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Blockade of Androgen-induced Malignant Phenotypes by Flutamide Administration in Human Salivary Duct Carcinoma Cells

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Abstract. *Background/Aim: Androgens are known to play a critical role in prostate cancer progression, but their effect on malignant phenotypes in salivary gland cancer is unclear. The androgen–androgen receptor (AR) axis may be involved in malignant phenotypes of salivary duct carcinoma (SDC) cells and therefore may be a new target for SDC treatment. To test this hypothesis, we investigated the effect of the androgen 5α-dihydrotestosterone (DHT) on proliferation, migration, and invasiveness of SDC cells. Materials and Methods: We used a wound-healing assay to measure cell migration and a Boyden chamber invasion assay to investigate SDC cell invasive capacity. Results: DHT treatment increased cell proliferation, migration, and invasion. However, treatment with flutamide, an AR inhibitor, blocked the effects of DHT. Conclusion: These results suggest that the androgen–AR axis is involved in SDC malignancy and may be an effective therapeutic target for treatment of human SDC.*

The development of malignant neoplasms is a multistep process that involves many genetic and epigenetic alterations. Identifying these alterations is essential in understanding the mechanisms of cancer progression and in developing more effective methods of diagnosis and treatment. In the case of prostate cancer, hormonal stimulation is also critically involved in carcinogenesis. The androgen–androgen receptor (AR) system plays an important role in prostate cancer progression, and AR is one of the targets for treatment of patients with prostate cancer (1, 2).

In the head and neck region, salivary duct carcinoma (SDC) is a rare and highly aggressive epithelial malignancy of the major and minor salivary glands, with limited evidence to guide standard treatment. Surprisingly, several immunohistochemical studies demonstrated AR expression in almost all SDCs (3, 4). The expression of AR is a strong prognostic factor in patients with prostate cancer and has long been used in clinical management as an indicator of endocrine responsiveness (5, 6).

Given the potential involvement of steroid hormone receptors in salivary gland cancer progression, we hypothesized that hormonal regulation may have an impact on SDC pathogenesis. Therefore, the strategy used for prostate cancer treatment may be effective for SDC treatment.

In this study, we determined whether or not the androgen–AR axis was functional in SDC cells and assessed the effect of flutamide, an anti-androgen agent, on SDC.

Materials and Methods

Cell culture. HSY salivary gland carcinoma cells were kindly provided by Professor N. Sato of Tokushima University. This cell line was established by Professor Sato (7) from an SDC of the parotid gland. The human prostate cell lines PC-3 and LNCaP were purchased from the American Tissue Culture Collection (Manassas, VA, USA) and used as negative and positive controls, respectively. Cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% (v/v) fetal bovine serum at 37˚C in the presence of 5% CO_2. Fetal bovine serum was omitted in experiments in which serum-free medium was used.

Chemicals. 5α-Dihydrotestosterone (DHT) solution (1.0 mg/ml in methanol; Sigma Chemical Co.) was used for the experiments. For each of the following assays, cells were treated once or twice daily

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with the DHT-methanol mixture (1 nM); control cells received 0.1% methanol only.

Flutamide (1000-fold stock; Sigma Chemical Co.) was added to ethanol to yield a final ethanol concentration of 0.1%. For each of the following assays, cells were treated once or twice daily with the flutamide–ethanol mixture (10 nM); control cells received 0.1% ethanol only for 2 days. In terms of the blockage experiment, DHT and flutamide were added to the medium at the same time; control cells received 0.1% ethanol for 2 days. Assays were performed in triplicate and the results were averaged.

Cell proliferation assay. Cell proliferative ability was determined by counting cells. Cells were digested with trypsin-ethylenediaminetetra-acetic acid at 37˚C for 5 min. Next, 8 ml of conditioned medium containing 5% (v/v) fetal bovine serum was added to the cells and the cells were collected. Then, 0.5 ml of the supernatant was diluted with 9.5 ml of balanced electrolyte solution. Cell number was quantified by particle counting and the analysis was performed by using a Multisizer™ 3 system (Beckman Coulter, Brea, CA, USA). Assays were performed in triplicate and the results were averaged.

