

Procaine Suppresses the Oncogenic Phenotypes of Ovarian Cancer Cells through modulating microRNA-204

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Liang *et al.*: Action of Procaine on Oncogenic Phenotypes of Ovarian Cancer Cells

To explore the action of procaine on ovarian cancer cell oncogenic phenotypes and its molecular mechanism. Ovarian cancer cells were divided into control group, procaine low, medium and high dose group, microRNA-204 group, microRNA-NC group, high dose group+anti-microRNA-ovarian cancer group, high dose group+anti-microRNA-204 group; 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide, clone formation and transwell assays detected cell proliferation, migration and invasion, Western blotting and quantitative reverse transcription polymerase chain reaction detected protein and ribonucleic acid expression. Procaine treatment dose-dependently impaired the proliferation, migration, invasion and epithelial-mesenchymal transition progression in ovarian cancer cells. Moreover, microRNA-204 levels were observed to be dose-dependently increased by procaine treatment. MicroRNA-204 overexpression suppressed the oncogenic phenotypes mentioned above in ovarian cancer cells. Importantly, the inhibition of microRNA-204 abolished the anticancer effects of procaine on ovarian cancer cells. In conclusion, procaine up-regulated microRNA-204 to ovarian cancer cell oncogenic phenotypes.

Key words: Procaine, microRNA-204, ovarian cancer, tumor, anesthesia

Ovarian Cancer (OC) is a highly invasive malignant tumor occurring in female reproductive system with high invasiveness, the postoperative metastasis and recurrence have been identified to be important causes of death in OC patient^[1]. Anesthetics have been exhibited that can affect tumor growth, metastasis, and patient prognosis, however, whether postoperative recurrence of tumors can be reduced from the perspective of anesthesia management is one of the research hotspots in recent years^[2,3]. Procaine is a local anesthetic, and research has shown that procaine has achieved certain effects in the treatment of various tumors, it can affect tumor progression by modulating tumor suppressor genes that not only serve as diagnostic markers, but also as potential therapeutic targets^[4]. For example, procaine could impair Hepatocellular Carcinoma (HCC) development by attenuating cell Epithelial-Mesenchymal Transition (EMT) process *via* mediating the phosphorylation of c-Met^[5]. Procaine has growth inhibition and apoptosis induction effects on gastric cancer cells^[6]. In addition, procaine restrained the survival and induced

death in colon cancer cells by modulating RhoA expression^[7]. However, the effects and mechanisms of procaine on OC are still unclear. Additionally, recent researches showed that microRNA (miR)-204 showed the anticancer action in OC by suppressing cancer cell oncogenic phenotypes^[8,9]. Through the preliminary experiments, we observed that procaine could elevate miR-204 expression in OC cells. Herein, this work probed the regulatory action of procaine and miR-204 on OC cell proliferation and metastasis, and further investigated whether procaine affect OC progression *via* modulating miR-204.

MATERIALS AND METHODS

Cells and main reagents:

OC cell line SKOV3 (American Type Culture Collection (ATCC)); Roswell Park Memorial

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Institute (RPMI)-1640 medium, 10 % Fetal Bovine Serum (FBS) (Gibco, United States of America (USA)); procaine (Shanghai Xinyu Biotechnology Co., Ltd.); transwell insert, Matrigel gel (Corning, USA); 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl Tetrazolium Bromide (MTT) colorimetric assay (Aimijie Technology Co., Ltd.); Trizol reagent and SYBR Green Taq Mix (Beijing Tiangen Biotechnology Co., Ltd.); Lipofectamine 2000 (Invitrogen, USA); miR-204 mimic (miR-204), inhibitor (anti-miR-204) and the contrasts (miR-NC or anti-miR-NC) (Gima Biol Engineering Inc., Shanghai, China), The antibodies against Matrix Metalloproteinase-2 (MMP-2) (ab37150, 1:500), Ki67 (ab16667, 1:1000), MMP-9 (ab137867, 1:500), N-cadherin (1:1000, ab18203), Vimentin (1:500, ab92547), E-cadherin (1:500, ab40772), β -actin (ab6276, 1:1000) and secondary Horseradish Peroxidase (HRP)-conjugated antibody (ab6721, 1:2000) (Abcam, United Kingdom).

