliferative marker, to correlate the expression of ER beta with cell proliferation. As shown in Figure 1b, both E2 and DHT hormones increased the expression of Ki67 mRNA levels at 30 min. Ki67 expression differs between E2 and DHT-treated cells at later points (18 and 24 hrs). Upregulated expression of Ki67 mRNA as compared to control is maintained throughout the experimental condition in E2 treated cells. Unlike E2, Ki67 mRNA expression levels in DHT-treated cells fell below the control at 18 and 24 hrs time points. It suggests that DHT increases the proliferation of cells independent of estrogenic receptors. E2 upregulated the expression of DDX3 mRNA levels at all the time points, highest levels observed at 30 min (2 fold) as compared to the control. DDX3 expression levels are consistent with ki67 expression levels in E2-treated cells. Although DHT induced the expression of DDX3 mRNA levels and its expression is independent of time and Ki67 mRNA expression (Fig. 1c). Overall, this data demonstrates that both hormones participate in the proliferation of cervical cancer cells, possibly by upregulating the expression of a well-known oncogene, DDX3.

Characterization of Human DDX3X UTR in SiHa Cell Line

To assess whether DDX3 expression in cancer cells is reg-

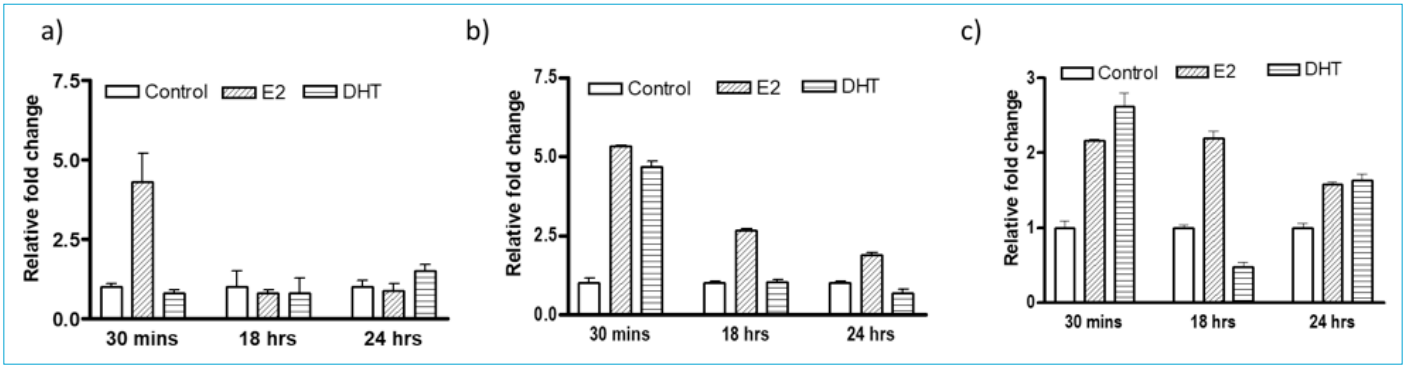


Figure 1. The Effects of Estradiol (E2) and Dihydrotestosterone (DHT) on Cervical Cancer Cell Growth Real-time PCR analysis of the mRNA expression levels at different incubation time points in SiHa cells following incubation with 100 nM of E2 and DHT. **(a)** ER beta, **(b)** Ki67, and **(c)** DDX3.