Cell migration assay. Cells were seeded in 6-well plates at a density of 5.0×10^5/well. The following day, the bottom of each well was scratched with a pipette tip. Wells were rinsed with medium to remove detached cells, and the medium was replaced with serum-free medium. DHT (1 nM) with or without flutamide (10 nM) was added to the cells for 12 h. Images of each well were acquired immediately following wound generation and again after 12 h. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to measure areas that were free of migrating cells. Experiments were performed in triplicate.

Boyden chamber invasion assay. Assays were performed in modified Boyden chambers with 8-μm-pore filter inserts for 24-well plates (Collaborative Research, Bedford, MA, USA). Filters were coated with 10-12 μl of ice-cold Matrigel® (Collaborative Research). HSY cells (40,000 cells/well) were added to the upper chamber in 200 μl of serum-free medium. Cells were pre-treated with DHT with or without flutamide for 2 days (once a day). Cells were assayed in triplicate or quadruplicate, and the results were averaged. The lower chamber was filled with 300 μl of conditioned medium from fibroblasts. After incubation for 20 h, cells were fixed with 2.5% (v/v) glutaraldehyde in phosphate-buffered saline and stained with 0.5% (v/v) toluidine blue in 2% (w/v) Na_2CO_3. Cells that remained in the Matrigel® or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy.

Western blotting analysis. Cells were lysed in 2× Laemmli buffer and stored at –70˚C. The protein concentration was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). Samples (20-30 μg of total protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Hybond P; Amersham Biosciences, Little Chalfont, Bucks, UK). Membranes were blocked for 1 h at room temperature with Tris-buffered saline with Tween-20 (20 mM Tris, 137 mM NaCl, 3.8 mM HCl, and 0.1% Tween-20) containing 5% nonfat milk, and blots were probed with anti-AR (AR441; DAKO, Glostrup, Denmark) or anti-actin (C4; Chemicon International, Temecula, CA, USA) antibodies for 1 h. Membranes were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed, and developed by enhanced chemiluminescence using the Amersham ECL-Plus kit (Amersham Biosciences) according to the manufacturer's instructions.

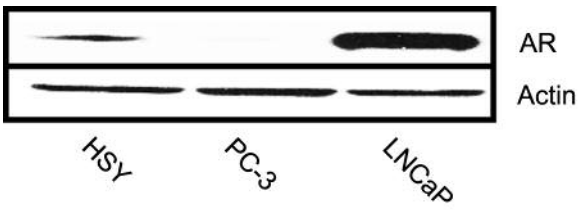

Figure 1. *Androgen receptor (AR) expression in HSY, PC-3, and LNCaP cell lines. Representation western blot is shown.*

Statistical analyses. Statistical comparisons were performed using Student's *t*-test as appropriate. A *p*-value less than 0.05 was considered statistically significant. All statistical tests were performed using Statcel2 software (OMS, Tokyo, Japan).

Results

AR expression profile in cell lines. As shown in Figure 1, AR was highly expressed in LNCaP cells, whereas PC-3 cells did not express AR. HSY cells weakly expressed AR when cultured in RPMI-1640 supplemented with 5% (v/v) charcoal-stripped serum.

Effects of DHT with and without flutamide on cell proliferation. DHT treatment induced the proliferation of HSY cells. Flutamide partially suppressed the effect of DHT on HSY cell proliferation. However, DHT–flutamide-treated cells still presented a proliferative activity similar to that of ethanol-treated control cells. The increase in proliferation induced by DHT was significantly different from that induced by ethanol or DHT plus flutamide treatment ($p<0.01$; Figure 2).

Cell migration assay. Pipet scratching produced wounds of similar size in monolayers of HSY cells and control cells (Figure 3). At 12 h after wound infliction, a higher percentage of the scratched area was filled with cells in cultures treated with DHT than in cultures treated with DHT–flutamide or control cells ($p<0.05$; Figure 3).

