# Androgens enhance the ability of intratumoral macrophages to promote breast cancer progression

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**Abstract.** Androgens are produced locally in breast carcinoma tissues by androgen-producing enzymes such as 5α-reductase type 1 (5αRed1) and affect not only breast cancer cells but the tumor microenvironment as well. Tumor-associated macrophages (TAMs) are primary components of the tumor microenvironment and contribute to tumor progression. Although previous studies suggest that androgen/androgen receptor (AR) signaling in macrophages has important roles in human diseases, androgen action on TAMs has remained largely unknown. We immunolocalized macrophage marker CD163 as well as AR and 5αRed1 in 116 breast carcinomas and correlated them with clinicopathological parameters and clinical outcomes. Moreover, we examined the roles of androgens on macrophages in breast cancer progression using cell lines 4T1 (mouse breast cancer) and RAW264.7 (macrophage) in a tumor-bearing female BALB/c mouse model. Double immunohistochemistry revealed that AR was sporadically expressed in the macrophages in breast carcinoma tissues. Macrophage infiltration was significantly correlated with an aggressive phenotype of breast carcinomas and worse prognosis, especially in the 5αRed1-positive group. In a sphere-forming assay using 4T1 and RAW-AR cells, which stably express AR, the sphere size was significantly increased due to androgens when 4T1 cells were cocultured with RAW-AR cells. Furthermore, *in vivo* experiments revealed that tumor growth and Ki67, a cell proliferation marker, were increased when androgens were stably produced in breast cancer cells and AR was expressed in macrophages. In conclusion, AR is expressed in intratumoral macrophages and is associated with an aggressive phenotype of breast carcinomas, especially when breast cancer cells actively produce androgens. Thus, androgens may enhance the ability of macrophages to promote breast cancer progression.

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## Introduction

Biologically active steroids such as estrogens and androgens are locally produced in breast cancer tissues by sex-steroid producing enzymes. Among these enzymes, aromatase is an important therapeutic target in estrogen receptor (ER)-positive breast cancers. However, *de novo* or acquired resistance to aromatase inhibitors (AIs) are often experienced and this is a key problem to be solved (1). On the other hand, androgen-producing enzymes such as 17β-hydroxysteroid dehydrogenase type 5 [17βHSD5, also known as aldo-keto reductase family 1 member C3 (AKR1C3); androstenedione to testosterone] and 5α-reductase type 1 [5αRed1; testosterone to dihydrotestosterone (DHT), biologically active androgens] are also frequently expressed in breast carcinoma tissues. These enzymes contribute to intratumoral androgen synthesis (2) and 5αRed1 is known as a potent regulator of *in situ* DHT production (3). Because the androgen receptor (AR) is also frequently expressed in breast cancer cells (4,5), it has been considered that intratumoral androgens have pivotal roles in the progression of breast cancers. However, the significance of androgen in breast cancer has not been fully understood and opposite findings have often been reported. For instance, numerous studies have pointed out that AR expression is associated with better clinical outcomes (6), while androgen action was recently highlighted in a distinct subset of ER-negative breast cancers [namely molecular apocrine subtype or luminal AR subtype (7,8)] or ER-positive breast cancers which have become resistant to AIs (9). It is therefore important to study the action of androgens in breast cancer.

The tumor microenvironment is composed not only of tumor cells but also contains various stromal cells including

macrophages, leukocytes and fibroblasts, which, together with cross-talks between tumor cells and other cellular components, is strongly associated with tumor progression (10). Tumor-associated macrophages (TAMs) are well known as the primary components of the tumor microenvironment and promote cancer progression (11-16). Monocytes in the blood are attracted to tumor tissues via soluble factors released from tumor tissues, where they differentiate into TAMs (17). Based on their function, macrophages are classified into M1 or M2 phenotypes. M1 macrophages are driven by cytokines such as tumor necrosis factor α (TNFα) and interferon γ (IFNγ), while M2 macrophages are driven by interleukin (IL)-4 and IL-13 (18). Each macrophage type displays different functions and expression profiles of cytokines and cell-surface markers. While M1 macrophages express CD80 and CD86 and have tumor-suppressive and pro-inflammatory effects, M2 macrophages express CD163, are the predominant phenotype of TAMs in human solid tumors (18,19) and contribute to tumor malignancy by promoting tumor growth, metastasis, angiogenesis, epithelial-mesenchymal transition (EMT) and drug resistance in breast cancers (11-16). Previous studies have reported that a high infiltration of M2 macrophages is correlated with lymph node metastasis, higher histological grade and cell proliferation, and contributes to worse prognosis in breast cancers (13,19).

It has been reported that AR in monocytes/macrophages plays key roles in the greater prevalence and severity of atherosclerosis in men (20,21). Moreover, it has also been reported that some macrophages express AR and that androgen/AR signaling in macrophages inhibits cutaneous wound healing (22). These findings suggest that androgen acts upon macrophages affecting various human diseases, although its role in human tumors, including breast cancers, remains unclear. We therefore focused on the relationship between androgen action in intratumoral macrophages and progression of breast cancers, with the purpose of a better understanding of the biological and/or clinical significance of androgens in breast cancers.

## Materials and methods

*Patients and tissues*. A total of 116 specimens of invasive breast carcinomas were obtained from Japanese female patients who had undergone surgical treatment from 2007 to 2008 at Tohoku University Hospital, Sendai, Japan. The clinical outcome was evaluated by disease-free and breast cancer-specific survival. Disease-free survival was defined as the time from the date of surgery to that of the first locoregional recurrence or distant metastasis within the follow-up time and the median was 59 (range 3-84) months. Breast cancer-specific survival was defined as the time from surgery to death from breast cancer and follow-up time was 61 (from 3-84) months. All specimens had been fixed with 10% formalin neutral buffer solution and embedded in paraffin wax. Experiments and analyses were performed in accordance with the Helsinki declaration and the research protocol of this study was approved by the Ethics Committee at the Tohoku University Graduate School of Medicine (approval no. 2016-1-697, 2019-1-219). This is a retrospective study and, therefore, informed consents had not been obtained.

*Immunohistochemistry*. The information regarding the antibodies is listed in Table SI. Immunohistochemistry for CD163, 5αRed1 and Ki67 was carried out using a Histofine kit (Nichirei Bio, Inc., Tokyo, Japan) as described previously (23,24). The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) and hematoxylin was used for counterstaining. Immunohistochemistry for ER and progesterone receptor (PR) was carried out with Ventana Benchmark XT system (Roche Diagnostics Japan). For human epidermal growth factor receptor 2 (HER2), the HercepTest (Dako) was used.