ulated by hormones at the posttranscriptional level, transient transfection experiments were performed in SiHa cervical cancer cells using three different UTR-reporter constructs. Figure 2a depicts a schematic representation of the different UTR –reporter and fusion constructs (BM1 – BM3) used. Results showed the activity of all the UTRs decreased as compared to the control plasmid. As shown in Figure 2b, a negligible difference in the reporter activity is seen between the UTR regions in the absence of hormone as compared to BM1 (3UTR) as a control. It suggests that neither 3' nor 5'UTR influenced each other in the absence of hormones. To identify putative miRNAs involved in the expression of DDX3, we generated deletion constructs using BM3 (5UTR-LUC-3UTR) as a template, which mimics the endogenous genome architecture of DDX3. As shown in Figure 2c, the left panel, depicts a schematic representation of the different UTR-reporter deletion constructs (BM4 – BM7) used. Our results indicate that BM6 and BM7 enhanced the UTR activity in the presence of both hormones as compared to untreated cells (Control). It suggests that there may be tumor suppressor miRNA binding sites in the 2135 to 4307 bp region compared to BM1-BM5. In addition, the deletion of 5UTR (BM7) does not change the reporter activity as compared to BM6. As a result, under the conditions used in SiHa cells, this region may contain putative miRNA binding sites, which could be used to downregulate the expression of DDX3 in pathological conditions.

Prediction of miRNA

Comparative genome analysis of the full-length DDX3 (X: NG_012830.1) and (Y: NG_012831.1) were aligned using vista (http://genome.lbl.gov/vista/index.shtml). The UTR regions of both X and Y were extracted and aligned using NCBI blast2 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=blast2seq). Results showed that the 3'UTR region is 82% (1866/2269) identical and 6% gaps (156/2269) between DDX3X and DDX3Y (Fig. 3a). Next, the 2234 bp region of the 3'UTR sequence (2073-4307) was analyzed for putative miRNA binding sites using the web interface tools (http://mirdb.org/custom.html and https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php). Based on the comparative genome and reporter deletion analysis, we have selected 5 miRNAs for further studies. Selected miRNAs are as follows: hsa-miR-671-5p, hsa-miR-361-5p, hsa-miR-140-5p, hsa-miR-564, and hsa-miR-769-5p. The target sites of all the selected miRNA are listed in Figure 3b.

Differential Expression of miRNAs as Biomarkers in Cervical Cancer Patient Samples

To identify whether the selected miRNAs can be used as biomarkers for the classification of cervical cancer, we validated

