

Ouabain Mediates Integrin Switch in Human Lung Cancer Cells

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Abstract. *Background:* Physiological effects of ouabain, an endogenous human hormone, are being intensively investigated. However, its role in regulation of integrin pattern in lung cancer is largely unknown. The switching in the expression pattern of integrins is recognized as an important factor facilitating metastasis of several cancers. *Materials and Methods:* Cytotoxicity and proliferative effects of ouabain on H460 lung cancer cells were evaluated by the MTT assay. The levels of integrin proteins in response to ouabain were determined by western blotting. Anchorage-independent growth and migration behaviors were performed by the wound healing assay and colony formation assay, respectively. *Results:* Herein, the results suggested that exposure of the lung cancer cells to physiological concentrations of ouabain significantly altered the level of integrins. Ouabain suppressed integrin α_4 , α_5 , α_v , β_3 and β_4 , whereas it had no significant effect on integrin β_1 and β_4 . According to the switch patterns of integrins, ouabain treatment resulted in a dramatic reduction of cell colony size and inhibition of cancer cell migration. However, ouabain-induced integrin switch had only a slight effect on chemotherapeutic drug susceptibility. *Conclusion:* Ouabain may have a role in suppressing cancer metastasis via integrin regulation.

Ouabain, a human endogenous hormone, has been found in plasma and tissue at concentrations ranging from 2 to 770 pM (1-3). The levels of ouabain in plasma was found to be up-regulated during exercise and pathological states,

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DMSO, dimethyl sulfoxide; OB, ouabain.

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including hypertension, myocardial infarction, heart and renal failure (4). Although the functions of this biological compound is largely unknown, certain investigations have pointed out that it may play a critical role for human body in regulating cardiac functions. Recently, studies have shown that ouabain possesses anti-tumor activities (5, 6). Our previous study showed that at physiological-related concentrations, ouabain could sensitize lung cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis (7). In addition, ouabain was shown to enhance lung cancer cell detachment (8).

Nowadays, attempts have been made to understand the molecular basis, which regulates cancer cell metastasis. Lung cancer has become the leading cause of cancer-related deaths worldwide and the death of lung cancer patients is tightly related to metastasis (9). Indeed, cancer metastasis is a complicated multistep process beginning with cancer cells detachment from the extracellular matrix (ECM), migration, invasion and extravasation to the circulation (10, 11). When the cancer cells reach secondary sites, they adhere, intravasate and form new tumors (11).

Interestingly, numerous studies reported that the metastatic processes of cancer cells are regulated by a large family of adhesion molecules termed integrins (12, 13). Integrins are heterodimeric transmembrane proteins consisting of 8α and 18β subunits. In general, integrins function as an important cell-cell and cell-extracellular matrix (ECM) interacting molecules, and such interactions contribute to the activation of intracellular signals (12, 13). Of note, studies have reported that an increase in migratory and invasive behavior of metastatic cancer cells is associated with the expression of integrin α_4 , α_5 , α_v , β_1 , and β_5 (14-21). Overexpression of integrin β_1 was found to enhance chemotherapy resistance of various cancer cells (22-24). Moreover, the depletion of β_3 , β_4 and β_5 integrins has been shown to reduce tumor growth and angiogenesis (12, 21, 25). Pathological studies of human cancers have provided evidence indicating that tumor cells switch their integrin types along with the progression of tumor (12-13, 26); switches in integrin expression frequently

enhance the aggressive behavior of cancer cells (12-13, 27-29). Since there is limited information regarding the role of ouabain on the expression pattern of integrins, as such knowledge could help fulfill the understanding of tumor cell biology, the present study aimed to investigate the effects of ouabain on integrin switch and related aggressive behavior in human lung cancer cells.

Materials and Methods

Cells and reagents. Human lung cancer epithelial H460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). H460 cells were cultured in RPMI 1640 medium in a 5% CO₂ environment at 37°C. The media was supplemented with 2 mmol/l L-glutamine, 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin (Gibco, Gaithersburg, MA, USA). Ouabain, cisplatin, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 nucleic acid stain were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Doxorubicin, paclitaxel and etoposide were obtained from Calbiochem (San Diego, CA, USA). Antibodies for integrin α_4 , integrin α_5 , integrin α_v , integrin β_1 , integrin β_3 , integrin β_4 , integrin β_5 , β -actin and peroxidase-labeled secondary antibodies were obtained from Cell Signaling Technology, Inc. (Denver, MA, USA).