*Double immunohistochemistry*. Immunoreactivity of AR was firstly visualized with DAB as described above, and then antigen retrieval was carried out again and immunoreactivity of CD163 was visualized with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP).

*Scoring of immunoreactivity*. 5αRed1 immunoreactivity was detected in the cytoplasm of breast carcinoma cells and considered positive when the cases had more than 10% of positive carcinoma cells (3). Infiltration of CD163-positive macrophages was scored into 4 categories (0, no focal areas; 1, small scattered focal areas; 2, numerous larger focal areas; 3, very numerous large focal areas) according to a previous study (25), and finally dichotomized for statistical analyses (0, 1: Low infiltration and 2, 3: High infiltration). ER, PR and Ki67 immunoreactivity were detected in the nucleus of carcinoma cells and the percentage of positive cells (labeling index; LI) was calculated by evaluating in more than 1,000 cells. According to a previous report (26), cases with LI of more than 1% were considered positive for ER and PR. HER2 immunoreactivity was determined according to the grading system proposed in HercepTest (Dako).

*Cell lines and chemicals*. Mouse breast cancer cell line 4T1 and mouse macrophage cell line RAW264.7 were obtained from American Type Culture Collection (ATCC) and RIKEN Bioresource Center (Japan), respectively. These cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum (FBS) (Biosera) and incubated at 37˚C under 5% $CO_2$. In experiments using androgen, these cells were cultured in phenol red-free RPMI-1640 with 10% dextran-coated charcoal-stripped FBS.

*Coculture experiment*. The 4T1 cells were cocultured with RAW264.7 cells using ThinCerts™ (pore size 0.4 $\mu$m, Greiner bio-one). RAW264.7 cells were seeded in the bottom plate ($3x10^5$ cells/well) and allowed to attach for 24 h. Then, the 4T1 cells were placed onto an upper Transwell ($2x10^5$ cells/well) and cocultured with RAW264.7 cells for 3 days.

*Quantitative real-time PCR*. Total RNA (500 ng) was extracted using TRI Reagent (Molecular Research Center, Inc.) and cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co. Ltd.). Real-time PCR was performed using the THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co. Ltd.) and LightCycler nano system (Roche Diagnostics Japan). Thermocycling conditions were as follows: 95˚C for 60 sec (initial denaturation), followed by 45 cycles at

95˚C for 15 sec and 60˚C for 30 sec. The sequences for the PCR primer sets are listed in Table SII. Relative mRNA levels of *Arg-1*, *Ar*, *Srd5a1* and *Akr1c6* were summarized as the ratio to *Rpl13a*.

*Plasmid construction*. Open reading frame of *Ar* and FLAG-tagged aldo-keto reductase family 1 member C6 (*Akr1c6*, mouse testosterone-producing enzyme) were amplified via PCR using KOD FX Neo (Toyobo Co. Ltd.) and cloned into pIRES2-AcGFP1 vector (Clontech Laboratories) and pcDNA3.1 (-) vector (Invitrogen; Thermo Fisher Scientific, Inc.), respectively, using DNA Ligation Kit <Mighty Mix> (Takara Bio) (pIRES-mAR and pAKR1C6-FLAG). Thermocycling conditions were as follows: 94˚C for 2 min, followed by 35 cycles at 98˚C for 10 sec, 62˚C for 30 sec and 68˚C for 90 sec. The sequences for the PCR primer sets are listed in Table SII.

*Plasmid transfection and establishment of stable clones*. Since 4T1 and RAW264,7 cells do not express *Akr1c6* and *Ar*, respectively, we tried to establish their sublines which stably express these genes in order to investigate the roles of androgens in intratumoral macrophages. According to the manufacturer's instructions for transfection, RAW264.7 cells were transfected with pIRES-mAR or pIRES2-AcGFP1 using GenomONE™-Neo (Ishihara Sangyo Kaisya, Ltd.) and 4T1 cells were transfected with pAKR1C6-FLAG (4T1-AKR1C6) or pcDNA3.1(-) (4T1-CT) using Avalanche®-Everyday Transfection Reagent (APRO Science). These cells were cultured in media containing 400 $\mu$g/ml G418 (Wako), and stable clones expressing AR (RAW-AR), AKR1C6 (4T1-AKR1C6) and corresponding negative control clones (RAW-CT and 4T1-CT) were established.

*Western blotting*. Western blotting was performed according to previous reports (27,28) and the information regarding the primary antibodies is listed in Table SI. The cells were lysed using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) containing Halt Protease Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA). The protein extracts (10 $\mu$g) were separated by SDS-PAGE (10% acrylamide gel) and then transferred to Hybond P polyvinylidene difluoride membrane (GE Healthcare). The membrane was blocked in 5% non-fat milk in TBS-T for 1 h at room temperature and then incubated with primary antibodies at 4˚C overnight. The blots were incubated with the appropriate HRP-conjugated secondary antibody (anti-mouse; 1:10,000, cat. no. NA931 or anti-rabbit; 1:10,000, cat. no. NA934, GE Healthcare) for 1 h at room temperature. Detection of antibody-protein complexes on the membrane was visualized using ECL-Prime Western blotting detection reagents (GE Healthcare) and LAS-4000 image analyzer (Fuji Photo Film Co.).

*Synthetic androgen R1881 treatment*. RAW-CT and RAW-AR cells ($1x10^5$ cells/well) were cultured in a 12-well plate with or without synthetic androgen R1881 (10 nM) for 48 h.

*Sphere-forming assay*. A sphere-forming assay was performed using EZ-BindShut®II micro plate (IWAKI). The 4T1 cells ($1x10^5$ cells/well) were cocultured in a well with RAW-CT or RAW-AR cells ($3.33x10^4$ cells/well) with or without R1881 (10 nM). After 3 days, spheres were photographed under a microscope and the size of 100 spheres was measured using ImageJ 1.53e (https://imagej.nih.gov/ij/).

*Cell proliferation assay*. The 4T1-AKR1C6 or 4T1-CT cells were seeded in a 96-well plate (3,000 cells/well) and the cell proliferation was measured by the WST-8 method using a Cell Counting Kit-8 (Dojindo Molecular Technologies) for 4 days. Absorbance was determined using a Bio-Rad iMark plate reader (Bio-Rad Laboratories Inc.).

