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Membrane Androgen Receptor(s) and their Role in Prostate Cancer

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Abstract

Androgens, the steroid hormones, typically mediates their action by binding to the cytosolic Androgen Receptor(s) (AR), via the classical or genomic pathway. Androgens can also act through a non-classical or non-genomic pathway interacting with receptors present on the plasma membrane of cells. Although the identity of the nuclear AR is well established, the identity of the membrane AR is still not clear. Through independent studies, three proteins have been identified that are present on plasma membranes of prostate cells and can mediate androgen signalling, viz, GPRC6A, AR8 and ZIP9. Although these proteins can mediate androgen signalling, the membrane receptor which is used most frequently and specifically for mediating androgen action in prostate cells is not confirmed. Recent research has shown that the non-genomic androgen signalling plays a key role in progression of prostate cancer (PCa). In this review, the potential of these three proteins for their ability to act as the membrane AR has been analysed. The use of membrane AR as a novel target for treatment of PCa has also been discussed.

Keywords: Androgen, Androgen Receptor, Genomic Pathway, Non-Genomic Pathway, Prostate Cancer

1. Introduction

Androgens play a pivotal role in the development of male reproductive organs[27]. The effects of androgens are primarily mediated by binding to the cytosolic AR. Upon binding of androgen to the AR, the AR dimer translocates into the nucleus where it binds to androgen responsive genes and modulates their transcription. This is the classical or genomic pathway which takes hours or days to manifest as it involves changes in gene transcription[7]. Recent research has shown that androgens also act through a non-classical or non-genomic pathway which takes place in seconds or minutes. This pathway is mediated through membrane AR and is not inhibited by routine inhibitors of androgen signalling[7].

Androgens also play an important role in development and progression of PCa[24]. For last several decades, androgen removal or castration has been the primary treatment mechanism for PCa patients. However, it was noted that eventually the tumor stops responding to androgens removal and starts growing in mass. Thus, castration alone is not enough to prevent the growth of tumor as it transits from the androgen dependent to independent-stage[6]. The androgens independent tumors are resistant to currently available treatments and, therefore, intensive studies are required to understand the mechanisms that lead to transition to castrations resistant growth[9]. Also, in androgen-independent cell lines genetic changes occur due to which the AR responds to a lower concentration of androgens or the mutated AR responds to non-androgenic signals. It was noted that the castrations resistant growth is not induced through the classical mechanism involving nuclear receptor rather through membrane receptors that lead to activation of rapid signalling pathways which are non-genomic in nature[2]. Well documented non-genomic signalling pathways activated by androgens include Mitogen-Activated Protein Kinase (MAPK) pathway, tyrosine kinase c-Src,

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and Protein Kinase A(PKA)[1,3,4]. Non-genomic signalling by androgens is often mediated through membrane receptors rather than the classical AR. In fact, a few membrane proteins namely GPRC6A, AR8 and ZIP9 have been identified through independent studies that can mediate androgen signalling in androgen-independent (castration) conditions[2,15,28]. It is however not clear if any/all of them are involved in mediating androgen signalling under normal circumstances in prostate cells. For a better understanding of membrane AR signalling and its role in PCa additional studies are needed. In this article, the membrane proteins which would mediate androgen signalling have been analysed for their suitability as the membrane AR. The expression and role of these three proteins in various PCa cell lines are also highlighted.

2. GPRC6A

GPRC6A belongs to class C of the G-Protein Coupled Receptor (GPCR) family. The class C receptors include the nutrient receptors derived from amino acids and nutrient transporters from bacteria[5,12]. GPRC6A protein is made of 926 amino acids and is located on chromosome band 6q22.31. It contains a seven transmembrane (7TM) domain and a long amino terminal domain of 590 amino acids[26].

GPRC6A exhibits 45% resemblance to odorant goldfish 5.24 receptor which suggests that it is the human orthologue of the goldfish receptor. It shows greater expression in peripheral tissues, brain, kidney, testis, skeletal muscle and white blood cells[26].

Studies by many groups on GPRC6A knockout mice suggested the role of GPRC6A in metabolism, inflammation and endocrine regulation[15,17].