Cytotoxicity assay. Cell viability was determined by the MTT colorimetric assay. Briefly, cells in a 96-well plate were incubated with 500 μ g/ml of MTT for 4 h at 37°C. The supernatant was then removed and DMSO was added to dissolve the formazan product. The intensity was spectrophotometrically measured at 570 nm using an ELISA reader (Anthros, Durham, NC, USA). All analyses were performed in at least three independent replicate cultures. The optical density ratio of treated to non-treated control cells was calculated and presented in terms of relative cell viability.

Apoptosis assay. Apoptotic cell death was detected by Hoechst 33342 staining. After specific treatments, cells were stained with 10 μ M of the Hoechst 33342 for 30 min at 37°C. The apoptotic cells having condensed chromatin and/or fragmented nuclei stained by Hoechst 33342 were visualized and scored under a fluorescence microscope (Olympus IX51 with a DP70 digital camera system, Tokyo, Japan).

Western blot analysis. After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) for 40 min on ice. The cell lysates were collected and the protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins from each sample (80 μ g) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and subsequently loaded onto a 10% SDS-PAGE. After separation, proteins were transferred onto 0.45 μ M nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl pH 7.5, 125 mM NaCl, and 0.05% Tween 20) and incubated with the appropriate primary antibodies at 4°C overnight. Then, the

membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-labeled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by enhancement with chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad).

Colony formation assay. The anchorage-independent growth assay was performed in two-layer soft agar. Briefly, aliquots of melted 1% agarose (1 g of agarose in 100 ml of sterile water) were cooled in 55°C water bath before use. To make the lower layer, an equal volume of melted 1% agarose (55°C) and complete RPMI medium (1:1) were mixed to give 0.5% agarose and 0.5% complete RPMI medium. Then, the mixture was put in 24-well plate at 500 μ l/well and allowed to solidify at 4°C for 5 min. To prepare the upper layer (0.33% agarose), melted 1% agarose (55°C) was mixed with complete RPMI medium containing trypsinized adherent cells at a density of 1×10^3 cells/well under various concentrations. Subsequently, it was added to the lower layer at 250 μ l/well and allowed to gel at room temperature before placing in an incubator at 37°C for 10 days. Fresh complete RPMI medium (250 μ l/well) was added every 3 days over the upper layer. After 10 days of growth, colonies were photographed in order to count the colonies and the colony size was measured.

Migration assay. Migration was determined by wound healing assays. For the wound healing assay, a monolayer of cells was cultured in a 96-well plate and a wound space was made with a 1-mm-wide tip. After rinsing with PBS, the cell monolayers were incubated with the indicated treatments and allowed migration for 24 h. Microphotographs were taken under a phase-contrast microscope (Olympus DP70, Melville, NY, USA) and the wound spaces were measured using the Olympus DP controller software. Quantitative analysis of cell migration was performed using the average wound space from those random fields of view, and the percentage of change in the wound space was calculated using the following formula: % change = (average space at time 0 h) - (average space at time 24 h) / (average space at time 0 h) \times 100. The relative cell migration was calculated by dividing the percentage of change in the wound space of treated cells by that of the control cells in each experiment.

Statistical analysis. All data were expressed as means \pm standard error of the mean (S.E.M.) from three or more independent experiments. Multiple comparisons were examined for significant differences of multiple groups, using analysis of variance (ANOVA), followed by individual comparisons with the Scheffe's *post-hoc* test. Statistical significance was set at $p < 0.05$.