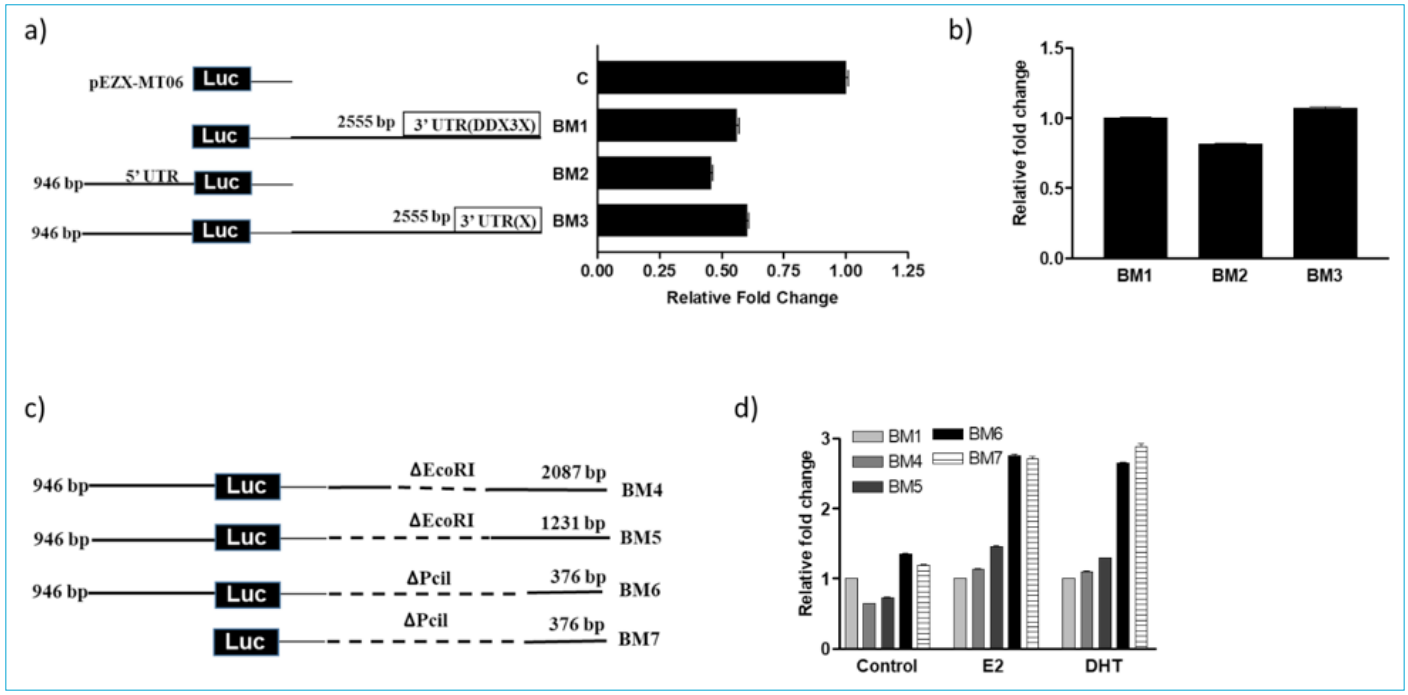
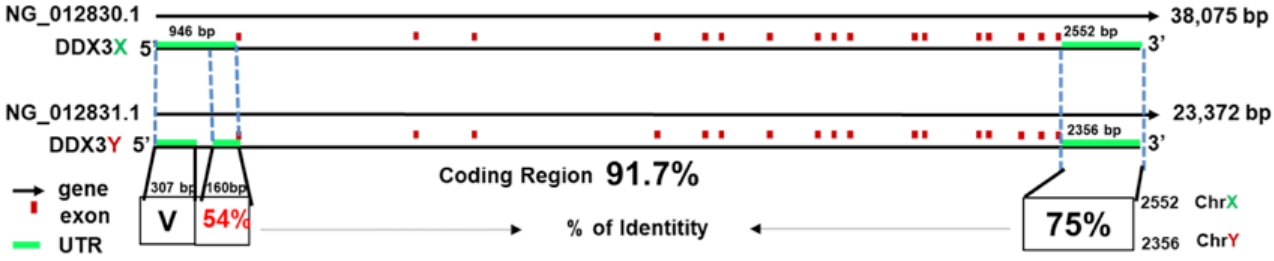


Figure 2. Human DDX3 UTR characterization. **(a)** Linear representation of the DDX3 UTR-reporter constructs and their relative luciferase (Luc) activities in SiHa cells. Luc activities are shown as bar graphs that indicate the fold of Luc activity as compared to control. **(b)** Comparative Luc activity between 5′ and 3′ UTR constructs. **(c)** A linear representation of various deletion constructs and their sizes in base pair. **(d)** Luc activities of UTR deletion constructs with and without E2 and DHT hormones. Error bars (standard deviation) from quadruplicate samples are shown.

a)



b)

miR name	Target site	ΔG Kcal/mol	Duplex Structure	UTR
hsa-miR-671-5p	2143	-14.00	GTCCAGATTCTCAGATGTTTGTT ||||| ::||| |:|| :| GAGGTCGGGGAGGTCCCGAAGGA	3
hsa-miR-361-5p	2063	-11.70	caTGGGGAC—CTCTAAGACTATT | :| || ||:||:|||| taAATCATGCCAAGGTTTTGATAc	3
hsa-miR-140-5p	102	-16.60	TTACCA-GCTGTGATTCTCCACTG :||||| ||:| ||||| GATGGTATCCCATT--TTGGTGAC	3
hsa-miR-140-5p	1865	-9.80	gaTGGTATCC---CA--TTTTGGTGAc |||| | | || |||| |||| tgACCAAAAGACAGTACAAAAACACTg	3
hsa-miR-564	1325	-16.30	GCCACTTTGAATTCTGTGCTA ||| :||| |:||||: CGG-ACGACT-GTGGCACGGA	3
hsa-miR-564	1436	-13.10	GCCACTTTGAATTCTGTGCTA ||| :||| |:||||: CGG-ACGACT-GTGGCACGGA	3
hsa-miR-769-5p	144	-17.00	TTAAGGGAGCTCA-AGGTCACA :||:|:|| ||||| || TCGAGTCTTGGGTCTCCAGAGT	3