Min Pi group, in 2010, examined the role of GPRC6A in mediating the rapid, non-genomic effects of androgen in various heterologous models[16]. GPRC6A, in response to testosterone (T), rapidly stimulated ERK activation (Figure 1). ERK, an intracellular protein kinase, functions in regulation of cell division and activate many transcription factors like ELK1 and other downstream protein kinases[11]. Impermeable T/BSA activates ERK in cells transfected with GPRC6A but not in un-transfected control cells[16]. The activation of these kinases finally leads to modulation in the transcription of genes which are involved in PCa progression (Figure 1). Cells isolated from GPRC6A null mice failed to show the rapid signalling responses to androgen. SiRNA-mediated knockdown of GPRC6A in prostate cells expressing endogenous GPRC6A attenuated their non-genomic responses to androgens. This rapid response was membrane-mediated and was not dependent on endogenous AR, as the heterologous cell culture models were devoid of nuclear AR. Also, the T-induced rapid response was not inhibited by the anti-androgens flutamide and bicalutamide.7« vivo experiments demonstrated that T treatment at a pharmacological dose stimulated both ERK activity and Egr-1 expression in bone marrow and testis of wild type mice, but the response was attenuated in GPRC6A null mice[16].

The expression of GPRC6A is much higher in cancer-derived prostate cell lines such as 22RV1, PC-3 and LNCaP than normal prostate cells such as RWPE-1[16]. However, RT-PCR studies with intron spanning primers for GPRC6A in prostate cell lines such as RWPE-1, 22Rv1, PC-3 and LNCaP resulted in a product of 428 bp in all prostate cells. The level of GPRC6A expression was five-fold higher in prostate cell lines viz., 22Rvl, PC-3 and LNCaP than in normal human prostate cell line RWPE-1[15].

Many research groups demonstrated GPRC6A activation by stimulation with T and osteocalcin in the presence of extracellular calcium[14-16]. Recent research in mice demonstrated that osteocalcin (OCN) regulates T production in males, but not in females[14]. HEK293 cells were transfected with GPRC6A and the cells were exposed to different concentrations of T ranging between 5 and 25 nM, to check for androgen binding. GPRC6A showed maximum binding to T between the range 1020 nM. To analyse the specificity of binding of GPRC6A to T, competition receptor binding assay was performed using cold T. Addition of cold T at concentrations 0, 25, 50, 100, 200 nM increasingly inhibited binding to labelled T demonstrating the specificity of binding[16].

Other steroids like dehydroandrosterone, dihydrotestosterone, 17 β-estradiol and progesterone were analysed for their potential to activate GPRC6A. Dehydroandrosterone, dihydrotestosterine and 17 (3-estradiol could elicit variable degrees of GPRC6A-mediated phosphorylation of ERK in HEK-293 cells (transfected with GPRC6A) at higher concentrations than T. Progesterone did not activate GPRC6A but inhibited ERK at concentrations upto 80nM[16]. GPRC6A was also activated by extracellular calcium, amino acids, osteocalcin and androgens[15]. Despite specific androgen binding

ability these variable sensing properties of GPRC6A make it a coordinator for nutritional and hormonal anabolic signals, rather than a sole effector for androgen[16].

3. AR8

AR8 is a newly identified isoform of AR[28]. It is a splice variant of the original full-length nuclear AR and consists of 573 amino acids[28]. The protein sequence of AR8 differs from that of other AR variants due to a substitution of all the amino acids after N-Terminal Domain (NTD) of a conventional AR with a unique C-terminal sequence[10]. AR8 is composed of exons 1,3 and 3b[28]. Due to usage of an alternative splice acceptor site in exon 3, this transcript encodes a protein containing the N-terminal transactivation domain and a 33-amino acid unique sequence at the C terminus. This C-terminal truncated variant of AR lacks the DNA Binding Domain (DBD) required for transcriptional activity and is primarily localised in plasma membrane due to palmitoylation of two cysteine residues within its unique C-terminal sequence. This membrane localisation of the protein is confirmed by over-expression studies in COS-1 or PCa cells (LNCaP and CWR-R1)[28].

Studies show that several AR splice variants (AR3, AR4 and AR5) lacking the ligand binding domain are upregulated in a hormone-resistant prostate cancer cell line (CWR22RV1) and promote castration-resistant growth[9]. The AR8 protein does not function as an independent transcription factor due to lack of DBD and probably functions through a non-genomic mechanism. The lack of transcription activity of AR8 was confirmed by testing a series of reporters driven by androgen response element-containing promoters. Immunofluorescence studies indicated that when AR8 was over-expressed in COS-1 cells, it mainly localized in the plasma membrane and only sparsely in some perinuclear compartments[28]. Studies show that it co-operates with AR to potentiate androgen and growth factor response in PCa cells by promoting AR association with EGFR on the plasma membrane and enhancing AR tyrosine phosphorylation[28].