Results

Effects of ouabain on viability of human lung cancer H460 cells. The plasma concentrations of ouabain and ouabain-like substances in humans are ranging from 2 to 770 pM (1-3). We first evaluated for cytotoxicity of such concentrations of ouabain in human lung cancer H460 cells. Cells were cultured in the presence of ouabain (0-40 pM) for 24 h and cell viability and proliferation were determined by the MTT assay at 24 h and 72 h, respectively. The results showed that

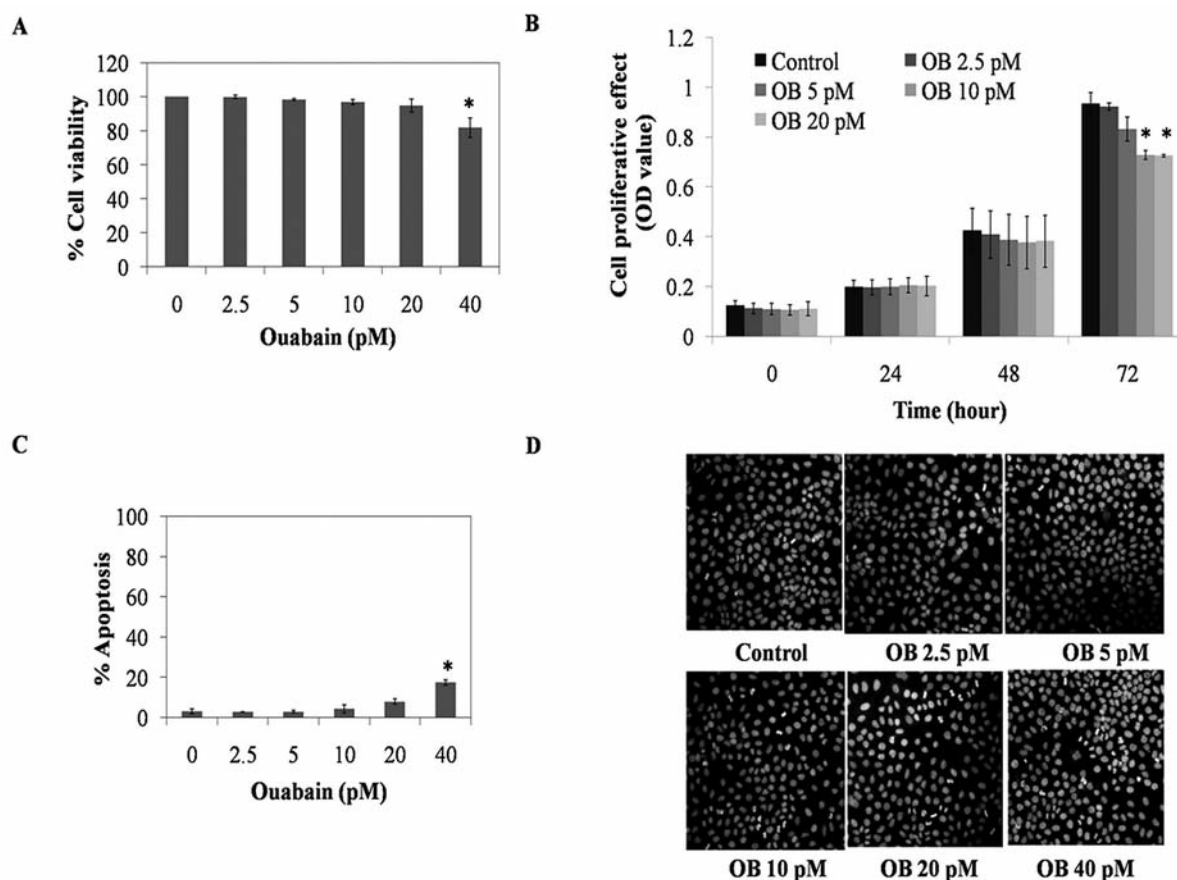


Figure 1. The cytotoxic effects of ouabain on lung cancer H460 cells. Cells are treated with various concentrations of ouabain (2.5-40 pM) for 24 h. (A) Percentage of cell viability determined by the MTT assay. Values are means of 3 independent triplicate experiments \pm SEM. * p <0.05 versus non-treated control. (B) Proliferative effect of ouabain on H460 cells. Cells were treated with ouabain (2.5-20 pM) for 0-72 h and analyzed by MTT assay. (C) Apoptotic cells were detected by Hoechst33342 staining. Values are means of 3 independent triplicate experiments \pm SEM. * p <0.05 versus non-treated control. (D) Nuclear morphology of the cells stained with Hoechst 33342 and visualized under a fluorescence microscope.