Effects of DHT with and without flutamide on cell invasion. To investigate the effects of DHT on invasive properties, we plated cells in Matrigel invasion chambers. After 48 h of treatment with DHT with and without flutamide, the invasive capacity of DHT-treated HSY cells was markedly increased. DHT–flutamide-treated cells and ethanol-treated control cells

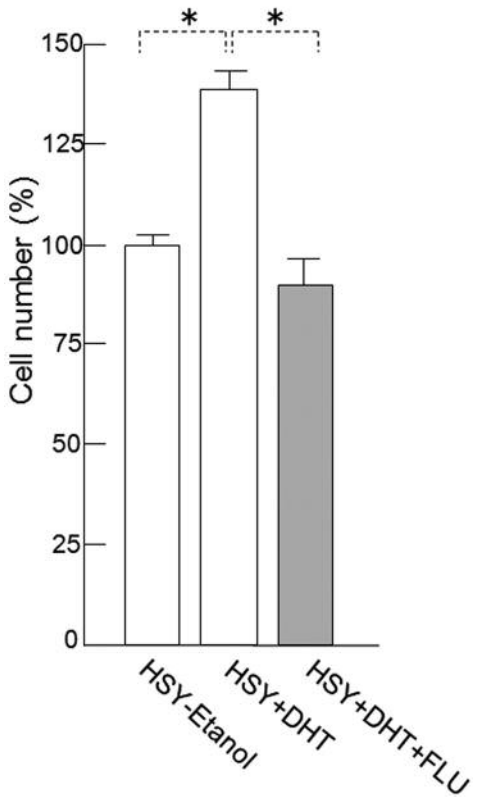

Figure 2. *Effect of 5α-Dihydrotestosterone (DHT) and flutamide (FLU) on HSY cell proliferation. DHT-treated HSY cells exhibited significantly higher proliferation than cells treated with DHT+FLU or with ethanol (*p<0.01). Addition of FLU inhibited the effects of DHT on HSY cell proliferation.*

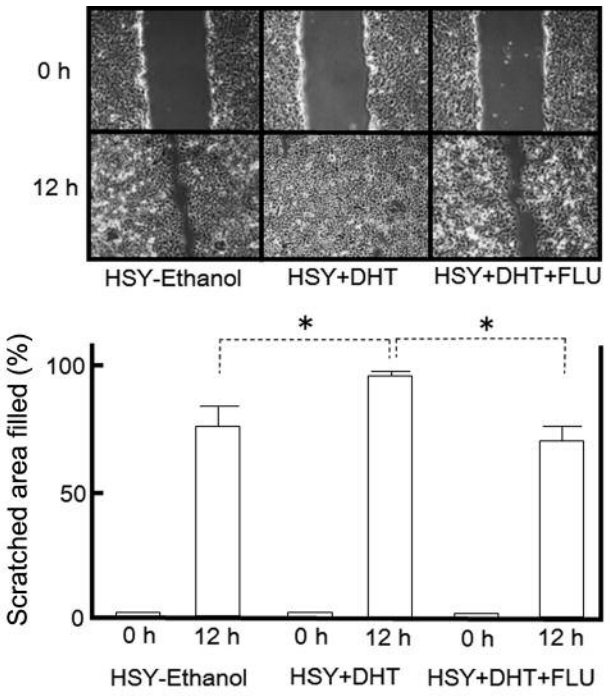

Figure 3. *Effects of 5α-Dihydrotestosterone (DHT) and flutamide (FLU) on migration of HSY cells. At 12 h after wound infliction, a greater percentage of the scratched area was filled in cultures of DHT-treated cells than in cultures of DHT-FLU-treated and control cells (*p<0.05).*

presented similar invasive capacities. DHT-treated cells exhibited significantly greater invasion than cells treated with DHT–flutamide or ethanol ($p<0.01$; Figure 4).

Discussion

Salivary gland tumors, which account for approximately 3% of all head and neck cancer, are heterogeneous, rendering the identification of their epidemiological characteristics difficult (8). SDC is a rare, aggressive salivary gland malignancy with limited evidence available to guide standard treatment (9). Differential expression of some genes has been investigated in the different histotypes of salivary gland carcinoma (10). However, salivary gland carcinomas are divided into 24 different entities by the World Health Organization, including mucoepidermoid, adenoid cystic carcinoma, adenocarcinoma, and SDC. Malignant salivary gland tumors are difficult to treat because of their poor sensitivity to chemotherapy and radiotherapy (11, 12). Therefore, new treatment modalities need to be developed.