*Tumor-bearing mouse model*. The protocols of all animal experiments were submitted, reviewed and approved in advance by the Institutional Animal Care and Use Committee at Tohoku University (2019MdA-172). The experiments were carried out according to ARRIVE guidelines and Guidelines for Animal Experiments at Tohoku University. A total of 11 4-week-old wild-type female BALB/c mice were obtained from CLEA Japan (Tokyo, Japan) and randomly assigned to two types of tumor-bearing models as described below with no inclusion criteria. They were housed in a specific pathogen-free facility with free access to water and MR stock food and environmental enrichment. In the present study, 4T1 and RAW264.7 sublines were subcutaneously injected in both the left and right side of mice in different combinations in order to avoid individual differences. In the first experiment (n=6), 4T1-CT or 4T1-AKR1C6 cells ($5x10^5$ cells) were subcutaneously injected together with RAW-AR cells ($1.25x10^5$ cells) into the left or right side of the back of mice, respectively, using 150 $\mu$l Matrigel (Corning, Inc.). In the mirror experiment (n=5), 4T1-AKR1C6 cells ($5x10^5$ cells) were injected together with either RAW-CT or RAW-AR cells ($1.25x10^5$ cells) into the left or right side of the back of mice, respectively. Mice were monitored for body weight and tumor volume changes twice a week. Tumor volume was measured using a digital caliper at least twice a week and calculated by modified ellipsoid formula 1/2 (length x width$^2$). Tumors were allowed to grow in mice until they reached the volume of 2,000 mm$^3$ or 10 weeks from injection had elapsed, whichever came first. No adverse events were observed and mice were euthanized 4 weeks after injection by cervical dislocation under anesthesia using isoflurane (4%) and thereafter tumors were resected, weighted, and then fixed in 10% formalin for immunohistochemistry.

*Statistical analyses*. Statistical analyses were performed using JMP pro 14.0.0 (SAS Institute). $\chi^2$ test or Mann-Whitney U test were applied for correlation between the infiltration of CD163-positive macrophages and clinicopathological factors. Breast cancer-specific and disease-free survival curves were generated by Kaplan-Meier method and statistical significance was evaluated by a log-rank test. In the *in vitro* and *in vivo* experiments, the statistical analyses were performed using paired or unpaired t-test. The data are presented as the mean ± SD. In this study, $P<0.05$ was considered as indicative of statistical significance.

## Results

*Expression of AR in macrophages within breast carcinoma tissues*. We first examined whether macrophages in breast

carcinoma tissues expressed AR or not. By double immunohistochemistry for AR and CD163, a marker of M2 macrophages, the macrophages with immunoreactivity for both CD163 (blue) in the cell membrane and AR (brown) in the nuclei were sporadically observed in the stroma (Fig. 1A).

We next immunolocalized CD163 as well as 5αRed1 in 116 breast carcinoma tissues in order to address the clinical significance of androgen action on macrophages in breast cancer. CD163 immunoreactivity was observed in the macrophages (Fig. 1B and C) and 34% (39 out of 116 cases) were categorized as high infiltration. 5αRed1 immunoreactivity was observed in the cytoplasm of breast carcinoma cells (Fig. 1D and E) and 56% (65 out of 116 cases) were considered positive for 5αRed1. Correlation between macrophage infiltration and clinicopathological parameters is presented in Table I. Macrophage infiltration was positively correlated with lymph node metastasis (P=0.0057), histological grade (P<0.0001) and Ki67 labeling index (LI) (P<0.0001), while it was negatively correlated with ER (P=0.0020) and PR (P=0.0014). No significant correlation was detected between macrophage infiltration and 5αRed1 immunoreactivity (P=0.74). When we compared the correlation between macrophage infiltration and clinicopathological parameters in the 5αRed1-negative group (n=51) and the 5αRed1-positive group (n=65) (Table II), macrophage infiltration was correlated with lymph node metastasis (P=0.011), PR (P=0.0061) and HER2 (P=0.015) only in the 5αRed1-positive group. On the other hand, it was negatively correlated with ER only in the 5αRed1-negative group (P=0.001), while it was correlated with histological grade and Ki67 LI in both the 5αRed1-positive and -negative group (histological grade; P=0.0013 in the 5αRed1-negative group and P=0.0069 in the 5αRed1-positive group, Ki67 LI; P=0.0017 in the 5αRed1-negative group and P=0.0020 in the 5αRed1-positive group). Furthermore, it is of interest that a significant correlation between macrophage infiltration and these parameters was detected only in the 5αRed1-positive group when the cases were limited to the ER-positive group.

We next examined the correlation between macrophage infiltration and clinical outcomes of breast cancer patients. As shown in Fig. 2A and B, macrophage infiltration was significantly correlated with an increased risk of recurrence (P=0.0049) and worse prognosis (P=0.016). In contrast, when we analyzed according to 5αRed1 status, macrophage infiltration was significantly correlated with increased risk of recurrence (P=0.01, Fig. 2C) and worse prognosis (P=0.045, Fig. 2E) only in the 5αRed1-positive group and no significant correlation was detected in the 5αRed1-negative group (Fig. 2D and F). This tendency was still observed when the cases were limited to the ER-positive group (Fig. 2G-I), although breast cancer-specific survival curve could not be generated because no patients had died in the ER-positive/5αRed1-negative group.

*Androgen is necessary for the pro-proliferative effects of TAMs*. We suggested that androgens may have important roles in macrophage-induced breast cancer progression by immunohistochemical analyses for CD163 and 5αRed1 as described above. We then tested this hypothesis in *in vitro* experiments using mouse breast cancer and macrophage cell lines, 4T1 and RAW264.7.

