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Effects of dihydrotestosterone on gene expression in mammary gland

D.H. Aboghe, C. Bolduc, M. Yoshioka, J. St-Amand*

Functional Genomics Laboratory, Molecular Endocrinology and Oncology Research Center, Québec Genome Center, Laval University Medical Center (CHUL), Department of Anatomy and Physiology, Laval University, 2705 Boul. Laurier, Québec G1V 4G2, Canada

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ABSTRACT

Breast cancer is the most common cancer among women. Androgens, the male sexual hormones produced by ovary, act as protector of mammary gland. To elucidate the possible effects of dihydrotestosterone (DHT) on the transcriptome of mammary gland, serial analysis of gene expression was carried out on three groups of gonadectomized mice. After gonadectomy (GDX), DHT was injected 3 or 24 h before sacrifice, whereas the control (GDX) group received vehicle solution. Approximately 42,000 tags were sequenced in each group. Genes involved in the cytoskeletal and extracellular matrix, such as troponin I skeletal fast 2 and keratin complex 1 acidic gene 14, were upregulated. In the immunity, complement component 1 q subcomponent gamma polypeptide and expressed sequence tag similar to lectin galactose binding soluble 3 were downregulated by DHT, whereas serine (or cystein) proteinase inhibitor clade A member 1a was upregulated. In the energy metabolism, the gene expression level of cytochrome *c* oxidase subunit I was upregulated by DHT, while NADH dehydrogenase subunit 2 was downregulated. In addition, transcripts involved in transport metabolism, such as apolipoprotein A-1, were upregulated by DHT, whereas retinol binding protein 4 plasma was downregulated. Several previously unknown sequence tags were identified, which may allow to characterize new molecules of interest. These results suggest the suppression of immune response in normal mammary gland after DHT injection. This study can assist in refining research on the role of androgens in mammary gland homeostasis and breast cancer.

1. Introduction

Breast cancer is the most common type of cancer and the first cause of cancer-related mortality among women across the world. However, a large proportion of breast cancer may be avoided with a better understanding of its characteristics, which may help developing a definitive and specific treatment for each type of cancer. In addition, according to its unique developmental aspects, its growth factors and its complex regulation by hormones, the mammary gland has been helpful to the understanding of the pathology of cancer. Recently, there is a particular interest for growth factors which seem to play an important role in apoptosis regulation, and may be involved directly or indirectly in the cancer process with a mechanism similar to the development and morphogenesis without activation by gene mutation [1].

Sexual steroid hormones play an essential role in the development, morphogenesis, growth and functions of mammary gland as well as in the development and treatment of breast cancer [2]. Many studies have shown that 70–80% of primary breast tumors express the estrogen receptor (ER), 50–70% express the progesterone receptor (PR), and 70–90% express the androgen receptor (AR) [3]. A normal ovary produces estrogens, progesterone and androgen, with an androgen production exceeding the two others [4]. Endogenous androgens normally inhibit estrogen-induced mammary epithelial proliferation (MEP), and therefore act as protector of mammary gland [4]. Moreover, the androgen receptor blockade results in the increase of the MEP. Furthermore, testosterone could reduce mammary epithelial ERα and increase ERβ expression [4].

Consequently, numerous studies suggest a treatment with a balanced formulation of ovarian hormones for treatment of breast cancer [4,5]. In addition, estrogens suppress gonadotrophins, reduce ovarian androgenesis and increase the levels of globulin which binds sex hormones, resulting in reduced androgen bioavailability [4]. Thus, a better understanding of the androgenic mechanisms involved in breast growth inhibition and the network complexity is necessary.

Several large-scale transcriptome studies have been conducted to identify the differentially expressed genes between normal cells and breast cancer cells by using microarray [6], differential display [7] and serial analysis of gene expression (SAGE) [8]. However, few studies have investigated the modulation of gene expression by androgens [9–11]. Moreover, taking into account the fact that androgens act on target tissues through the androgen receptor by

* Corresponding author. Tel.: +1 418 654 2296; fax: +1 418 654 2761.
E-mail address: Jonny.St-Amand@crchul.ulaval.ca (J. St-Amand).

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transcriptional regulation of androgen-regulated genes [12], an *in vivo* model is necessary in order to maintain tissue properties stable during experimental treatment, and to realise a system which respects the tissue–tissue and/or cell–cell interactions.

SAGE is a powerful strategy to analyze quantitatively, simultaneously and differentially the expression of all the mRNAs of the cells in a given tissue, with notably the expression of known and unknown genes [13]. In addition, it can constitute a great tool for the characterization of molecular mechanisms of interest [14]. Therefore, this study has used this powerful strategy to analyze the effects of dihydrotestosterone (DHT) injection, the most potent androgen, on mammary gland of gonadectomized (GDX) mice.