Figure 3. Prediction of miRNA binding sites **(a)** Diagram depicting the comparative analysis of the DDX3X and DDX3Y genomes between 5 and 3 UTR regions, a percentage of identity and variable (v) regions are labeled. **(b)** A tabular representation of the selected miRNAs and their duplex formation at the target site in the 3 UTR.

their expression in cervical cancer patients. Real-time PCR results showed that the miRNA levels of hsa-miR-671-5p, hsa-miR-361-5p, hsa-miR-140-5p, hsa-miR-564, and hsa-miR-769 were downregulated in cancer patients (Fig. 4, top panel). Further ROC analysis was carried out to evaluate the differential expression between the controls vs. the patients. The ROC curves were graphically represented by the ΔCT values and the AUC was determined (Fig. 4, bottom panel). The AUC, COP, and Youden index for the upregulated and downregulated markers were listed in Table 3. Relatively, a good pre-

Table 3. Statistical analysis based on the ΔCT values of the control vs. the patient group of miRNAs.

S.No	miRNA	AUC	Y-index	COP (ΔCT)	p	95% Confidence Interval
1	hsa-miR-671-5p	0.825	0.675	9.75	0.057	0.574 to 1.076
2	hsa-miR-361-5p	0.838	0.70	6.566	0.016	0.64 to 1.035
3	hsa-miR-140-5p	0.815	0.611	7.453	0.045	0.572 to 1.058
4	hsa-miR-564	0.852	0.611	8.613	0.006	0.683 to 1.021
5	hsa-miR-769-5p	0.792	0.638	14.71	0.034	0.547 to 1.036

dictive value AUC > 0.75 was obtained in patient samples. The ROC analysis showed that COP was over the ΔCT values for hsa-miR-671-5p with a p-value of 0.057, hsa-miR-361-5p with a p-value of 0.016, hsa-miR-140-5p with a p-value of 0.045, hsa-miR-564 with a p-value of 0.006, and hsa-miR-769 with a p-value of 0.034 are considered tumor suppressor in the test group. Upregulated expression of DDX3 is found in mRNA of the same patients (Fig. 4b). Overall, it suggests that selected miRNAs can be used as biomarkers for the diagnosis of cervical cancer.

Identification of Functional miRNAs in Responsive to Cisplatin

To identify whether selected miRNAs are functionally responsive to cisplatin, which is a commonly used chemotherapy drug against cervical cancer. As a first step, we measured the cytotoxicity of cisplatin in SiHa cells. The IC50 value of Cisplatin for SiHa cells was found to be 89.7μM (Fig. 5a). Following the observation, cells were treated with 89.7μM of cisplatin, and total RNA and miRNA were extracted at 24 hrs. As shown in Figure 5b, Cisplatin significantly