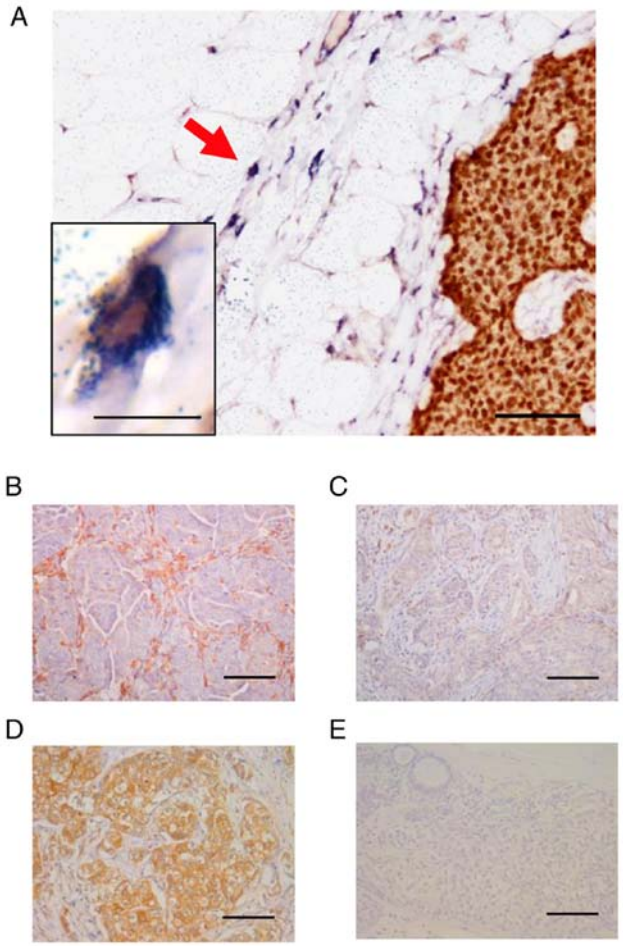


Figure 1. Immunohistochemistry for CD163, androgen receptor (AR) and 5α-reductase type 1 (5αRed) in human breast carcinoma tissues. (A) Representative image of double immunohistochemistry for CD163 (deep blue, NBT/BCIP) and AR (brown, DAB). Bar, 100 $\mu$m (low magnification) and 10 $\mu$m (high magnification). (B and C) Representative image of CD163-positive macrophages infiltration (B, high infiltration; C, low infiltration). (D and E) Representative image of 5αRed1 immunostaining, showing immunoreactivity in the cytoplasm of breast cancer cells (D, positive case; E, negative case). Scale bar, 100 $\mu$m.

When RAW264.7 cells interacted with 4T1 cells in a coculture chamber, mRNA and protein expression of *Arg-1*, an M2 macrophage marker was significantly increased when compared to the control (monoculture group), indicating that RAW264.7 cells were successfully polarized into an M2 phenotype (Fig. 3A). We next investigated mRNA expression, in 4T1 and RAW264.7 cells, of mouse AR (*Ar*) as well as 5αRed1 (*Srd5a1*) and *Akr1c6*, the ortholog of human *AKR1C3* and involved in androgen synthesis in the mouse (29). Real-time PCR analysis showed that *Ar* (Fig. 3B) and *Akr1c6* (Fig. 3C) mRNA was almost negligible in both cell lines, while *Srd5a1* mRNA was detected in both (Fig. 3D). In addition, the mRNA level of these genes was almost comparable in coculture condition (Fig. 3E and F). To investigate the effects of androgens on macrophages, we generated RAW264.7 sublines expressing AR (RAW-AR) and the corresponding negative control (RAW-CT) (Fig. 3G). When we treated RAW-CT and RAW-AR cells with synthetic androgen R1881, mRNA expression of M2 macrophage marker *Arg-1* was not affected by R1881 (Fig. 3H). We confirmed that AR protein was not

Table I. Association between CD163-positive macrophages and clinicopathological parameters in 116 breast carcinomas.

| | CD163 positive macrophages | | |
|---|---|---|---|
| | Low (n=77) | High (n=39) | P-value |
| Age[a] (years) | 55 (27-87) | 58 (37-76) | 0.3700 |
| Menopause | | | |
| Premenopause | 30 | 11 | 0.2500 |
| Postmenopause | 47 | 28 | |
| Stage | | | |
| I | 49 | 18 | 0.1000 |
| II | 19 | 11 | |
| III | 9 | 10 | |
| Pathological T factor | | | |
| pT1 | 56 | 22 | 0.0770 |
| pT2-4 | 21 | 17 | |
| Lymph node metastasis | | | |
| Negative | 59 | 20 | **0.0057** |
| Positive | 18 | 19 | |
| Histological grade | | | |
| 1 (well) | 39 | 6 | **<0.0001** |
| 2 (intermediate) | 30 | 16 | |
| 3 (poor) | 8 | 17 | |
| ER | | | |
| Negative | 9 | 14 | **0.0020** |
| Positive | 68 | 25 | |
| PR | | | |
| Negative | 17 | 20 | **0.0014** |
| Positive | 60 | 19 | |
| HER2 | | | |
| Negative | 69 | 30 | 0.0680 |
| Positive | 8 | 9 | |
| 5αRed1 | | | |
| Negative | 33 | 18 | 0.7400 |
| Positive | 44 | 21 | |
| Ki67 LI[a] (%) | 8 (1-44) | 21 (1-60) | **<0.0001** |

[a]Data are presented as median (minimum-maximum). All other values are presented as the number of cases. P<0.05 is considered indicative of statistical significance and are presented in bold print. 5αRed1, 5α-reductase 1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index; PR, progesterone receptor.

detected in 4T1 cells (Fig. 3G), and 4T1 cells were cocultured with RAW-CT or RAW-AR cells and cell proliferation was investigated by sphere-forming assay. As shown in Fig. 3I, sphere size was significantly increased by R1881 when the cells were cocultured with RAW-AR (P<0.01), while R1881 had no effect on monocultured 4T1 cells or those cocultured with RAW-CT.

*Androgen acts on macrophages to enhance breast cancer progression in a tumor-bearing mouse model*. In order to further address whether androgen acts on macrophages to promote breast cancer progression, we performed *in vivo* experiments using tumor-bearing mice. We generated 4T1 cells stably expressing AKR1C6 (4T1-AKR1C6) using pAKR1C6-FLAG vector or control vector (Fig. 4A). When we compared *in vitro* cell proliferation of these cells, it was comparable in 4T1-AKR1C6 and 4T1-CT cells (Fig. 4A). 4T1-CT or 4T1-AKR1C6 cells were subcutaneously injected together with RAW-AR cells on the left or right side of the back of mice, respectively (Fig. 4B; left image). The tumors on the right side (4T1-AKR1C6+RAW-AR) grew more rapidly compared with those on the left side (4T1-CT+RAW-AR) (Fig. 4C and D). In addition, immunohistochemistry demonstrated that Ki67 LI was higher in the tumor on the right side (70%) compared to those on the left side (30%) (Fig. 4E). We then performed the mirror experiment, where

Table II. Association between CD163-positive macrophages and clinicopathological parameters according to 5αRed1 and ER status in 116 breast carcinomas.

