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Dacarbazine inhibits proliferation of melanoma FEMX-1 cells by up-regulating expression of miRNA-200

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Abstract. – OBJECTIVE: Melanoma is a highly aggressive tumour, and treatment efficacy depends on the stage of the tumour. Early stage cutaneous melanoma is efficiently treated by surgical excision. In contrast, late-stage melanoma requires chemotherapy with dacarbazine (DTIC). Unfortunately, advanced melanoma can often be resistant to DTIC. The mechanisms of anti-melanoma effects of DTIC are still poorly understood, which hinders development of more potent therapies.

In this study, we examined the effects of DTIC on growth inhibition of FEMX-1 melanoma cell line, expression of apoptosis-related proteins, and expression of micro (mi)RNA-200 (miRNA-200a, miRNA-200b, miRNA-200c, and miRNA-141).

MATERIALS AND METHODS: DTIC was used at 50 (low dose) or 100 (high dose) mg/ml. Cell growth inhibition was documented by MTT assay. Cell apoptosis was quantified by propidium iodide staining and caspase 3-8 activity assay. Expression of apoptosis-related proteins Bim, Bak, BAX, and Bad were documented by Western blot analysis, while expression of miRNA-200 by PCR.

RESULTS: DTIC dose-dependently inhibited growth of FEMX-1 melanoma cell line, induced cell apoptosis, modulated the levels of apoptosis-related proteins, and up-regulated expression of miRNA-200 family members.

CONCLUSIONS: DTIC inhibits the growth of melanoma cells by up-regulating expression of miRNA-200.

Key Words:
Melanoma, Dacarbazine, microRNA.

Introduction

Melanoma is a malignant tumour whose incidence has increased over the past 30 years. Furthermore, mortality rates due to melanoma rise faster than those of most other types of cancer[1]. This tumour is highly aggressive, and prognosis depends on the developmental stage at the time of the diagnosis[2]. Surgical excision of early stage cutaneous melanoma can produce excellent results, but chemotherapy is required for late-stage melanomas[3]. Dacarbazine (DTIC), an anti-cancer drug used to treat fibrosarcoma[4], Hodgkin's disease[5], and lung cancer[6], is a standard medication for advanced melanoma[7]. DTIC treatment of malignant melanoma was reported to be effective in about 10% of cases[8]. DTIC promotes apoptosis in melanoma cells, and inhibits growth and invasion of melanoma[9,10]. However, advanced melanoma can show resistance to DTIC[11]. Despite some advances, the mechanisms of DTIC suppression of melanoma are still poorly understood. A better understanding of these mechanisms will be helpful to achieve more pronounced therapeutic effects.

MicroRNA (miRNA) are endogenous small (18-25 long nucleotides) non-coding RNA that account for only 1% of the human genome[12]. miRNA appear to regulate expression, modification, transcription and translation of more than a third of human genes[13,14]. The miRNA-200 family, which includes four members (miRNA-200a, 200b, 200c, and miRNA-141), has been intensively studied[15]. Expression of miRNA-200 family members is time and tissue specific, and there is a highly conserved gene clustering phenomenon[16]. The miRNA-200 family is expressed at low levels in liver cancer and renal cell carcinoma[17,18], but is overexpressed in bladder and cervical cancers[19,20]. miRNA-200 expression in melanoma cells regulates morphological plasticity and the mode of melanoma invasion[21,22]. Specifically, miRNA-200c causes cells to exhibit the amoeboid invasion mode[22]. In contrast, upregulation of miRNA-200a changes the cell invasion mode to elongated intrusion mode[22].

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A relationship between expression of the above miRNA and DTIC efficacy was reported. This indicates potential involvement of miRNA-200 family in anti-melanoma effects of DTIC. Specifically, DTIC may suppress melanoma by regulating miRNA-200 levels. In this research, we used FEMX-1 melanoma cell line to examine an association between DTIC effects on melanoma and miRNA-200 family.