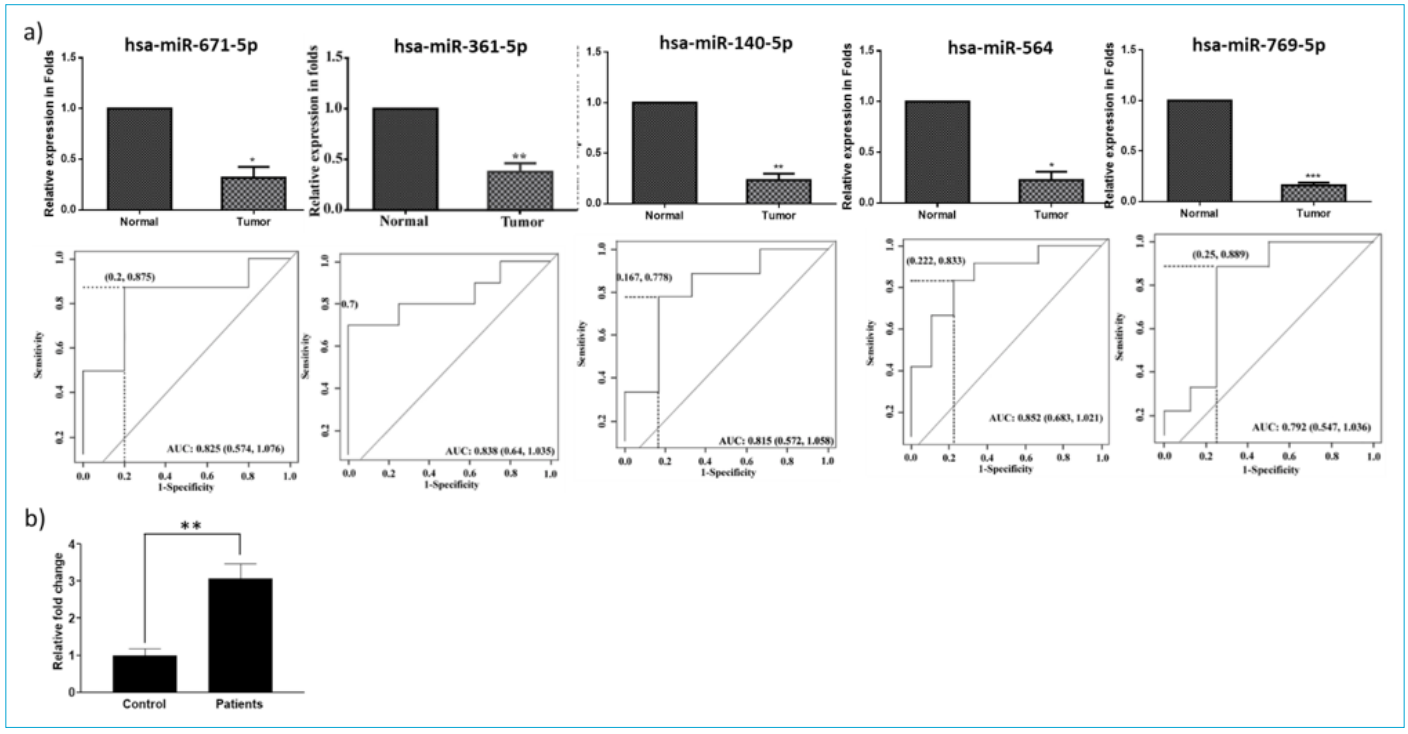


Figure 4. miRNA analysis in cervical cancer patients A. Real-time PCR analysis of the various miRNA expression levels in cervical cancer patients (top panel). Receiver operating curve (ROC) based on the RT-PCR data (bottom panel). The figure e represents a plot of the sensitivity (true positive rate) vs. 1 specificity (false positive rate) for all the CT values. The point on the dotted line shows the highest Youden (Y) indices associated with the COP.