| | All cases (n=116) | | ER-positive cases (n=93) | |
|---|---|---|---|---|
| Parameters | 5αRed1-negative (n=51) | 5αRed1-positive (n=65) | 5αRed1-negative (n=41) | 5αRed1-positive (n=52) |
| Age | 0.42 | 0.73 | 0.53 | 0.61 |
| Menopause | 0.14 | 0.81 | 0.40 | 0.69 |
| Stage | 0.20 | 0.15 | 0.67 | 0.26 |
| Pathological T factor | 0.14 | 0.29 | 0.28 | 0.36 |
| Lymph node metastasis | 0.18 | **0.011** (Positive) | 0.63 | **0.043** (Positive) |
| Histological grade | **0.0013** (Positive) | **0.0069** (Positive) | 0.14 | **0.0011** (Positive) |
| ER | **0.0010** (Negative) | 0.23 (Negative) | - | - |
| PR | 0.082 | **0.0061** (Negative) | 0.18 | **0.0059** (Negative) |
| HER2 | 0.92 | **0.015** (Positive) | 0.23 | **0.0079** (Positive) |
| Ki67 LI (%) | **0.0017** (Positive) | **0.0020** (Positive) | 0.091 | **0.0009** (Positive) |

P-values of statistical analyses for the correlation between macrophage infiltration and clinicopathological parameters are provided. P<0.05 is considered significant and is represented in bold print. 5αRed1, 5α-reductase 1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index; PR, progesterone receptor.

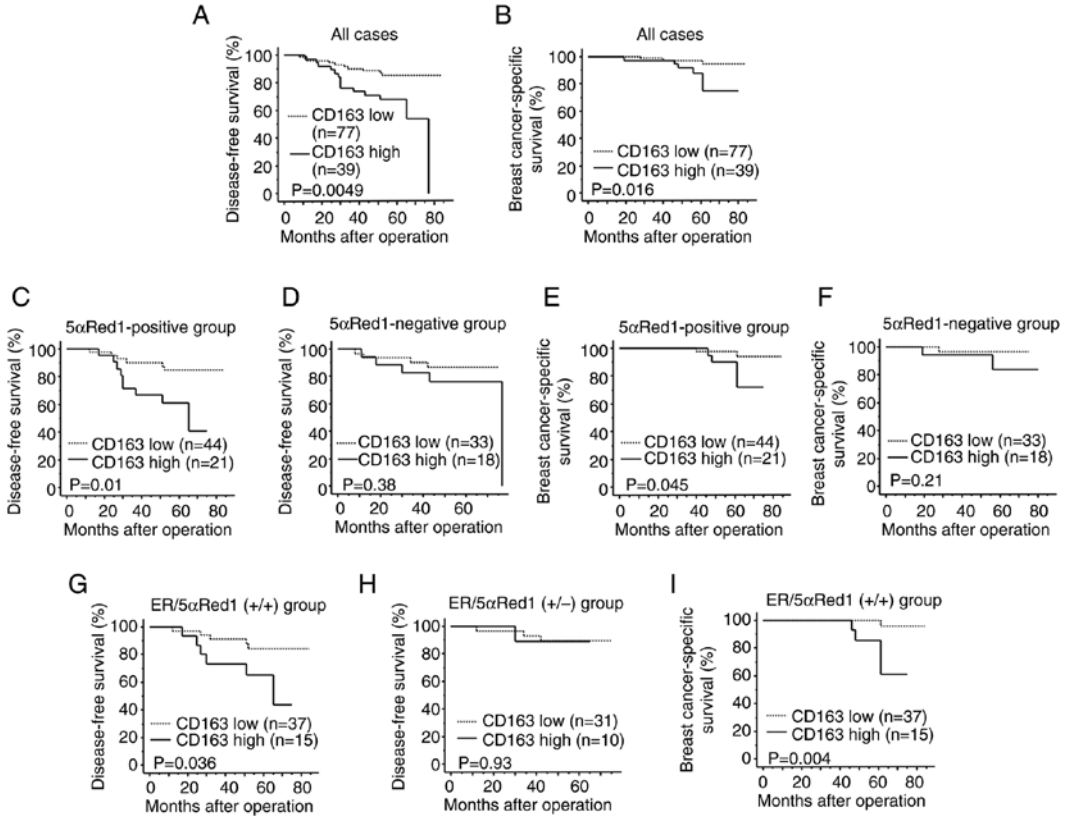


Figure 2. Association between CD163-positive macrophages and disease-free (A, C, D, G and H) or breast cancer-specific survival (B, E, F and I). (A and B) All 116 cases. (C-F): Survival curves were divided according to 5αRed1 status [C and E, 5αRed1-positive group (n=65) and D and F, 5αRed1-negative group (n=51)]. (G-I): Survival curves in ER-positive breast cancers are shown according to 5αRed1 status [G and I; 5αRed1-positive group (n=52) and H; 5αRed1-negative group (n=41)]. No patients had died within the follow-up period in the ER/5αRed1 (+/-) group.