treatment of the cells with ouabain at concentrations ranging from 2.5 to 20 pM caused neither cytotoxicity nor proliferative effects on the H460 cells (Figure 1A). A significant decrease in cell viability was detected in the 40 pM ouabain-treated cells with approximately 82% of the cells remaining viable. Also, the proliferative effect of ouabain was investigated by culturing the cells in ouabain-containing media for 0-72 h. The results of the proliferation assay indicated that 10 and 20 pM of ouabain significantly inhibited cell proliferation at 72 h (Figure 1B). To confirm the above cytotoxic results, apoptosis evaluation using Hoechst33342 staining was performed; cells were exposed to ouabain and apoptosis and necrosis were determined. Figure 1C and D show that apoptotic cells containing condensed or fragmented nucleus were not detectable in response to 2.5-20 pM ouabain. Taken together, the non-cytotoxic concentrations of ouabain (2.5-20 pM) will be used for further experiments.

Ouabain induces integrin switch in human lung cancer H460 cells. As an integrin switch was shown to enhance ability of cancer cells to metastasis (12), we therefore investigated whether treatment of the cells with ouabain could alter the pattern of integrin expression. The cells were treated with ouabain at concentrations of 0-20 pM for 24 h and the expression levels of integrin α_4 , α_5 , α_v , β_1 , β_3 , β_4 and β_5 were evaluated by western blot analysis. Figure 2A and C show that ouabain caused the reduction of integrin α_4 , α_5 , α_v , β_3 and β_4 , whereas had no effect on integrin β_1 and β_5 . As expression of integrin α_5 , α_v and β_3 was shown to enhance the metastasis potential of lung cancer, the suppression of such integrins mediated by ouabain may be responsible for its anti-metastasis activities.

Effects of ouabain on anchorage-independent growth and migration of human lung cancer H460 cells. The ability of the tumor cells to grow in anchorage-independent condition and to migrate away from its original tumor are linked with