Materials and Methods

Cell Culture

The FEMX-1 cell line was kindly provided by the Third Military Medical University (Chongqing, China). FEMX-1 cells were maintained in RPMI 1640 medium (Life Technologies, San Francisco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 2 mM L-glutamine (Life Technologies) at 37º C / 5% CO_2 in a humidified atmosphere. The cells were routinely checked for mycoplasma contamination.

Experimental Exposure to DTIC

FEMX-1 cells were exposed to three different concentrations of DTIC (0, 50 or 100 mg/ml). These experimental conditions are respectively defined as control, low-dose DTIC, and high-dose DTIC.

Apoptosis Quantification with Propidium Iodide

Cells stained with propidium iodide (PI) were analyzed by flow cytometry. Briefly, cells were seeded onto 6-well plates at a concentration of 2 × 10^5 cells/ml and stained with the PI apoptosis detection kit (Kaiji Co, Nanjing, China) according to the manufacturer's instructions. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 hour after staining.

MTT Assay

FEMX-1 cells were seeded onto 96-well tissue culture plates at a density of 1 × 10^4 cells per well. Cell proliferation was quantified by MTT assay after 72 hours of treatment. Absorbance was read using an ELx800 Absorbance Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) at 570 nm. Proliferation of DTIC-treated cells was calculated as percentage of control cells.

Caspase Activity Assay

The activity of caspase 3/8 in FEMX-1 cells was measured using Caspase-Glo 3/8 assay from Promega (Madison, WI, USA). Cells were seeded onto 96-well white plates with optical bottom (Nunc, Roskilde, Denmark) at a concentration of 5,000 cells per well and treated as described above. Caspase activity was measured after 48 hours of treatment in accordance with the manufacturer's instructions. Assays were performed in triplicate and repeated at least three times.

Western Blot Analysis of Apoptotic Proteins

Western blot assay was performed to determine protein expression levels of Bim, Bak, BAX, and Bad. Cells were obtained as above, centrifuged in 15 ml of 18% (w/v) dextran solution (10,000 g, 4° C, 10 min), and were immediately frozen and stored in liquid nitrogen. Cells were then homogenized in 10 volumes of lysis buffer (2 mM EDTA, 10 mM EGTA, 0.4% NaF, 20 mM Tris-HCl, 1% NP-40, 1% Triton X-100, protease inhibitors, pH 7.5). The lysates were subsequently centrifuged for 1 hour at 17,000 g and 4° C, and protein concentrations in supernatants were determined using Coomassie G250 binding assay (Shfeng Biological Technology, Shanghai, China). Equal amounts of proteins (10-20 mg) were separated on 15% SDS-PAGE, followed by transfer to nitrocellulose membranes (Abcam, Cambridge, UK). The membranes were blocked with blocking buffer (5% nonfat dairy milk dissolved in Tween-TBS) overnight at 4° C. The blots were then incubated with rabbit polyclonal Bim antibody (1:800 dilution; Santa Cruz Biotechnology, TX, USA), rabbit polyclonal Bak antibody (1:800; Santa Cruz Biotechnology), rabbit polyclonal BAX antibody (1:400; Abcam, Cambridge, MA, USA), goat polyclonal Bad antibody (1:400; Santa Cruz Biotechnology), or mouse polyclonal anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology). Expression of β-actin was used as a gel-loading control. The HRP-conjugated goat anti-mouse IgG (1:500; Abcam) was used as a secondary antibody. The proteins were visualized by enhanced chemiluminescence kit (Abcam). The membranes were scanned using Chemi Imager 5500 V2.03 (Alpha Innotech, San Leandro, CA, USA). The relative integrated density values were calcu-