2. Materials and methods

2.1. Sample preparation

Forty-two female C57BL6 mice (12–15 weeks old) were obtained from Charles River Laboratories (St. Constant, Canada). The mice had access to Lab Rodent Diet No. 5002 and water *ad libitum*. On day 1 of the study, animals were bilaterally ovariectomized (GDX) under isoflurane anesthesia. Prior to the necropsy performed on day 8 of the study, mice have received a single subcutaneous injection (0.2 ml/mouse) of the vehicle alone (0.4% (w/v) to Methocel A15LV Premium/5% ethanol; group 1) or DHT (groups 2 and 3). The injection of vehicle was performed 24 h prior the necropsy for animals in group 1 while DHT was injected 3 h (group 2) or 24 h (group 3) prior to the necropsy. The experimentation was performed according accepted standards of human animal care. A mammary gland portion was dissected, and the samples from all mice of the same group were pooled to eliminate interindividual variations and to extract sufficient amount of mRNA. The samples were stored at −80 °C until the analysis. The serum levels of hormones were determined by gas chromatography-mass spectrometry from GDX (14.12 ng/ml), DHT3h (5.45 ng/ml) and DHT24h (0.08 ng/ml).

2.2. Transcriptome analysis

The SAGE method was performed as previously described [13,15,16]. Polyadenylated RNA was annealed with the biotin-5′-T_{18}-3′ primer and converted to cDNA with the cDNA synthesis kit (Invitrogen). The resulting cDNA library was digested with NIaIII (anchoring enzyme), and the 3′ restriction fragments were isolated with streptavidin-coated magnetic beads (Dynal) and separated into two populations. Each population was ligated to one of the two annealed linker pairs and extensively washed to remove unligated linkers. The tag beside the most 3′ NIaIII restriction site (CATG) of each transcript was released by digestion with BsmFI (tagging enzyme). The blunting kit from Takara Co. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR with an initial denaturation step of 1 min at 95 °C, followed by 22 cycles of 20 s at 94 °C, 20 s at 60 °C and 2 s at 72 °C with 27 bp primers [16]. The PCR products were digested with NIaIII and the band containing the ditags was extracted from acrylamide gel. The purified ditags were self-ligated to form concatemers of 500–1800 bp isolated by agarose gel. The resulting DNA fragments were ligated into the SphI site of pUC19 and cloned into UltraMAX DHT5αFT (Invitrogen). White colonies were screened by PCR to select long inserts for automated sequencing.

2.3. Validation by quantitative real-time PCR (Q_RT-PCR)

First-strand cDNA was synthesized using 5 μg of pooled RNA of each experimental group (GDX, DHT3h and DHT24h) in a reaction containing 200 U of Superscript III Rnase H-RT (Invitrogen Canada Inc.), 300 ng of oligo-dT_{18}, 500 mM deoxynucleotides triphosphate, 5 mM dithiothreitol and 34 U of human RNase inhibitor (Amersham Pharmacia, Piscataway, NJ) in a final volume of 50 μl. The resulting products after treating with RNase A were purified with Qiaquick PCR purification kits (Qiagen Inc.). The cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler real-time PCR apparatus (Roche Inc., Nutley, NJ). Reagents were obtained from the same company and were used as described by the manufacturer. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, AB) and their specificity was verified by blast in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following: lectin galactose binding soluble 3 (Lgals3), NM_010705, 554/667; retinol binding protein 4 plasma (Rbp4), NM_011255, 417/664. The expression levels of mRNA (number of copies/μg total RNA) were calculated using a standard curve of crossing point (Cp) versus logarithm of the quantity. The standard curve was established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) and a LightCycler 3.5 program provided by the manufacturer (Roche Inc.). The Q_RT-PCR was performed in triplicates. Data are expressed as a ratio to the Hprt1.

2.4. Statistical analysis

The transcript identification of differentially expressed genes with more than a twofold change ($p \leq 0.05$) was carried out by using the comparative count display (CCD) test between DHT and GDX groups [17]. A normalisation of the level of gene expression was set to 50,000 in order to facilitate visual comparison in tables. The data of Q_RT-PCR were analyzed by ANOVA with Dunnett's post hoc test to identify the significant DHT effects in female GDX mice ($p < 0.05$).

3. Results

Using SAGE method, it was possible to quantify the expression frequency of all known or unknown genes. Three SAGE libraries (GDX, DHT3h and DHT24h) were constructed to characterize the effects of DHT on the mammary gland transcriptome. A total of 131,695 tags were sequenced, representing the expression level of 48,196 tag species. Table 1 shows all the genes significantly regulated by DHT in mammary gland. The majority of the tags represent known genes ($n = 9$), whereas several others represent novel transcripts ($n = 6$). Among 15 regulated transcripts, there were 9 transcripts upregulated by DHT, while 6 transcripts were downregulated.

Genes involved in the cytoskeletal and extracellular matrix, such as keratin complex 1 acidic gene 14 (Krt14) and troponin I skeletal fast 2 (Tnni2), were upregulated by DHT. In the immunity, complement component 1 q subcomponent gamma polypeptide (C1qc) and expressed sequence tag (EST) similar to lectin galactose binding soluble 3 (Lgals3) were downregulated, while serine (or cystein) proteinase inhibitor clade A member 1a (Serpina1a) was upregulated by DHT. In energy metabolism, the gene expression level of cytochrome *c* oxidase subunit I (MtCo1) was upregulated by DHT, while NADH dehydrogenase subunit 2 (MtNd2) was downregulated by DHT. In addition, transcripts involved in transport metabolism, such as apolipoprotein A-1 (Apoa1) was upregulated by DHT, whereas retinol binding protein 4 plasma (Rbp4) was