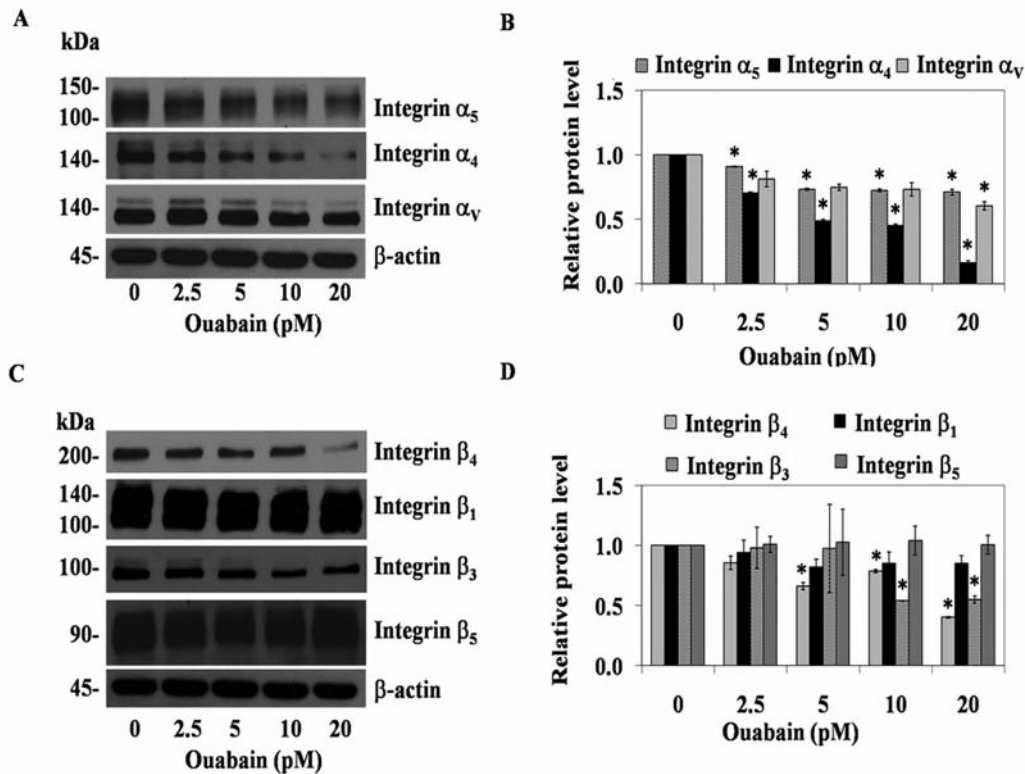


Figure 2. Effects of ouabain on integrin expression. (A) and (C) Cells were treated with ouabain (2.5-20 pM) for 24 h and integrin α_4 , α_5 , α_v , β_1 , β_3 , β_4 and β_5 protein levels were determined by western blotting. β -actin was used as loading control to confirm equal loading of the samples. (B) and (D) The protein signals were quantified by image densitometry and mean data from 3 independent experiments were normalized to the results. Values are means of 3 independent triplicate experiments \pm SEM. * $p < 0.05$ versus untreated control.

cancer aggressiveness, as well as metastatic potential (14, 17, 21-22, 30). We, thus, further investigated whether an ouabain-mediated alteration in integrin could regulate such behavior of cancer cells. H460 cells were cultured in the absence or presence of ouabain (2.5-20 pM) for 24 h and subjected to soft agar anchorage-independent growth assay and migration assay, as described in Materials and Methods. For anchorage-independent growth, cells were subjected to soft agar as a single-cell suspension and let colonies form for 10 days. Figure 3 shows that treatment with 10-20 pM of ouabain significantly decreased the colony size in comparison to that of non-treated control cells, while had no significant effects in terms of number of colonies. These results pointed out that ouabain has no effect on the survival of the cells in detached condition; however, it has a negative effect on cell growth. Because several types of integrins are essential for cell migration (14, 17, 21, 30), we thus tested whether ouabain-induced integrin switch has an impact on H460 cell migration. A monolayer of cells were scratched and treated with various non-toxic concentrations of ouabain for 0-48 h. Figure 4A and B show that ouabain, at the concentration of

10-20 pM, significantly inhibited migration of the cells with approximately 35-40% inhibition as compared to that of non-treated control. The results suggested that the switch of integrin α_4 , α_5 and α_v in response to ouabain treatment may affect the capacity of lung cancer cell migration.

Ouabain elicits no significant effects on chemotherapeutic drug-induced lung cancer H460 cell death. Since the susceptibility of cancer cells to chemotherapeutic agents has been shown to be an important hallmark of cancer aggressiveness, we tested whether ouabain affects cellular responses to cisplatin, etoposide, paclitaxel and doxorubicin-mediated death. Figure 5 shows that cells treated with 100 μ M cisplatin, 200 μ M etoposide, 1 μ M paclitaxel and 10 μ M doxorubicin for 24 h exhibited approximately 56.48%, 44.90%, 51.47% and 56.37% reduction in cell viability, respectively. Interestingly, addition of non-cytotoxic concentrations (0-20 pM) of ouabain to the cells treated with the aforementioned anticancer agents did not alter the cytotoxic effect of these drugs. The results suggest that ouabain, at indicated concentrations, exerts no impact on chemotherapeutic drug susceptibility.