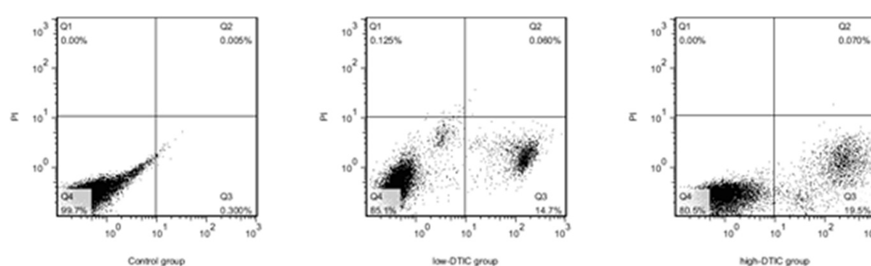

Figure 1. Apoptosis in FEMX-1 cells exposed to DTIC. Cells were exposed to 0 (control), 50 (low does) or 100 (high dose) mg/ml DTIC, and stained with propidium iodide (PI). Apoptosis rates were higher in cells exposed to low or high dose DTIC. Shown are representative flow cytometry images.

lated using an image analysis software Fluor Chen 2.0 (Fluor Corporation Irving, TX, USA) and normalized to expression of β-actin.

Expression of miRNA-200

Total RNA extraction and reverse transcription were performed as previously reported[22] using cells treated for 24, 48, or 72 hours. PCR reaction was as follows: 15-sec denaturation step at 95° C and 1-min annealing step at 60°C, repeated for 45 cycles. PCR products were separated on a 2.5% agarose gel and visualized with ethidium bromide. The gels were imaged using a FluorImager 595 (LI-COR Biosciences, Lincoln, NE, USA). Specificity of amplified product was identified by the dideoxy sequencing methods using Thermo Sequenase Cycle Sequencing Kit, with a DSQ-1000L sequencer (Shimazu Co., Shiga, Japan). Primer sequences were as follows: miR-200a reverse primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATCGT and forward primer GGCCCGTAACACTGTCTGGTAA, miR-200b reverse primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCAT and forward primer GCCGCTTTAATACTGCCTGGTA, miR-200c reverse primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCATCA and forward primer GCCGATTTAATACTGCCGGGT, and miR-141 reverse primer GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCATCTT and forward primer GCCGCTAACACTGTCTGGTAAA.

Statistical Analysis

The SPSS 16.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were presented as mean ± SD. Differences were tested for significance using ANOVA and Dunnett's post-hoc tests. Differences at a p value of < 0.05 were considered statistically significant.

Results

Growth of Melanoma FEMX-1 Cells is Inhibited After Exposure to DTIC

PI-stained cells were analyzed by flow cytometry to test whether exposure to DTIC inhibits cell growth and induces apoptosis. As shown in Figure 1, apoptosis rates were very low in control cells (0.3 ± 0.02%). In contrast, apoptosis rates of melanoma FEMX-1 cells in cells exposed to low or high-dose DTIC were markedly increased (respectively, 14.7 ± 0.4% and 19.5 ± 0.8 %). Cells exposed to both DTIC conditions showed significantly ($p < 0.05$) higher rates of apoptosis; furthermore, there was also a statistically significant difference ($p < 0.05$) in apoptosis rates between cells treated with low vs. high doses of DTIC.

The MTT test showed decreased viability of melanoma FEMX-1 cells exposed to DTIC (Figure 2). Specifically, we observed low cytotoxicity in control cells, and marked and dose-dependent growth inhibition by DTIC (Figure 2).

DTIC Promotes Caspase-Dependent Apoptosis in Melanoma FEMX-1 Cells

Subsequently, we tested for caspase activation in cells treated with DTIC (Figure 3). We observed that DTIC dose-dependently induced activation of both caspase 3 and 8 (Figure 3).

Expression of Apoptosis-related Proteins After Treatment with DTIC

To further confirm cell apoptosis, we examined the expression of pro-apoptotic proteins Bak, Bax and Bad, as well as cleavage of Bid to BimEL, BimL, and BimS (Figure 4). Control cells showed low levels of Bid cleavage. In contrast, Bid cleavage was enhanced by DTIC (Figure 4). Similarly, expression of other tested apoptosis-related proteins was markedly altered by DTIC (Figure 4).