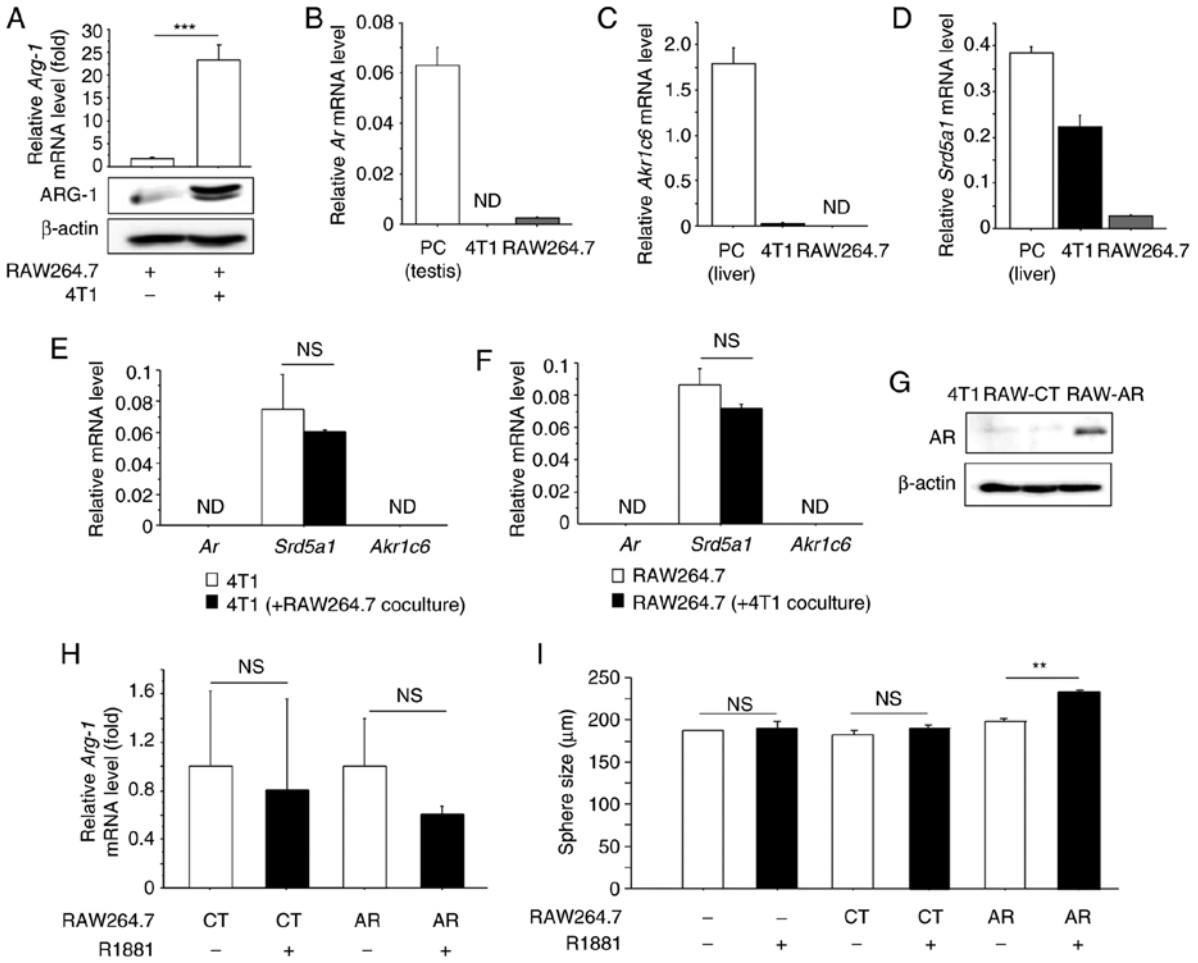


Figure 3. Effects of androgen action in macrophages on breast cancer cell proliferation. (A) RAW264.7 cells were cocultured with 4T1 cells for 3 days and Arginase-1 (*Arg-1*) mRNA expression (M2 macrophage marker) was evaluated by real-time PCR in RAW264.7 cells. *Arg-1* mRNA was evaluated as the ratio to ribosomal protein L13A (*Rpl13a*) mRNA level and relative *Arg-1* mRNA level was presented as the fold change compared to the control (monoculture) group. The statistical analysis was carried out using unpaired t-test and the data are presented as the mean ± SD (n=3). $^{***}$P<0.001 vs. the control group. Expression of ARG-1 protein in RAW264.7 cells was evaluated by immunoblotting. β-actin was used as a loading control. (B-D) Androgen receptor (*Ar*), (B) aldo-keto reductase family 1 member C6 (*Akr1c6*) (C) and 5α-reductase 1 (*Srd5a1*) (D) mRNA expression in 4T1 and RAW264.7 cells was evaluated by real-time PCR as the ratio to *Rpl13a*. The data are presented as the mean ± SD (n=3). (E and F) *Ar*, *Akr1c6* and *Srd5a1* mRNA expression was evaluated by real-time PCR in 4T1 (E) and RAW264.7 cells (F) in coculture for 3 days. These mRNAs were evaluated as the ratio to *Rpl13a* mRNA level. The statistical analysis was carried out using unpaired t-test and the data are presented as the mean ± SD (n=3). (G) Expression of AR in 4T1, RAW-CT and RAW-AR cells was assessed by immunoblotting. β-actin was used as a loading control. (H) RAW-CT and RAW-AR cells were treated with synthetic androgen R1881 (10 nM) for 48 h and *Arg-1* mRNA expression was evaluated by real-time PCR in RAW264.7. *Arg-1* mRNA was evaluated as the ratio to *Rpl13a* mRNA level and relative *Arg-1* mRNA level was presented as the fold change compared to control (without R1881). The statistical analysis was carried out using unpaired t-test and the data are presented as the mean ± SD (n=3). (I) 4T1 cells were cocultured with either RAW-CT or RAW-AR cells, and then exposed to R1881 (10 nM) in the low attachment surface plate for 3 days. Sphere size was evaluated by measuring 100 spheres in each well using ImageJ and averages were calculated. The statistical analyses were carried out using unpaired t-test and the data are presented as the mean ± SD (n=3). $^{**}$P<0.01 vs. the control group (without R1881). ND, not detected; NS, not significant.

4T1-AKR1C6 cells were subcutaneously injected together with RAW-CT and RAW-AR cells on the left or right side of the back of mice, respectively (Fig. 4B; right image). The tumors on the right side (4T1-AKR1C6+RAW-AR) grew more rapidly compared with those on the left side (4T1-AKR1C6+RAW-CT) (Fig. 4F and G). Ki67 LI was higher in the tumors on the right side (80%) compared to those on the left side (30%) (Fig. 4H).

## Discussion

This is the first report demonstrating expression of the androgen receptor (AR) in macrophages in human solid tumors, including breast carcinomas. To date, the focus of study for the action of androgen in breast cancers has been mainly its mediation by AR in breast carcinoma cells, while AR in stromal cells has not been explored. On the other hand, AR signaling in cancer stromal cells has been implicated in prostate carcinomas and associated with higher proliferation and invasion of prostate carcinoma cells (30,31). Moreover, expression of AR in macrophages has been so far reported in several human diseases. For instance, macrophages with AR expression contribute to the greater prevalence and severity of atherosclerosis in men, by upregulating cholesteryl ester content (20). In addition, testosterone has been reported to induce TNFα, NO and IL-6 in a polycystic ovary syndrome rat model and contribute to the pathogenesis of polycystic ovary syndrome (32). Therefore, it is reasonably speculated that androgens affect breast cancer progression through AR, not only in breast cancer cells but also intratumoral macrophages.