The level of AR8 transcript was two-three folds elevated in castration-resistant LNCaP derivatives such as C4-2, C4-2B and CWR22 xenograft tumors compared with its hormone-sensitive counterparts[28]. The level of AR8 transcript relative to nuclear AR appeared to be increased in CWR-R1 hormone resistant prostate cancer cells in response to treatment with androgen suggesting that the relative ratio of AR8 to AR is higher when cell proliferation is enhanced[28]. To examine the role of AR8 in promoting DHT induced AR transcriptional activity, LNCaP cells were infected with lentivirus encoding AR8.

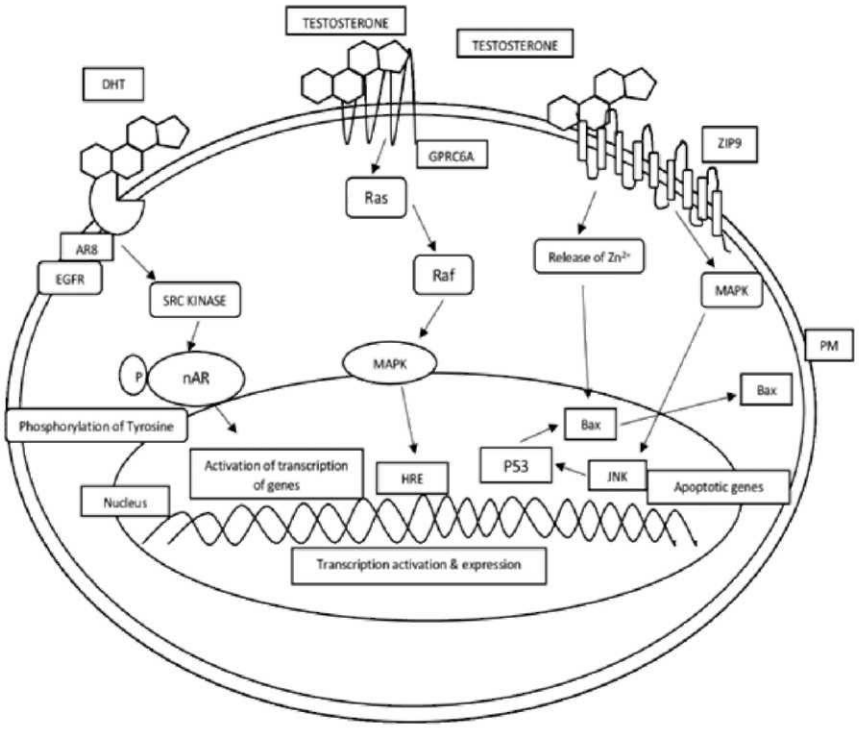

Figure 1. Schematic representation of putative membrane androgen receptors and their mechanism of action in the prostate cells.

At 16 hrs post-infection the cells were transfected with ARR2 Luciferase reporter and treated with 1nM DHT. Thirty-fold higher ARR2 driven reporter readout was observed in presence of AR8 indicating that AR8 potentiates AR transcriptional activity[28]. Studies show that AR8 activates SrcKinase (tyrosine kinase) pathway inside the cells[28].

To study the role of AR8 in PCa cells, biotin-conjugated peptides were synthesized (containing the AR8 C-terminal sequence) and used to identify proteins interacting with AR8 in CWR-R1 cells[28]. Mass spectrometric analysis revealed that multiple proteins (actin and tubulin) are associated with AR8 unique C-terminal sequence demonstrating that AR8 is associated with cytoskeletal proteins in PCa cells[28]. Binding studies to assess sensitivity and specificity of AR8 to androgens would further clarify its role in mediating androgen signalling in prostate cells[28].