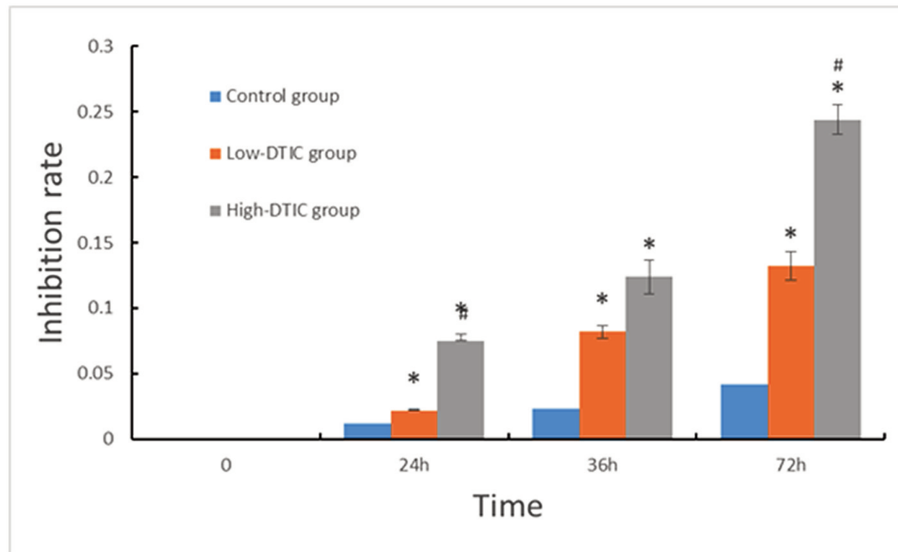

Figure 2. Inhibition of FEMX-1 cells by DTIC treatment. Cells were exposed to 0 (control), 50 (low dose), or 100 (high dose) mg/ml DTIC for 36 hours. Then, cells were analyzed by MTT test. Data are mean ± SD of 10 experiments. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. low dose DTIC.

Up-regulated Expression miRNA-200 Family is Associated with DTIC Treatment

Expression of miRNA-200 family (miRNA-200a, miRNA-200b, miRNA-200c, and miRNA-141) was tested in control cells or cells exposed to DTIC (respectively, 0, 50, or 100 mg/ml; Figure 5). All of the tested miRNA-200 family members were significantly up-regulated by DTIC ($p < 0.05$ vs. control cells; Figure 5).

Discussion

In this report, we tested whether DTIC was effective in inhibiting melanoma FEMX-1 cells. Our findings showed that both low dose of 50 mg/ml and high dose of 100 mg/ml of DTIC suppress the growth of melanoma FEMX-1 cells. The effects of DTIC were dose-dependent. Moreover, high dose DTIC up-regulated expression of pro-apoptotic proteins and activated caspases in melanoma FEMX-1 cells. In addition, expression of the members of the miRNA-200 family was also up-regulated by treatment with DTIC.

Melanoma is a tumour originating from melanin cells of neural ectoderm[23]. The treatment of melanoma is challenging because of aggressive early metastasization[24]. Lesion resection is a very effective intervention but is only applicable in early stages of the disease[25]. DTIC has long been used to treat metastatic melanoma and is still the first line treatment for late-stage malignant melanomas. DTIC can be used as a single agent or in combination with other drugs[7,9,24]. The mechanisms of anti-melanoma effects of DTIC are still not fully understood. In our work, DTIC showed a considerable and dose-depen-