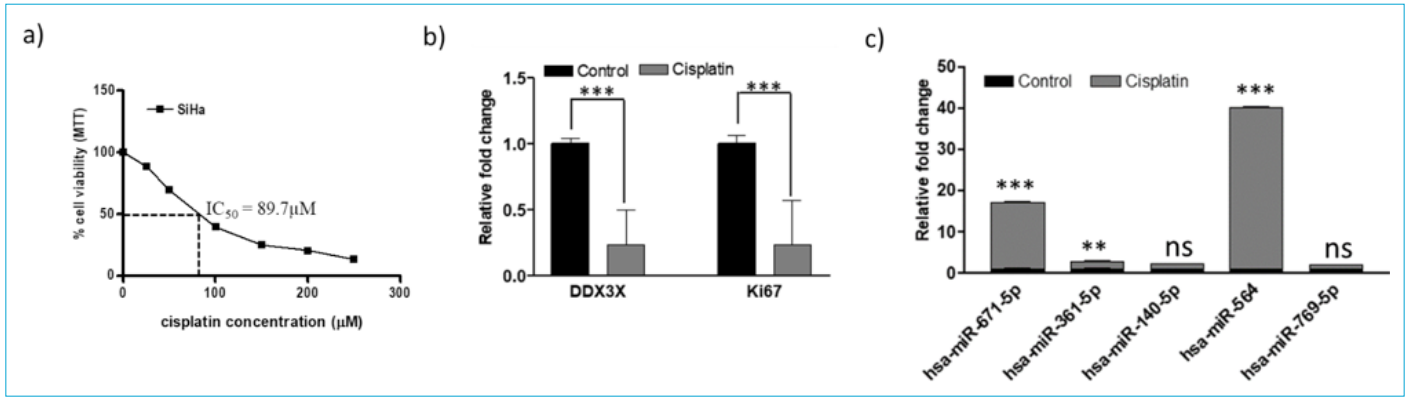


Figure 5. Cisplatin inhibits DDX3 expression and reduces cell viability in cervical cancer cells. **(a)** Cytotoxicity assay of cisplatin on the SiHa cell line at various concentrations. IC50 values for cisplatin are represented as dotted lines. **(b)** Fold changes in DDX3 and Ki67 expression between cisplatin-treated and untreated cells. **(c)** Real-time PCR analysis of miRNAs in drug-treated cells versus control SiHa cells.

reduced the expression of DDX3 as well as Ki67. It suggests that DDX3 is a cisplatin-responsive gene in cervical cancer cells and to check whether identified miRNAs are responsive to cisplatin therapy, we compared the expression profile of miRNA in treated vs. untreated cells. As shown in Fig. 5c, hsa-miR-671-5p, hsa-miR-361-5p, hsa-miR-140-5p, hsa-miR-564, and hsa-miR-769 were upregulated more than fivefold relative to that of untreated cells. Among all, hsa-miR-671 and hsa-miR-564 showed greater than fifteen-fold upregulation relative to control. No significant correlation was detected for miR-140 and miR-769 following treatment with cisplatin. Overall, it suggests that all the selected miRNAs are responsive to cisplatin therapy and these miRNAs can be used as therapeutic targets.

Discussion

The human DDX3 gene is located on the X chromosome and it plays a critical role in cell homeostasis. Deregulation of the DDX3 protein is shown to be associated with many disorders. No studies were carried out to understand the status of DDX3 under the influence of sex steroids. Sex steroids have been shown to participate in the progression of cervical cancer, which is the second most common female-specific cancer after breast cancer.[31] Estradiol (E2) and dihydrotestosterone (DHT) are sex steroids. It is very well-known that E2 is shown to participate in hormone dependent and independent cancers in females. Conversely, androgen excess has also been shown to be associated with cervical cancer in female subjects.[32] Very little is known about the interaction between androgens and estrogens in influencing cervical cancer risk. To understand the role of sex steroids in the pathogenesis of cervical cancer we exposed the SiHa cells to a 100 nm concentration and monitored the expression of ERα and ERβ and AR expression by quantitative real-time PCR. Our data analysis showed that ERβ is upregulated in SiHa cells following treatment with both E2 and DHT, whereas the CT for ERα is found to be greater than 30 cycles. Our data is in accordance with the downregulated expression of ERα in cervical malignant cells.[33] Expression of the AR is not detectable in SiHa cells. In a time course experiment, it is clear that the expression of ERβ is transient, maximum expression is noticed at 30 minutes and gradually declines by the end of 24 hrs. Conversely, DHT enhanced the expression of ERβ in the 24 hrs period. It suggests that upregulated expression of miRNAs is independent of the AR or transduced signal via the estrogen receptor.[34] In line with our findings, miR-130a-3p is shown to contribute to tumor progression by suppressing ERα and AR and serves as a promising candidate target for the treatment of patients with cervical cancer.[35] The role of E2 and DHT in the proliferation of cervical cancer is calculated by measuring the expression of Ki67. Both hormones enhanced the expression of Ki67 in a time-dependent manner. At early exposure (30 min) maximum levels of Ki67 are noted by both the hormones and DHT downregulated its expression. It appears that DHT inhibited the proliferation of cervical cancer cells in the same way that it inhibited the proliferation of Hela Cells.[36] To identify whether proliferation is hormone dependent or independent, we monitored the expression of X chromosome-specific oncogene, DDX3. DHT and E(2) both induced the expression of DDX3 in cervical cancer cells. This phenomenon is similar to the induction of PSA by E2 in prostate cancer cells.[37] Surprisingly, in our study, we noticed that DHT enhanced upregulation of DDX3 over E2 at 30 min. This might be due to the attenuation of estrogenic effects by DHT.[38] Based on our observations, we confirm that E2 and DHT both participate in cancer cell proliferation by enhancing the expression of a well-known oncogene, DDX3.