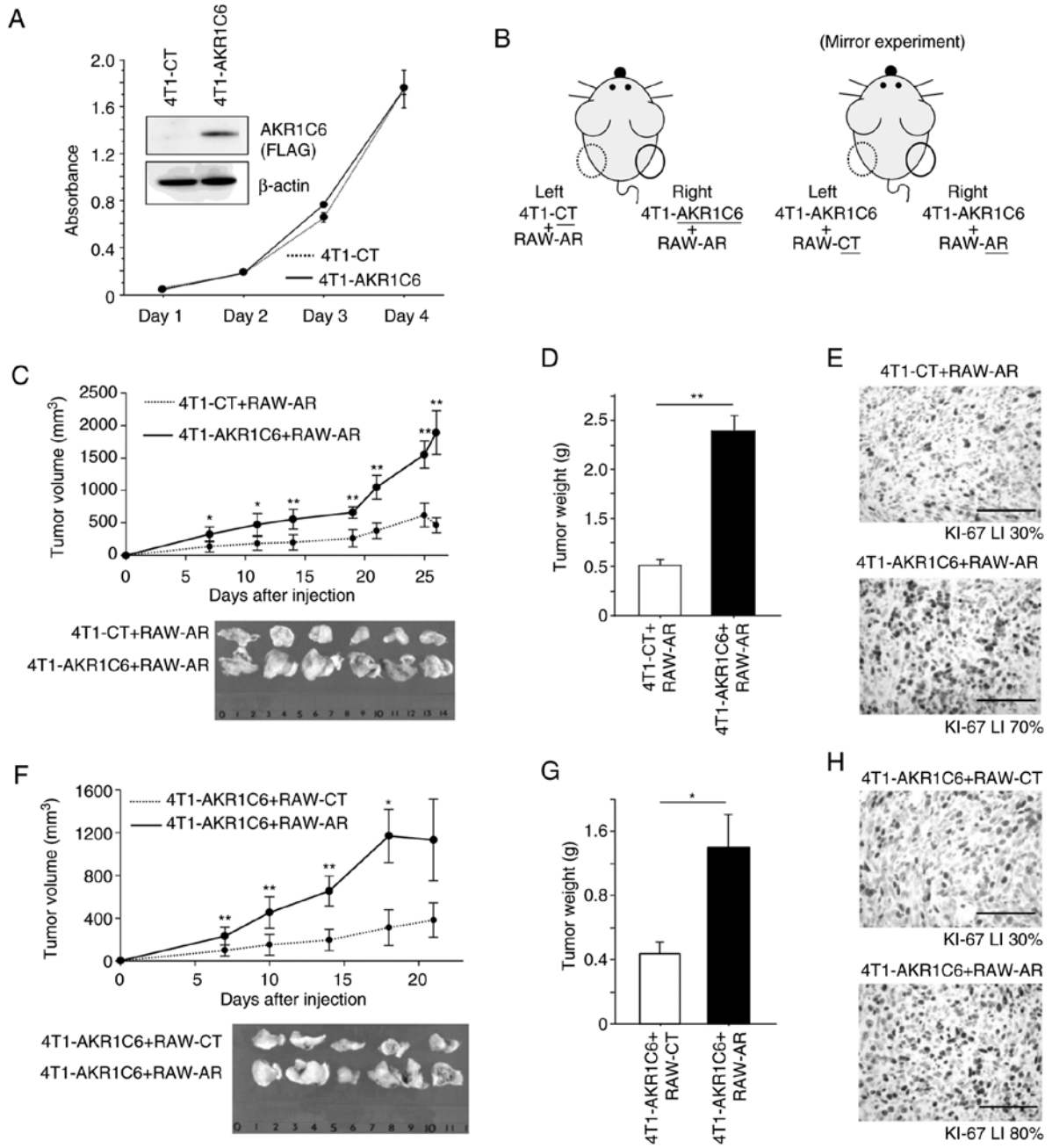


Figure 4. Effects of androgen action in macrophages on breast cancer progression were examined using tumor-bearing mice. (A) Aldo-keto reductase family 1 member C6 (AKR1C6)-FLAG protein was detected by immunoblotting for FLAG-tag. Immunoblotting for β-actin was carried out as a loading control. 4T1-AKR1C6 or 4T1-CT cells were seeded in a 96-well plate, and *in vitro* cell proliferation was measured by WST-8 method for 4 days. The statistical analyses were carried out using unpaired t-tests and the data are presented as the mean ± SD (n=4). (B) Outline images of the *in vivo* experiments. 4T1 cells were subcutaneously injected together with RAW264.7 cells in the left and right side of each mouse in various conditions. (C and F) Tumor volume was measured and calculated by modified ellipsoid formula 1/2 (length x width$^2$). Images of representative resected tumors: top; 4T1-CT+RAW-AR (C) or 4T1-AKR1C6+RAW-CT, (F) bottom; 4T1-AKR1C6+RAW-AR. (D and G) Comparison of resected tumor weight. (E and H) Representative images of Ki67 immunoreactivity in tumors. Scale bar, 100 $\mu$m. The statistical analyses were carried out using paired t-test and the data are presented as the mean ± SD. (C and D: n=6, F and G: n=5). $^{*}$P<0.05, $^{**}$P<0.01 vs. the control, respectively.

Breast carcinoma cells express androgen-producing enzymes such as 5α-reductase type 1 (5αRed1) and locally produce dihydrotestosterone (DHT), a bioactive androgen (2). We therefore hypothesized that intratumoral androgens possibly affect macrophages which express AR in a paracrine-manner. Then, we correlated macrophage infiltration with characteristics of breast carcinomas in all pathological samples. Macrophage infiltration was positively correlated with lymph node metastasis, higher histological grade and Ki67 LI while negatively correlated with the estrogen receptor (ER) and progesterone receptor (PR) in all 116 cases, showing agreement with previous reports (13,19). When we further analyzed them according to 5αRed1 status, correlation between macrophage infiltration and clinicopathological parameters showed differences between 5αRed1-positive and 5αRed1-negative groups, and this tendency was more prominent when the cases were limited to ER-positive breast cancers. Macrophage infiltration was in particular correlated with a more aggressive

phenotype of breast carcinomas with lymph node metastasis, higher histological grade, PR negativity, HER2 positivity and higher Ki67 LI in the 5αRed1-positive group but not in the 5αRed1-negative group. These findings indicate that the action of androgen in macrophages is important for the pro-tumorigenic roles of macrophages in breast cancers, especially in ER-positive breast cancers. This may be partly explained by the high diversity of cytokine receptor expression in breast carcinomas. For instance, it has been reported that the expression profile of cytokine receptor genes is quite different between the luminal type and basal-type breast cancer cell lines. Rearranged during transfection (RET), a receptor for glial cell line-derived neurotrophic factor (GDNF), is highly expressed in luminal-type breast cancer cell lines (33) and while production of GDNF is accelerated by androgens in testicular peritubular cells (34). Therefore, a future challenge will be to identify the soluble factors regulated by androgens in macrophages and their receptors in breast carcinoma tissues.