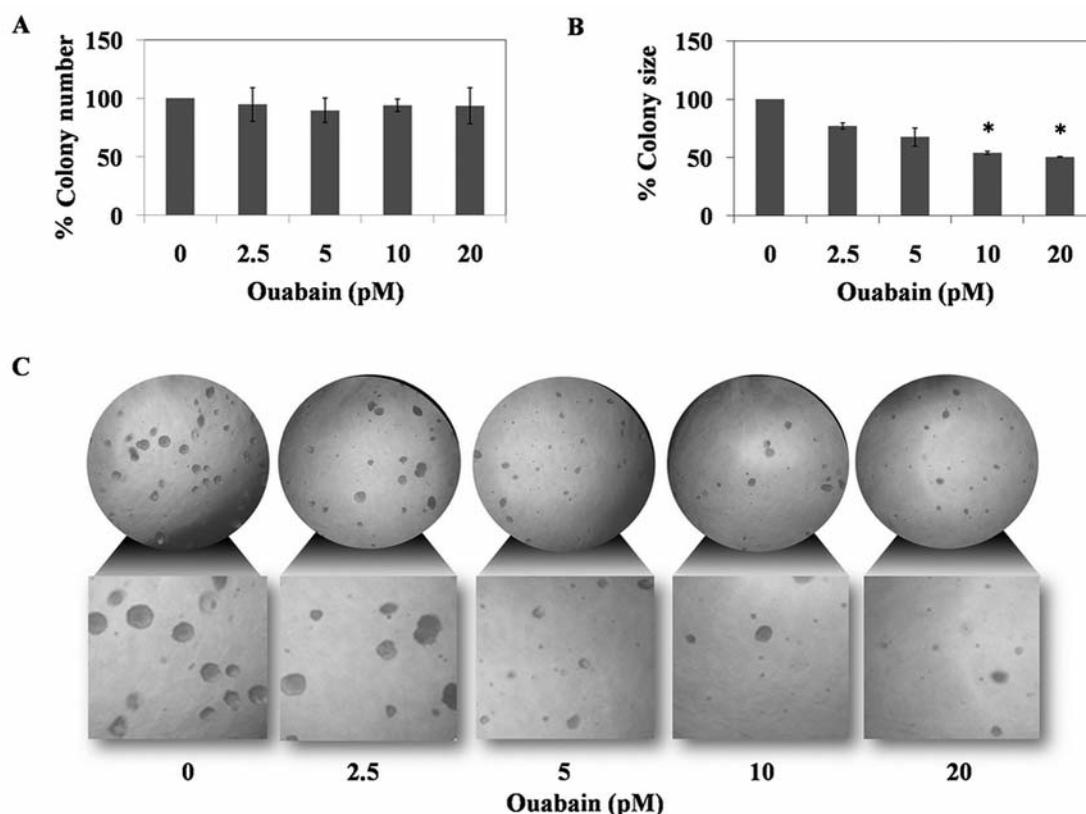


Figure 3. Ouabain inhibits anchorage-independent growth. After pre-treatment of the cells with ouabain (2.5-20 pM) for 24 h, cells were subjected to soft agar colony formation assay as described in Materials and Methods. (A) Colony number and (B) colony size were determined by image analyzer. Values are means of 3 independent triplicate experiments \pm SEM. * $p < 0.05$ versus non-treated control. (C) Representative fields from three independent experiments were photographed and presented.

Discussion

Cancer metastasis is a complicated process involving multiple alterations in cellular mechanisms. Some alterations are caused by the response of the cancer cell receiving signals from their microenvironment. Recent evidence demonstrated that deregulation or change in the pattern of integrins, termed “integrin switch” implicates cancer metastasis (12). Integrins are cell-adhesion molecules that play a key function in the interaction of the cells to the component of extracellular matrix (12). Indeed, by modulating the profile of integrins, cancer cells are able to adapt themselves to adhere on different types of basement (12), overcome anoikis (31), enhance migration as well as invasion (12) and resist chemotherapy (22-23, 32). Focusing on cancer metastasis, a dual role of $\alpha_v\beta_3$ was demonstrated, as the down-regulation of integrin $\alpha_v\beta_3$ in human intestine carcinoma was shown to inhibit the anoikis process (33, 34); however, these integrins exhibited an opposite effect in melanoma cells by protecting detached cells from apoptosis (31, 35). Likewise, integrin $\alpha_v\beta_5$ was shown to

mediate epithelial-mesenchymal transition (EMT) and facilitate cell migration in many cancers (12, 17, 36-37). An increasing attempt has been made for investigating the possible anti-cancer activities of endogenous compounds like the recently identified human hormone, ouabain, that has garnered significant attention as it possesses several anticancer activities. Herein we provide evidence indicating that exposure of lung cancer cells to ouabain at physiological concentrations caused an alteration in the expression pattern of integrins. Treatment of the cells with ouabain resulted in the reduction of integrin α_4 , α_5 , α_v , β_3 and β_4 expression, but had no effect on integrin β_1 and β_4 . In terms of anchorage-independent growth, previous studies suggested that the ability of the cells to grow in anchorage-independent conditions determined the success of metastasis (31). We found that treatment of the cells with ouabain significantly suppressed the levels of integrin α_v and α_5 that have been previously shown to be important factors for cell growth induction (38-39). Therefore, the significant decrease in cell proliferation (Figure 1B) and colony size (Figures 3B and C) of ouabain-treated cells may be explained