Cell culture and treatment:

SKOV3 cells were cultured in 10 % FBS-contained RPMI-1640 medium and incubated at 37° with 5 % Carbon dioxide (CO₂). For procaine treatment, SKOV3 cells were incubated with completed medium plus 3, 6, and 12 mmol/l procaine. Untreated cells were used as the control.

Cell transfection:

SKOV3 cells were placed onto a 6-well plate (1×10⁵ cells/ml per well). After washing with Opti-MEM I medium without serum, each well was added with 100 nM Opti-MEM containing 0.4 ml miR-204, anti-miR-204 or the contrasts (miR-NC or anti-miR-NC) and 10 μ g/ml Lipofectamine 2000, and incubated with cells for 48 h. Then cells were treated with 12 mmol/l procaine for further analysis.

MTT assay:

SKOV3 cells were reacted with 20 μ l of MTT for 4 h in 96-well plates (5×10³ cells/well). Then each well was incubated with Dimethyl Sulfoxide (DMSO) to dissolved MTT crystals, and spectrophotometric absorbance at 490 nm was examined to assess cell viability.

Colony formation assay:

SKOV3 cells were incubated in 12-well plates at 500 cells per well for (10-14) d. Then colonies

(\geq 50 cells) were visualized and counted with crystal violet staining.

Transwell assay:

For migration analysis, 100 μ l cell suspension (in serum-free medium) were transferred into the upper chamber of transwell inserts. 24 h later, migrating cells in bottom surface was dyed with crystal violet for 30 min, then an invert microscope was used to capture and count cells. For invasion analysis, the upper chamber of Transwell inserts were pre-coated with Matrigel gel and the other steps are the same as described above.

Western blotting:

The total protein of cells in each group was extracted, quantified, and then subjected to polyacrylamide gel electrophoresis (100 V, 2 h), and then transferred to the Polyvinylidene Difluoride (PVDF) membranes, followed by incubating at 4° overnight with primary antibodies, and secondary antibody at 37° for 2 h. The gray value of bands was analyzed.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR):

TRIzol[®] reagent was applied for the isolation of total Ribonucleic Acid (RNAs), which were then reverse-transcribed into complementary Deoxyribonucleic Acid (cDNA). Thereafter, qRT-PCR was performed with primers. miR-204, forward 5'-AACCTGATCCCGTGCTGAGATTG-3', reverse 5'-GCGGATCAAGATTGAGTTCGGTT-3' and U6, forward 5'-CGCTTCGGCAGCACATATACTA-3', reverse 3'-CGCTTCACGAATTTGCGTGTCA-3'. The fold changes were normalized to U6 Threshold Cycle (CT) values adopting the 2^{- $\Delta\Delta$ CT} method.

Statistical analysis:

Data are presented as the $x \pm s$. The t-test and analysis of variance with Least Significant Difference (LSD)-t were used for comparison between two and multiple groups. $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

SKOV3 cell viability and the number of colonies formed by SKOV3 cells, and Ki67 protein levels

in SKOV3 cells were suppressed in low, medium and high procaine treatment groups as shown in fig. 1 and Table 1.

Relative to the NC group, the migratory and invasive SKOV3 cells were markedly reduced in low, medium and high procaine treated SKOV3 cells; moreover, the related markers were measured, the low, medium and high procaine treatment suppressed the expression of MMP2 and MMP9 relative to untreated cells as shown in fig. 2 and Table 2.

The low, medium and high procaine treatment, relative to NC group, notably elevated miR-204 levels in SKOV3 cells as shown in Table 3.

miR-204 mimic introduction markedly elevated miR-204 expression relative to miR-NC as shown in Table 4. Moreover, miR-204 overexpression suppressed the viability and colonies formation in SKOV3 cells, reduced the levels of Ki67, MMP2 and MMP9, as well as the number of migratory and invasive SKOV3 cells as shown in fig. 3 and Table 4.