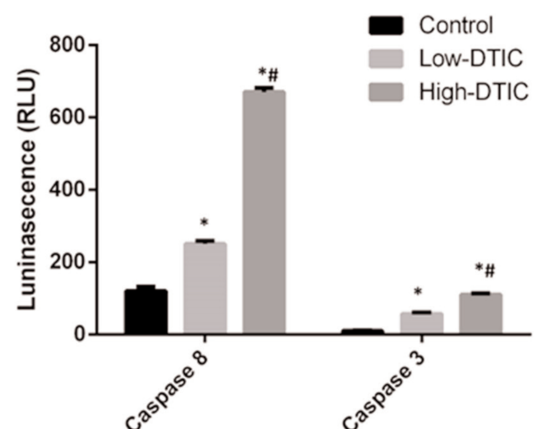

Figure 3. Caspase activation in FEMX-1 cells after DTIC treatment. Cells were exposed to 0 (control), 50 (low dose), or 100 mg/ml (high dose) DTIC for 36 hours. The caspase-Glo 3/8 assay from Promega was used to quantify caspase activity. Data are mean ± SD of 3 experiments. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. low dose DTIC.

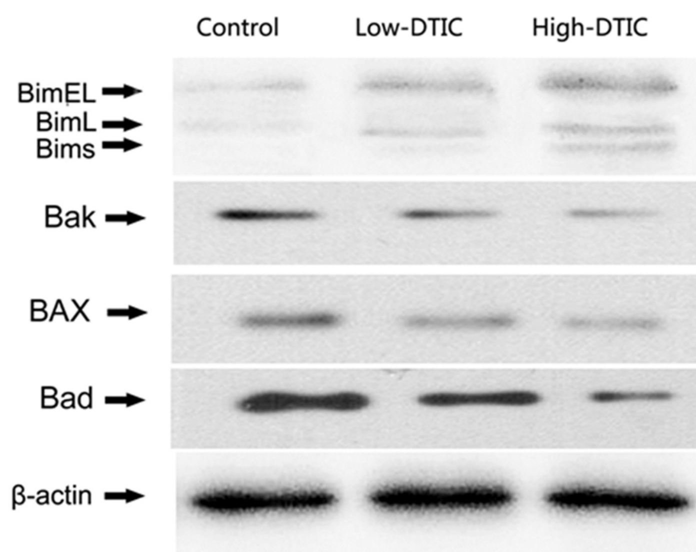

Figure 4. Western blot analysis of expression of apoptosis-regulating proteins in FEMX-1 cells after DTIC treatment. Cells were exposed to 0 (control), 50 (low dose), or 100 mg/ml (high dose) DTIC for 36 hours. Expressions of Bak, Bax, Bad, BimEL, BimL, and BimS were normalized to expression of β-actin (gel-loading control).

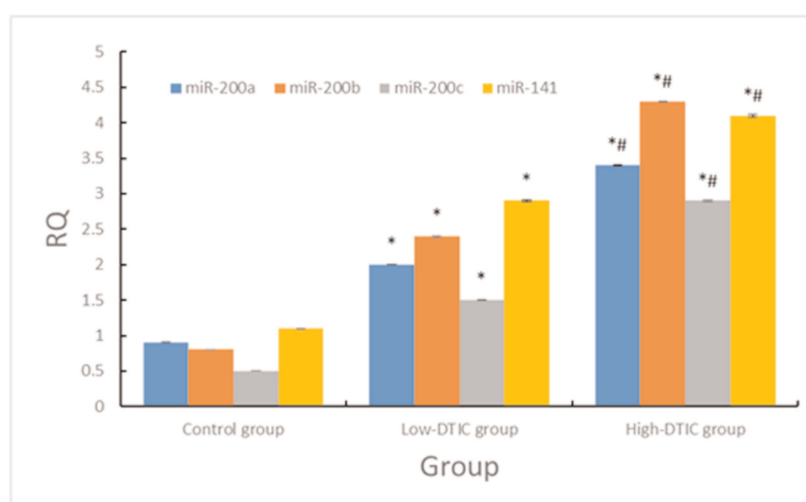

Figure 5. Expression of miRNA-200a, miRNA-200b, miRNA-200c, and miRNA-141 in FEMX-1 cells after DTIC treatment. Cells were exposed to 0 (control), 50 (low dose), or 100 mg/ml (high dose) DTIC for 36 hours. Data are mean ± SEM of 12 experiments. $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$ vs. low dose DTIC.