4. ZIP9

ZIP9, a zinc transporter protein, was first identified in Atlantic croaker as a membrane protein which can bind androgens[2]. Its homologs were subsequently identified in human PCa cells[2]. In the normal prostate, high zinc concentration serves to inhibit citrate oxidation thereby promoting the production and secretion of citrate, a major constituent of prostatic fluid. Decrease in zinc level indicates risk of developing PCa as zinc inhibits prostate cancer cell growth by induction of cell cycle arrest and apoptosis. These results gave the first indication that a zinc transporter protein can function as specific steroid membrane receptor[2]. By cloning and expression of cDNA from Atlantic croaker (Micropogonias undulates) ovaries, a seven-transmembrane protein of 33-KDa (307-312 amino acids) was obtained which demonstrated binding and signalling characteristics of a membrane AR[2]. This protein has 81-93 % amino acid sequence identity with zinc transporter ZIP9 (SLC39A9) subfamily member indicating that it is the ZIP9 protein. It has high affinity (dissociation constant, Kd, 12.7 nM), limited capacity, single binding site-specific for androgens, characteristic of steroid receptors[2].

ZIP9 protein can increase cAMP levels and activate G-Protein-Coupled Receptor (GPCR) in response to treatment with T[2]. Specific androgen binding to an ovarian plasma membrane fraction was demonstrated by radioreceptor assay protocol which consisted of short-term incubation with [3H]T. Saturation and scatchard analysis of T binding to an ovarian plasma membrane fraction indicated the presence of a single, high affinity (Bmax= 2.81=+- 0.31pmol/mg protein) or (.00281 nmol) androgen binding site. Specific androgen binding was readily displaceable, and the association and dissociation kinetics were rapid with half time under 5 min. Competitive binding studies revealed that progesterone showed the same high relative binding affinity as T

(Figure 1.) whereas bicalutamide, cortisol and estradiol-17|3 were ineffective. Androstenedione, mibolerone and hydroxyflutamide had lower affinities[2].

ZIP9, a zinc transporter, plays a role in mediating zinc flux into and out of cells and subcellular organelles[22]. ZIP9 shows 2-fold greater expression in biopsies of human malignant prostate tissues than in normal prostate tissues[2]. Also, ZIP9 shows 12-15 fold greater expression in androgen sensitive cell lines e.g., LNCaP cells than in androgen insensitive cell lines e.g., PC-3 and DU-145 cells[2].

In the ovaries of the Atlantic croaker, treatment with DHT caused a 50% reduction in in vitro estradiol production compared to controls, which was not changed by co-incubation with a transcription blocker actinomycin D at a concentration of 10ug/ml indicating that nuclear AR was not involved. This is the first evidence of a non-genomic, rapid, cell surface mediated, specific androgenic action on in vitro ovarian steroidogenesis[19]. Incubation with DHT-BSA, which is unable to enter the cell causes an inhibition of estradiol production similar to unconjugated DHT, indicating that androgen can decrease estradiol production through a nongenomic mechanism via a binding site located on the cell surface[23].

Knockdown studies in LNCaP cells indicate that this protein mediates androgen-induced apoptosis and increase in intracellular zinc ion concentration. Treatment of AR-positive LNCap cells with testosterone causes significant increase in intracellular zinc concentrations and apoptosis in cells transfected with non-target siRNA, whereas these effects were completely blocked after transfection with ZIP9 siRNA[2].

5. Discussion

The three membrane proteins analysed in this review exhibit different features with respect to androgen

signalling and binding to androgens[2,16,28]. Completely different binding kinetics are observed for the three proteins. GPRC6A gets activated by T in the concentration range 0-25nM[16] whereas ZIP9 shows maximum T binding at much lower androgen concentration of 0.00281nM[2]. Although specific binding studies have not been carried out for AR8 yet AR8 promotes AR transcriptional activity at 1nM DHT[28]. Also, studies suggest that any AR transcript that does not contain DBD is constitutively active[13]. Ligand specificity of each of these proteins is different. While GPRC6A responds to many ligands like calcium, amino acids, osteocalcin and androgens[17], ZIP9 shows specific binding towards androgen[2]. AR8 being a truncated version of nuclear AR co-operates with the prototype AR to potentiate androgen and growth factor response in PCa cells by promoting AR association with EGFR on the plasma membrane[28]. Thus, ZIP9 exhibits highest binding affinity and sensitivity among the three receptors indicating its suitability as a potential mAR.

The expression of the three membrane proteins varies widely in PCa cell lines and normal prostate cells. In general expression of all three proteins is higher in PCa cell lines as compared to normal prostate cells. It is however noteworthy that while GPRC6A and AR8 lead to enhancement of AR activity and cell proliferation ZIP9 expression promotes apoptosis.