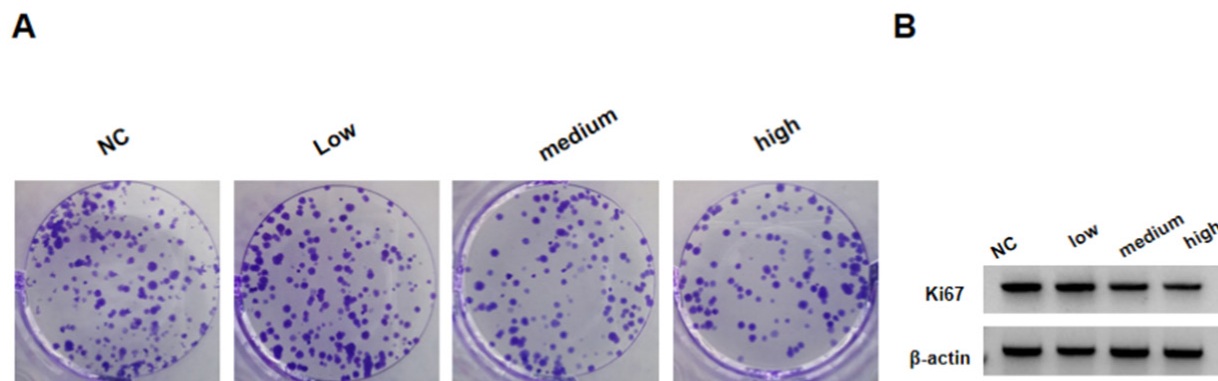


Fig. 1: The effects of procaine on SKOV3 cell proliferation, (A): Colony formation assay and (B): Western blotting analysis for Ki67 protein level

TABLE 1: THE EFFECTS OF PROCAINE ON SKOV3 CELL PROLIFERATION

Group	A value	Colonies	Ki-67
NC	1.055±0.09	128±10.53	0.91±0.09
Low	0.816±0.08*	98±8.46*	0.78±0.07*
Medium	0.643±0.06*	71±6.46*	0.61±0.06*
High	0.517±0.05*	53±5.08*	0.49±0.04*
F	94.881	154.23	67.698
p	0.000	0.000	0.000

Note: *p<0.05 vs. NC group

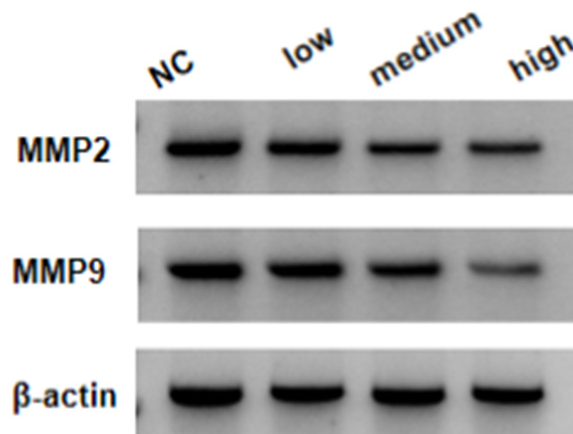


Fig. 2: Western blotting for MMP2 and MMP9 protein levels

TABLE 2: PROCAINE TREATMENT SUPPRESSES SKOV3 MIGRATION AND INVASION

Group	MMP2	MMP9	Migratory cells	Invasive cells
NC	0.88±0.07	0.75±0.07	231±18.91	181±16.03
Low	0.72±0.07*	0.62±0.06*	197±15.18*	144±12.68*
Medium	0.57±0.05*	0.51±0.04*	153±12.18*	105±9.76*
High	0.45±0.04*	0.40±0.03*	119±9.03*	81±6.91*
F	89.871	73.527	106.221	124.179
p	0.000	0.000	0.000	0.000

Note: *p<0.05 vs. NC group

TABLE 3: PROCAINE PROMOTES miR-204 EXPRESSION IN SKOV3 CELLS

Group	miR-204
NC	1.00±0.11
Low	1.57±0.13*
Medium	1.94±0.16*
High	2.31±0.19*
F	123.903
p	0.000