dent antitumor effect in melanoma FEMX-1 cells, which involved induction of apoptosis.

miRNA are non-coding RNA and are important regulators of gene expression[26]. It was reported that miRNA-200 family is involved in suppressing invasion and metastasis of melanoma cells[22]. Moreover, expression of miRNA-200 levels is associated with DTIC treatment in patients with melanoma. In our study, we documented that expression of miRNA-200 family members is up-regulated by DTIC in a dose-dependent manner. Thus, up-regulation of miRNA-200 expression may represent a pathway through which DTIC induces apoptosis in melanoma FEMX-1 cells. At present, such therapies may be too expensive, but future drugs may more closely utilize epigenetic regulation of cancer cell apoptosis for melanoma treatment.

Conclusions

Treatment with DTIC inhibits the growth of melanoma FEMX-1 cells, possibly by up-regulating expression of miRNA-200.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Zhu F, Liang Y, Chen D, et al. Melanoma Antigen Gene Family in the Cancer Immunotherapy. CancerTranslational Medicine 2016; 2: 85.
2) Schreiber MM, Bozzo PD, Moon TE. Malignant melanoma in southern Arizona. Increasing incidence and sunlight as an etiologic factor. Arch Dermatol 1981; 117: 6-11.
3) Agredano YZ, Chan JL, Kimball RC, Kimball AB. Accessibility to air travel correlates strongly with increasing melanoma incidence. Melanoma Res 2006; 16: 77-81.
4) Halperin DM, Phan AT, Hoff AO, Aaron M, Yao JC, Hoff PM. A phase I study of imatinib, dacarbazine, and capecitabine in advanced endocrine cancers. BMC Cancer 2014; 14: 561.
5) Law MF, Ng TY, Chan HN, Lai HK, Ha CY, Leung C, Ng C, Yeung YM, Yip SF. Clinical features and treatment outcomes of Hodgkin's lymphoma in Hong Kong Chinese. Arch Med Sci 2014; 10: 498-504.
6) Hassler MR, Pfeifer W, Knocke-Abulesz TH, Geissler K, Altorjai G, Dieckmann K, Marosi C. Temozolomide added to whole brain radiotherapy in patients with multiple brain metastases of non-small-cell lung cancer: a multicentric Austrian phase II study. Wien Klin Wochenschr 2013; 125: 481-486.
7) Kim G, McKee AE, Ning YM, Hazarika M, Theoret M, Johnson JR, Xu QC, Tang S, Sridhara R, Jiang X, He K, Roscoe D, McGuinn WD, Helms WS, Russell AM, Pope Miksinski S, Fourie Zirkelbach J, Earp J, Liu Q, Ibrahim A, Justice R, Pazdur R. FDA approval Summary: vemurafenib for treatment of unresectable or metastatic melanoma with the BRAF V600E mutation. Clin Cancer Res 2014; 20: 4994-5000.
8) Miller DM, Flaherty KT, Tsao H. Current status and future directions of molecularly targeted therapies and immunotherapies for melanoma. Semin Cutan Med Surg 2014; 33: 60-67.
9) Liu JD, Chen SH, Lin CL, Tsai SH, Liang YC. Inhibition of melanoma growth and metastasis by combination with (-)-epigallocatechin-3-gallate and dacarbazine in mice. J Cell Biochem 2001; 83: 631-642.
10) Engesaeter B, Engebraaten O, Florenes VA, Maelandsmo GM. Dacarbazine and the agonistic TRAIL receptor-2 antibody lexatumumab induce synergistic anticancer effects in melanoma. PLoS One 2012; 7: e45492.