In the present study, macrophage infiltration was significantly correlated with increased risk of recurrence and worse prognosis, similarly as previous reports (35,36). Interestingly, this correlation was observed in the 5αRed1-positive group but not in the 5αRed1-negative group, suggesting that androgen action in intratumoral macrophages contributes to breast cancer progression and worse prognosis. Similar results were obtained even when the cases were limited to the ER-positive group, who had received adjuvant endocrine therapy. Although endocrine therapy targeting estrogen synthesis or ER has successfully improved clinical outcome of ER-positive breast cancer patients, *de novo* or acquired resistance is still frequently experienced in the clinical setting. We previously reported that intratumoral androgen production is increased following aromatase inhibitor (AI) treatment (37), and increased androgen signaling in breast cancer cells possibly causes resistance to AI (9). In addition, macrophages infiltrate more in metastatic breast carcinoma tissues than in primary breast carcinoma tissues (15). Considering our present findings and these previous reports, androgen action in macrophages as well as breast carcinoma cells seems to play pivotal roles for acquisition of endocrine resistance. Our recent findings may thus provide important clues to overcome endocrine resistance.

We next constructed *in vitro* and *in vivo* experiments using mouse breast cancer and macrophage cell lines, 4T1 and RAW264.7. Real-time PCR analysis showed that *Ar* and *Akr1c6* mRNA was negligible in both 4T1 and RAW264.7 cells. However, cell lines could not necessarily mimic the microenvironment of human breast carcinoma tissues. We demonstrated the expression of AR in breast cancer-associated macrophages by double-immunohistochemistry, and androgen-producing enzymes such as 17βHSD5 and 5αRed1 are frequently expressed in human breast carcinomas and contribute to intratumoral androgen synthesis (2,3). We therefore tried to mimic this situation using mouse cell lines by establishing AR- and AKR1C6-overexpressing sublines. In the sphere-forming assay using 4T1 and RAW-AR cell lines, which stably express AR, sphere size was significantly increased by androgens when 4T1 cells were cocultured with RAW-AR cells. Because 4T1 cells did not express AR, increased sphere size was due to androgen action in RAW-AR cells. Furthermore, *in vivo* experiments using a tumor-bearing mouse model showed that tumor volume as well as Ki67 LI were increased significantly when androgens were stability produced in breast cancer cells and AR was expressed in macrophages. Intratumoral macrophages produce numerous kinds of soluble factors and they are strongly linked to pro-tumorigenic roles for macrophages (38), although little is known about androgen-regulated soluble factors in intratumoral macrophages. However, some cytokines such as TNFα and IL-6 are induced by androgens in macrophages in both pathological and physiological conditions (17,22,39) and finasteride, a potent 5α-reductase inhibitor suppresses TNFα secretion from prostatic macrophages (40). In breast cancer, TNFα enhances invasiveness of breast cancer cells through matrix metalloproteinase (MMP) induction in macrophages (41). Moreover, IL-6 expression in macrophages isolated from hepatocellular carcinoma (HCC) is markedly upregulated by interaction with HepG2 HCC cell line and IL-6 promotes stemness of HCC via the signal transducer and activator of transcription (STAT)3 pathway (42). Recently, AR signaling in macrophages has been shown to promote migration and invasion of prostate cancer cell lines by increased triggering receptor expressed on myeloid cells-1 (TREM-1) signaling, regulating the expression of its downstream cytokines such as CCL2, CCL7, CXCL8 and CCL13 (31). On the other hand, there was no significant correlation between macrophage infiltration and 5αRed1 in human breast carcinoma tissues and R1881 did not alter *Arg-1* mRNA expression in RAW-AR cells. Taken together, these results show that androgen action may not necessarily correlate with macrophage polarization but androgen activates macrophages by inducing soluble factors which have pro-tumorigenic roles in human malignancies. Further examination is needed to identify androgen-induced soluble factors in intratumoral macrophages, in order to explore the relationships between androgens and the breast cancer microenvironment.

One of the limitations of the present study is the lack of quantitative data on AR-positive macrophages due to the relatively weak immunoreactivity of AR in macrophages, making it impossible for us to directly evaluate the significance of androgen action upon them. Alternative quantitative methods such as flow cytometry will be required in the future. Moreover, we did not compare the tumor formation of 4T1-CT and 4T1-AKR1C6 cells without RAW264.7 cells, although we concluded that androgen action may not correlate with macrophage polarization as described above. In addition, the effect of finasteride, 5α-reductase inhibitor on intratumoral macrophages was not investigated in the present study. Further *in vivo* examination is needed to reveal in more detail the action of androgen in macrophages. Finally, *in vitro* and *in vivo* experiments were conducted using 4T1 mouse breast cancer cells, which do not express ER and we could not experimentally verify the role of androgen action of macrophages in ER-positive breast cancers. The reason why we used 4T1 is that the mouse models of ER-positive breast cancer cells are lacking (43) and 4T1 cells are widely used for mouse breast cancer models in many previous studies (44-46). In addition, we could not differentiate human monocytic THP-1 cells into the M2 phenotype by coculture with human breast cancer cell lines including MCF-7 cells, which express high levels of ER. Therefore, the present study is the starting point of further investigation into novel androgen action

focusing on intratumoral macrophages in breast carcinoma tissues, which may help understanding the complex breast cancer microenvironment.

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**Availability of data and materials**

All data and material presented in this article are available from the corresponding author upon reasonable request.

**Author's contributions**

MY performed cell and animal experiments and prepared the manuscript. KT contributed to the study conception and design and performed statistical analysis. MS performed the immunohistochemistry. AS assisted with the animal experiments. YM and YO gave skillful suggestions in regards to the immunohistochemistry. MM and HS collected the clinical samples and clinicopathological information. TS performed the pathological analysis. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

The study using clinical samples was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (approval no. 2016-1-697, 2019-1-219). Experiments and analyses were performed in accordance to the Helsinki declaration. This is a retrospective study and therefore informed consent had not been obtained. All animal experiments were performed in accordance with ARRIVE guidelines with the approval of the Institutional Animal Care and Use Committee at Tohoku University (2019MdA-172).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors have no competing interests.

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