Note: *p<0.05 vs. NC group

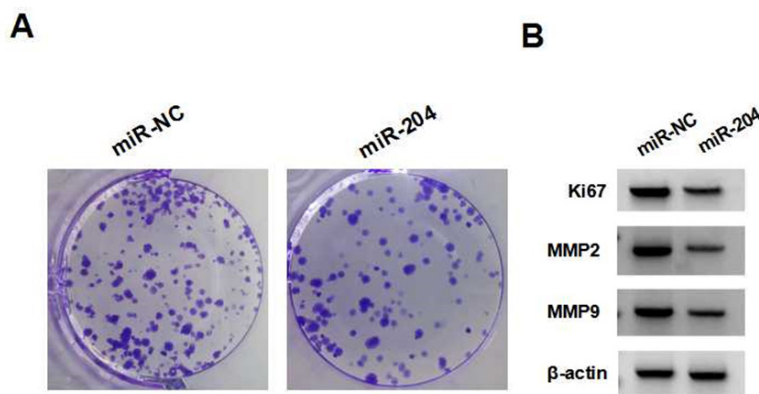


Fig. 3: Effects of miR-204 on SKOV3 cells, (A): Colony formation assay and (B): Western blotting analysis for Ki67 protein level

TABLE 4: EFFECTS OF miR-204 OVEREXPRESSION ON SKOV3 CELLS

Group	miR-204	A value	Colonies	Ki67	MMP2	MMP9	Migratory cells	Invasive cells
miR-NC	1.00±0.10	1.059±0.09	125±9.83	0.93±0.09	0.86±0.07	0.76±0.07	228±19.17	185±14.91
miR-204	3.15±0.25*	0.527±0.05*	62±5.34*	0.46±0.04*	0.41±0.03*	0.35±0.03*	108±8.36*	73±6.19*
t	23.955	15.502	16.895	14.316	17.726	16.151	17.214	20.813
P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05 vs. miR-NC group

SKOV3 cells in high+anti-miR-204 group relative to high+anti-miR-NC group showed decreased miR-204 levels as shown in Table 5. Functionally, high+anti-miR-204 group exhibited increased viability, colonies, protein levels of Ki67, MMP2 and MMP9, as well as the number of migratory and invasive SKOV3 cells relative to high+anti-

miR-204 group (Table 5 and fig. 4).

Relative to NC group, E-cadherin levels were boosted, and N-cadherin and vimentin levels were declined in high procaine-treated SKOV3 cells, while these effects mediated by high procaine were abolished after anti-miR-204 introduction (Table 6 and fig. 5).

TABLE 5: miR-204 KNOCKDOWN REVERSES THE EFFECTS OF PROCAINE ON SKOV3 CELLS

Group	miR-204	A value	Colonies	Ki-67	MMP2	MMP9	Migratory cells	Invasive cells
High+anti-miR-NC	1.00±0.09	0.513±0.05	55±5.27	0.48±0.04	0.44±0.04	0.39±0.03	121±10.19	83±7.68
High+anti-miR-204	0.43±0.03*	1.089±0.09*	119±9.73*	0.97±0.09*	0.84±0.08*	0.71±0.06*	247±21.61*	194±18.04*
t	18.025	16.784	17.351	14.926	13.416	14.311	15.821	16.984
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05 vs. high+anti-miR-NC group

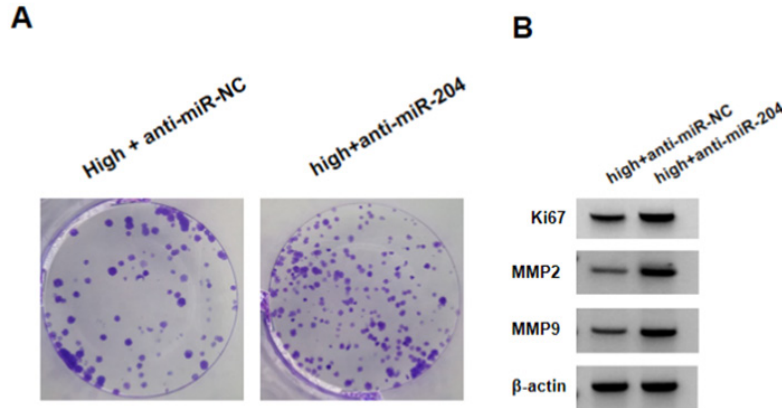


Fig. 4: miR-204 knockdown reverses the effects of procaine on SKOV3 cells, (A): Colony formation assay and (B): Western blotting analysis for Ki67 protein level