Three completely different signalling pathways are activated by these proteins. GPRC6A activates ERK pathway[16]. When GPRC6A is coupled to Gai and Gaq it activates intracellular calcium and inhibits cAMP pathway. The most reliable readout for GPRC6A activation is ERK and SRE (Steroid Response Element)-Luciferase promoter-reporter activity. Pi et al used these readouts and biochemical inhibitors to demonstrate that Gai, P13K, PKC, Src and Ras/RAF/MEK/ERK pathways are downstream effectors of GPRC6A[16]. On the other hand, AR8 activates Src Kinase (Tyrosine Kinase) pathway. Although the activated Src Kinase is localized to plasma membrane and the nuclear AR is present in the cytosol or nucleus, the membrane-anchored AR8 mediates Src-induced AR activation. EGF treatment promotes Src association with EGFR and induced Src-kinase activity which were further increased by AR8. AR8 knockdown diminished the EGF induced Src kinase activation[28]. ZIP9 causes the Zinc signalling activation and activates apoptotic pathway[2]. ZIP9 is expressed in malignant prostate biopsy samples which indicates its up-regulation in malignant cells. Although, other ZIP proteins like ZIP1, ZIP2 and ZIP3 are DOWN-REGULATED in PCa tissues as decreased zinc is hallmark for PCa. ZIP9 is the only protein which is expressed in PCa which suggested that it is related to an increase in zinc requirement in rapidly dividing cells. Also, its expression is seen highest in the sites for proliferation in PCa i.e., glandular epithelial cells[18]. Thus, ZIP9 is activated through other compensatory pathways through which it functions as mAR by mediating zinc signalling activation and apoptotic pathway. The comparison of signalling attributes of these three additional membrane proteins have not revealed activation of PKA pathway. Separate studies on non-genomic androgen signalling have indicated activation of PKA by testosterone in prostate cells[1]. Activation of PKA has also been correlated with malignancy in PCa cells and neuroendocrine trans-differentiation (NED) of PCa cells[20]. Thus, the presence of membrane proteins/ GPCRs within PCa cells that may mediate androgen signalling cannot be ruled out. Additional studies are required to identify the mARs (membrane Androgen Receptors) which may activate alternate signalling pathway upon androgen stimulation, as they may provide new insight into the mechanism of androgen signalling and development of PCa.

6. Membrane AR and Prostate Cancer

In this review, three membrane proteins that are totally different from each other in terms of structure and function have been analysed for their potential to act as mAR. GPRC6A which is expressed at low levels in normal prostate tissues and prostate cell lines showed marked elevation in its expression in PCa cells[15]. In fact, expression was 20-fold greater in human PCa cell lines like 22RV1, LNCaP and PC-3 cells[15]. Also, GPRC6A is reported to be upregulated in many primary and metastatic cancers[21] and promotes tumor formation and cancer progression[25]. GPRC6A is a strong activator of ERK signalling[17], a possible mechanism by which the receptor directly regulates PCa growth, since ERK pathway has a central role in PCa cell proliferation[8]. Further, GPRC6A indirectly influences the development and progression of PCa through its effect on sex steroid metabolism[17].

AR and its membrane localized spliced variant i.e. AR8, play important roles in regulating the transcription program essential for castration resistance[28]. AR8 shows upregulation in castration-resistant PCa cells like

C4- membrane localized Src Kinase to phosphorylate AR potentiating AR signalling. Thus, EGFR, Src, AR and AR8 form a dynamic signalling complex in response to EGF[28]. Specific knockdown of AR8 expression in PCa cells leads to decrease in EGF-induced Src activation and AR phosphorylation[28]. This causes the attenuation of proliferation and increase in apoptosis in PCa cells cultured in androgen-depleted medium[28] depicting the critical role of this AR splice variant in the progression of PCa.

Over-expression of ZIP9 in PC-3 and LNCaP cells and knockdown studies with ZIP9 siRNA show that ZIP9 might play a role in PCa development[18]. It is predicted that unlike GPRC6A and AR8, ZIP9 - transfected LNCaP and PC-3 cells potentiate cellular apoptosis pathway[18]. When LNCaP cells transfected with ZIP9 were treated with T there was 100-fold increase in relative intensity of intracellular zinc concentration[18]. Additional studies are required for throwing light on the exact roles of each of these membrane proteins and their roles in AR signalling and PCa. The identification of a suitable mAR could provide a potential target for PCa diagnostics and therapeutics.

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8. Conflict of Interest

None of the contributing authors has any conflict of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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