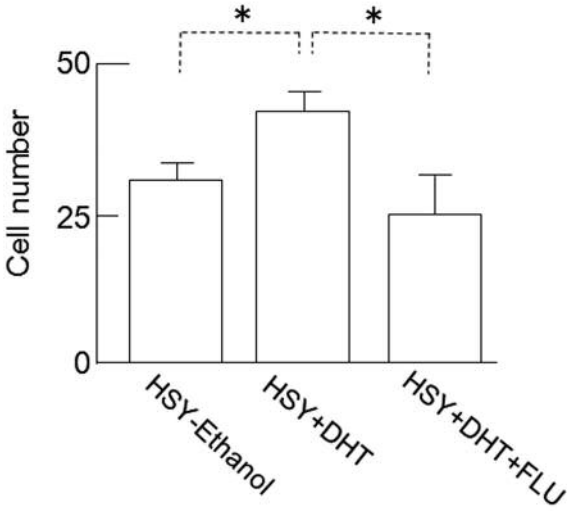

Figure 4. *Effects of 5α-Dihydrotestosterone (DHT) and flutamide (FLU) on the invasive capacity of HSY cells. After 48 h of treatment with DHT with or without FLU, the invasive capacity of DHT-treated HSY cells was significantly higher than that of the other two groups (*p<0.01). The invasive capacity of DHT+FLU-treated cells was similar to that of ethanol-treated control cells.*

Some biomarkers associated with salivary tumors have been reported. Minichromosome maintenance 3 might be a useful proliferation marker for differential diagnosis and recognition of clinical behavior of salivary gland tumors (13). Inhibitor of differentiation 1 has been reported as a target because it reduced proliferation and invasion in aggressive human salivary gland cancer cells (14). However, application of these molecular targets for treatment is difficult. Radiotherapy immediately after surgery has been reported to improve the prognosis of patients with malignant salivary gland tumors (15).

SDC is extremely aggressive, and postoperative radiotherapy and systemic chemotherapy are not effective (11, 12). To date, surgery is considered the only treatment modality available for SDC. However, SDC is known to overexpress AR (3, 4), which may thus be a therapeutic target.

Given the potential involvement of steroid hormone receptors in salivary gland cancer progression, we hypothesized that hormonal regulation may impact SDC pathogenesis. Therefore, a strategy used for prostate cancer treatment may be effective for SDC treatment. Some other treatments using chemotherapy agents and proteomic analyses have been applied to prostate cancer (16, 17), but other treatment modalities have to be applied for SDCs.

The present study showed that the androgen–AR axis does function in SDC cells. DHT-treated HSY cells exhibited significantly higher proliferation than ethanol-treated cells. In HSY cells, AR might play a role as a transcription factor inducing proliferation. Flutamide, an AR inhibitor, suppressed the effect of DHT on proliferation. However, the inhibition was only partial and the level of proliferation after combined treatment with DHT and flutamide was similar to that of ethanol-treated cells.

In prostate cancer, an association between gene polymorphism and prostate cancer risk has been reported. For example, *p27* V109G polymorphism is related to prostate carcinoma risk, and the regulatory effect of mutant *p27* on cell proliferation and apoptosis has been shown to be stronger than that of wild-type *p27* ($p<0.05$) (18). AR mutations have also been reported (19). However, such molecular characteristics were not investigated in the present study.

Several studies have reported that malignant salivary gland tumors present a higher rate of angiogenesis and cellular proliferation than benign tumors (20). Therefore, the inhibitory effect of flutamide on HSY cell proliferation in the present study was somewhat surprising.

In addition, we assessed other malignant phenotypes, cell migration and cell invasion. Both showed the same tendency as cell proliferation. Cell migration and invasion were induced by DHT treatment and flutamide inhibited the effect of DHT on cell invasion and migration, levels of which were similar to those of control cells.

In summary, our results using HSY cells indicate that AR is weakly expressed in SDC cells and functional, as DHT increased the proliferation, invasion, and migration of these cells, and AR inhibitor flutamide inhibited the effects of DHT. Although further experiments are needed to confirm our results, we believe that anti-AR therapy should be investigated as a hormonal therapy for SDC.

Conflicts of Interest

None.

Acknowledgements

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