11) Azimi A, Pernemalm M, Frostvik Stolt M, Hansson J, Lehtio J, Egyhazi Brage S, Hertzman Johansson C. Proteomics analysis of melanoma metastases: association between S100A13 expression and chemotherapy resistance. Br J Cancer 2014; 110: 2489-2495.
12) Liu H, Zhang S, Lin H, Jia R, Chen Z. Identification of microRNA-RNA interactions using tethered RNAs and streptavidin aptamers. Biochem Biophys Res Commun 2012; 422: 405-410.
13) Moore KB, Vetter ML. MicroRNA maintenance of cone outer segments. Neuron 2014; 83: 510-512.
14) Oom AL, Humphries BA, Yang C. MicroRNAs: novel players in cancer diagnosis and therapies. Biomed Res Int 2014; 2014: 959461.
15) Bracken CP, Li X, Wright JA, Lawrence D, Pillman KA, Salmanidis M, Anderson MA, Dredge BK, Gregory PA, Tsykin A, Neilsen C, Thomson DW, Bert AG, Leerberg JM, Yap AS, Jensen KB, Khew-Goodall Y, Goodall GJ. Genome-wide identification of miR-200 targets reveals a regulatory network controlling cell invasion. EMBO J 2014; 33: 2040-2056.
16) Howell PM, Jr., Liu S, Ren S, Behlen C, Fodstad O, Riker AI. Epigenetics in human melanoma. Cancer Control 2009; 16: 200-218.
17) Kulda V, Pesta M, Topolcan O, Liska V, Treska V, Sutnar A, Rupert K, Ludvikova M, Babuska V, Holubec L, Jr., Cerny R. Relevance of miR-21 and miR-143 expression in tissue samples of colorectal carcinoma and its liver metastases. Cancer Genet Cytogenet 2010; 200: 154-160.
18) Castro-Vega LJ, Jouravleva K, Liu WY, Martinez C, Gestraud P, Hupe P, Servant N, Albaud B, Gentien D, Gad S, Richard S, Bacchetti S, Londono-Vallejo A. Telomere crisis in kidney epithelial cells promotes the acquisition of a microRNA signature retrieved in aggressive renal cell carcinomas. Carcinogenesis 2013; 34: 1173-1180.
19) Wang G, Chan ES, Kwan BC, Li PK, Yip SK, Szeto CC, Ng CF. Expression of microRNAs in the urine of patients with bladder cancer. Clin Genitourin Cancer 2012; 10: 106-113.
20) Yu J, Wang Y, Dong R, Huang X, Ding S, Qiu H. Circulating microRNA-218 was reduced in cervical cancer and correlated with tumor invasion. J Cancer Res Clin Oncol 2012; 138: 671-674.
21) van Kempen LC, van den Hurk K, Lazar V, Michiels S, Winnepenninckx V, Stas M, Spatz A, van den Oord JJ. Loss of microRNA-200a and c, and microRNA-203 expression at the invasive front of primary cutaneous melanoma is associated with increased thickness and disease progression. Virchows Arch 2012; 461: 441-448.

22) Elson-Schwab I, Lorentzen A, Marshall CJ. MicroRNA-200 family members differentially regulate morphological plasticity and mode of melanoma cell invasion. PLoS One 2010; 5: pii: e13176.

23) Chinembiri TN, du Plessis LH, Gerber M, Hamman JH, du Plessis J. Review of natural compounds for potential skin cancer treatment. Molecules 2014; 19: 11679-11721.

24) Sayanjali B. Genome-wide transcriptome analysis of prostate cancer tissue identified overexpression of specific members of the human endogenous retrovirus-K family[J]. Cancer Translational Medicine 2017; 3: 1.

25) Ren YQ, Li QH, Liu LB. USF1 prompt melanoma through upregulating TGF-β signaling pathway. Eur Rev Med Pharmacol Sci 2016; 20: 3592.

26) Ruggieri M, Scocchera F, Genderini M, Mascaro A, Luongo B, Paolini A. Hyperthyroidism and concurrent thyroid carcinoma. Eur Rev Med Pharmacol Sci 1999; 3: 265-268.