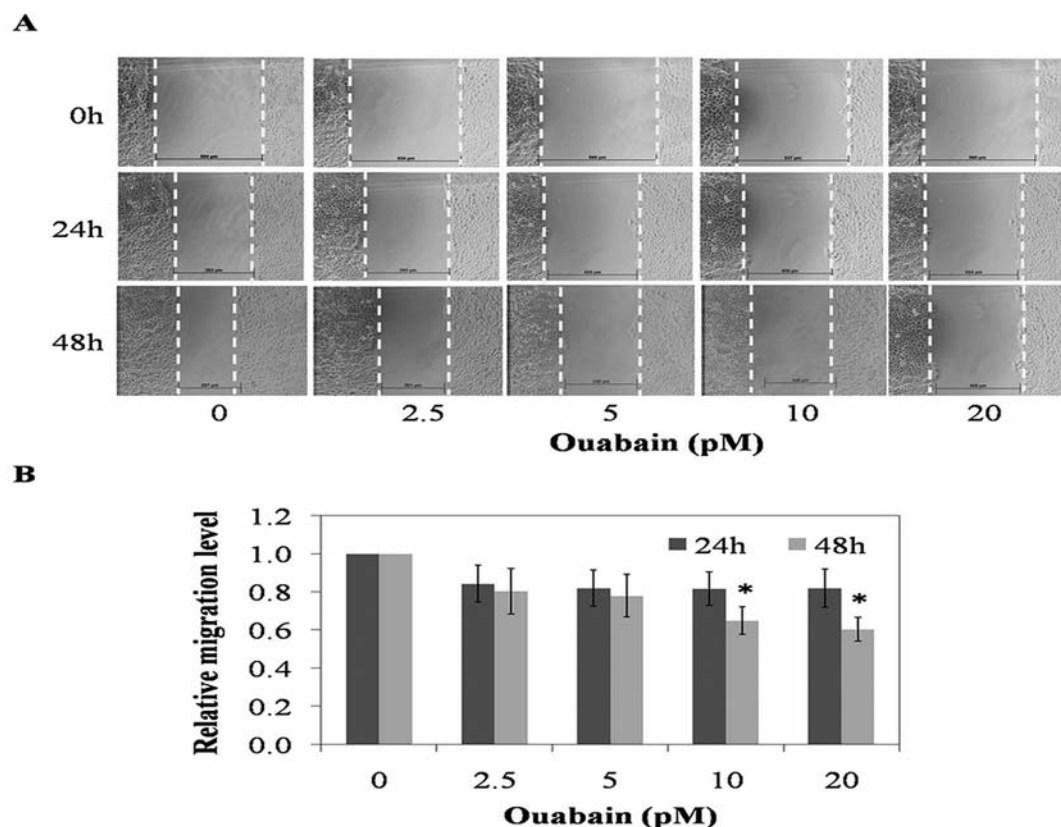


Figure 4. Effect of ouabain on H460 lung cancer cell migration. After pre-treatment of the cells with ouabain (2.5-20 pM) for 24 h, cells were subjected to migration assay. (A) and (B) After indicated treatment, wound space was analyzed and represented as a relative migration level. The relative cell migration was determined by comparing the relative change of those in untreated cells. Values are means of 3 independent triplicate experiments \pm SEM. * $p < 0.05$ versus untreated control.

by the decrease of the above-mentioned integrins. Studies have indicated that integrins α_4 , α_5 , α_v , β_1 and β_5 play a key role in cancer cell migration (14-21). Our results are consistent with those of previous studies, where the decrease in integrin α_4 , α_5 and α_v levels caused by ouabain was associated with the reduction of cell migration. The overexpression of integrin β_1 has been shown to inhibit paclitaxel- and vincristine-induced apoptosis, two microtubule-directed chemotherapeutic agents widely used in the therapy of breast cancer *via* activation of the PI3-kinase/AKT pathway (23, 32).