Comparative genome analysis of the UTR region of DDX3 is performed using bioinformatics approaches. The 3'UTR region was found to be well conserved between the X and Y chromosomes. To identify enhanced expression of DDX3 by hormones at the posttranscriptional level, we performed a luciferase-based reporter assay using a 3.5 kb UTR gene. In our reporter studies with SiHa cells, we observed that the distal region of the UTR (4307 to 4635bp) showed the highest activity in comparison to the proximal region of the UTR. It implies that miRNAs bound at the region ((2072 to 4306 bp) control gene expression and may act as tumor suppressors. This indicates that both E2 and DHT directly or indirectly can induce DDX3 expression via miRNAs located within the 328 bp region (4307 to 4635 bp) of 3'UTR. In search of tumor suppressor miRNAs, we searched potential miRNA binding sites of the 2.2 kb DDX3 3'UTR (2072–4306 bp) using miRDB and miRTarBase. Based on bioinformatics and UTR-based reporter assays, we selected miR-671-5p, miR-361-5p, miR-140-5p, miR-564, and miR-769-5p. Expression status of the selected miRNAs validated in clinical samples of cervical cancer. miRNAs are highly conserved small noncoding RNA molecules, used for the understanding of the pathogenesis of different malignancies. Except, for miR-6753-5p, all other selected miRNAs were downregulated in cervical cancer patient samples. miR-671-5p was found to be a tumor suppressor whose upregulated expression was shown to inhibit epithelial-to-mesenchymal transition by downregulating FOXM1 expression in breast cancer (BC) [39] and inhibit tumor proliferation by blocking the cell cycle in osteosarcoma.[40] Furthermore, miR-671-5p has been proposed as a novel biomarker for early breast cancer detection as well as a therapeutic target for breast cancer management.[41] Moreover, the miR-671-5p/DDX5 axis is

involved in the progression of gastric cancer.[42] It suggests the involvement of this miRNA in regulating DEAD box RNA helicases. Similar to miR-671, hsa-miR-361-5p is also downregulated in multiple cancer types and DDX3 is found to be one of the targets.[43] Hsa-miR-564 and miR-769 suppressed cell proliferation in various cancers. Hsa-miR-140-5p is a widely studied miRNA and its upregulated expression enhances doxorubicin-mediated cell death by targeting Wnti1 mRNA in breast cancer stem cells.[44] To validate that the selected miRNAs have tumor-suppressive properties, we treated SiHa cells with well-known anticancer drug cisplatin. Results suggested cisplatin downregulated the expression of DDX3 and upregulated the expression of miRNAs. It suggests that the selected miRNAs directly or indirectly regulate the expression of DDX3 in SiHa cells. Overall, we conclude that sexual hormones regulate the expression of DDX3 in SiHa cells, and the selected miRNAs may be used as potential therapeutic targets to control the expression of DDX3 in the progression of cervical cancer. Furthermore, establishing links between DHT and cancer risk may inform the development of potential therapeutic options in the future.

Disclosures

Ethics Committee Approval: The study was approved by the Local Ethics Committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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