TABLE 6: PROCAINE SUPPRESSES EMT PROCESS IN SKOV3 CELLS *via* REGULATING miR-204

Group	E-cadherin	N-cadherin	Vimentin
NC	0.31±0.03	0.82±0.08	0.73±0.08
High	0.69±0.06*	0.43±0.04*	0.35±0.03*
High+anti-miR-NC	0.68±0.07	0.41±0.04	0.36±0.02
High+anti-miR-204	0.33±0.03#	0.85±0.08#	0.76±0.07#
F	155.505	129.656	145.333
p	0.000	0.000	0.000

Note: *p<0.05 vs.NC group, and #p<0.05 vs. high+anti-miR-NC,

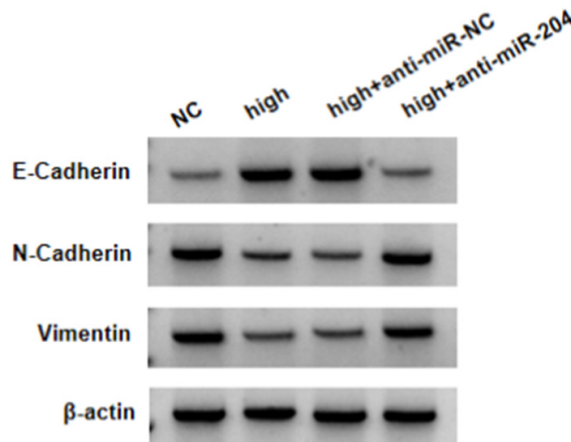


Fig. 5: Western blotting analysis

The high incidence and mortality rate of malignant tumors are major challenges that threaten human life and health. In recent years, the anti-tumor effects and mechanisms of local anesthetics are constantly being revealed^[10,11]. For instance, sevoflurane impeded the growth of OC cells by decreasing the content of stanniocalcin 1^[12]. Sevoflurane hindered the growth *in vivo*, and impaired cell survival and mobility *in vitro* in breast cancer^[13]. Propofol restrained the growth and mobility of colon cancer cells^[14]. Xia *et al.*^[15] showed that lidocaine suppressed Epidermal Growth Factor Receptor (EGFR) expression by elevating miR-520a-3p, and then suppressed the tumorigenesis of retinoblastoma. Procaine is a local anesthetic with less toxicity, and has been reported to possess anticancer activity^[4]. Herein, whether procaine performed antitumor effects in OC was explored. We found that procaine treatment suppressed the viability and colony formation ability, and reduced the invasive and migratory number in OC cells. Ki67 is a nuclear proliferation-associated protein involving in cell cycle progression^[16]. MMP2 and MMP9 are proteolytic enzymes involving in extracellular matrix degradation, the deregulation of them is associated with invasion and migration^[17]. Here, we also observed that procaine treatment declined the Ki67, MMP2 and MMP9 protein levels. The increase of E-cadherin, and decrease of vimentin and N-cadherin are the hallmark of EMT^[18]. Then we further confirmed that procaine treatment repressed EMT progression in OC cells.

It has been identified that anesthetics are able to affect tumor cell biology by a variety of cellular pathways regulated through the modification of miRNAs^[2]. Through the preliminary experiments, we observed that procaine could elevate miR-204 content in OC cells. As a functional miRNA, miR-204 promoted drug sensitivity and hampered metastasis in HCC cells^[19]. It also evoked gastric cancer cell apoptosis and restrained cell migration^[20]. In our study, as previously reported, we also discovered that miR-204 exhibited the anticancer action in OC cells by repressing cancer cell growth, invasion, migration and EMT process. More importantly, we found inhibition of miR-204 could abolish the suppressing activity of procaine on above oncogenic behaviors; implying procaine might modulate OC tumorigenesis *via* miR-204.

In conclusion, this work confirmed that procaine restrained OC cell proliferative, invasive and migratory abilities and EMT process *via* elevating miR-204 expression, suggesting a new therapeutic strategy for OC patients.

Conflict of interests:

The authors declared no conflict of interests.

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