In small-cell lung cancer, integrin β_1 is the predominant integrin that causes cellular resistance to chemotherapeutic agents, such as cisplatin, doxorubicin and etoposide (22). In this study we found that the expression of integrin β_1 could only marginally altered in response to ouabain. We also observed that the susceptibility of the cells to chemotherapeutic drugs, including cisplatin, etoposide, paclitaxel and doxorubicin, could not be altered. These results suggest that ouabain may have no effect on

chemotherapeutic resistance in these cells since it has no effect on integrin β_1 .

In summary, we provide novel information regarding the regulatory role of ouabain on integrin switch. This information may help fill the knowledge gap concerning the role of this hormone in the regulation of cancer behavior and encourage the use of this endogenous compound in further anticancer approaches.

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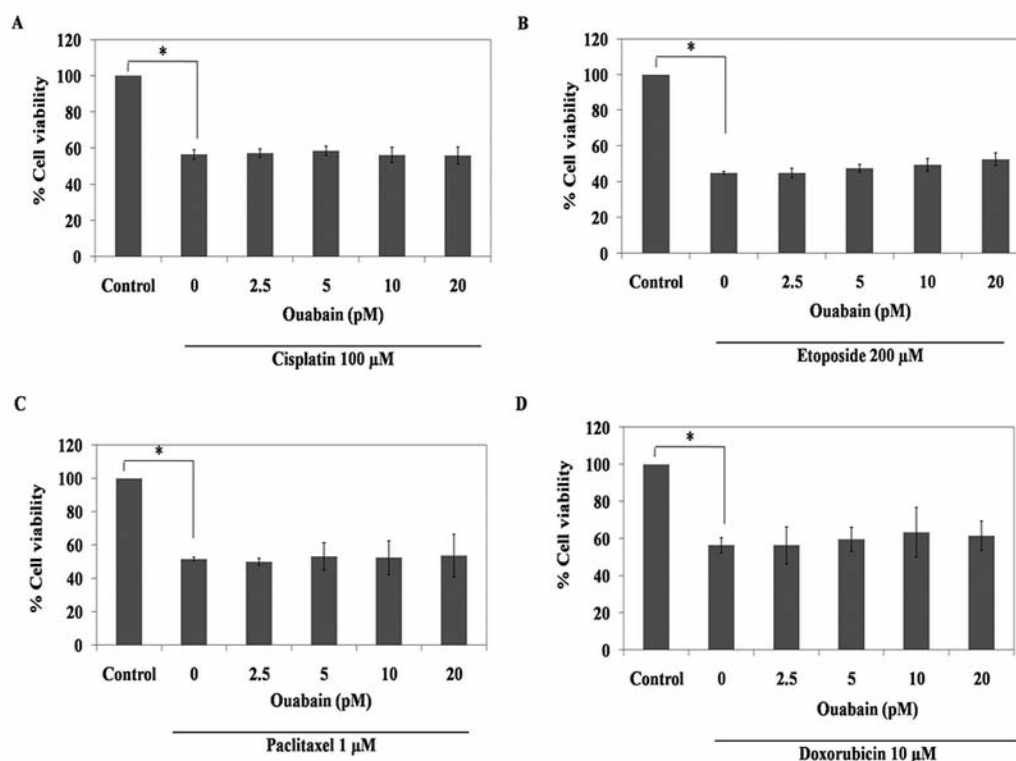


Figure 5. Ouabain has no significant effect on anti-cancer activity of chemotherapeutic drugs. Cells were pre-treated with various concentrations of ouabain (2.5-20 pM) for 24 h and subsequently treated with 100 μ M cisplatin, 200 μ M etoposide, 1 μ M paclitaxel or 10 μ M doxorubicin. The percentage of cell viability was determined after 24 h by MTT. Values are means of 3 independent triplicate experiments \pm SEM. * p <0.05 versus untreated control.

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