UNIVERSITÉ DE STRASBOURG

Thesis

Submitted for the degree of Philosophy Doctor of the University of Strasbourg

Récepteurs des androgènes constitutivement actifs dans le cancer de la prostate: interconnections avec les voies de signalisation

Constitutively active androgen receptor in prostate cancer: interconnection with kinases pathways

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Ms Gemma Marcias was a member of the European Doctoral College of Strasbourg during the preparation of her PhD, from 2006 to 2009, class name Virginia Wool. She has benefited from specific financial supports offered by the College and, along with her mainstream research, has followed a special course on topics of general European interests presented by international experts. This PhD research project has been led with the collaboration of two universities: the University of Strasbourg, Strasbourg, France and the Medical University of Innsbruck, Innsbruck, Austria.

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Index

Introduction

5αR : 5α-reductase

a.a.: amino acids

AA: antiandrogens

ADR3: androgen direct repeat separated by 3 bp of spacer

ADT: androgen deprivation treatment_ACTH: adrenocorticotropic hormone

AF-1: activation function 1

AIPC: androgen-independent prostate cancer

Akt: activated protein-serine/threonine kinase, also known as protein kinases B (PKB)

AP-1: activator protein- 1

AR : androgen receptor

AR*: amphiregulin

ARA70 : nuclear receptor coactivator 4 (NCOA 4)

ARE: androgen response element

ATF: activation transcription factor

ATP: Adenosine-5'-triphosphate

AWS: Androgen withdrawal syndrome

BAD: Bcl-2-associated death promoter

BCL2: B-cell lymphoma 2

BPH: benign prostatic hyperplasia

BPSA: benign PSA

BTC: betacellulin

CAB: complete androgen blockade

CAM: calmodulin

cAMP: cyclic adenosine monophosphate

CARM1: coactivator associated arginine methyltransferase 1

CBP: CREB binding protein

Cdk: cyclin-dependent kinase

CK1: casein kinase

COOH: acide carboxylique

COS-1: CV-1 in Origin, with SV40

CSAP: cryosurgical ablation of the prostate

CTE: C-terminal end

CTMP: carboxy-terminal modulator protein

CXC: chemochine

Cy-5: cyanine 5

DAG: diacylglycerol

DBD: DNA-binding domain

DHEA: dehydroepiandrosterone

DHT: dihydrotestosterone

DNA: deoxyribonucleic acid

DOCK: JNK docking region

DRE: digital rectal examination

ECM: extracellular matrix

EGF: epidermal growth factor

EGFP: enhanced green fluorescent protein

EGFR: epidermal growth factor receptor

EMT: epithelial-mesenchymatous transition

EPCA: early prostate cancer antigen

EPR: epiregulin

ER: estrogen receptor

ERK: extracellular signal-regulated kinases

f/t PSA: free/total PSA

FGF: fibroblast growth factor

FITC: fluorescein isothiocyanate

FKBP: FK506 binding protein

FOXO1: forkhead box O1

FSH: follicle-stimulating hormone

FSK: forskolin

G-protein: guanine nucleotide-binding proteins

GDP: guanosine diphosphate

GnRH: gonadotropin releasing hormone

GPCR: G protein-coupled receptors

GR: glucocorticoid receptor

GRIP-1: GR-interacting protein 1

GSK: Glycogen synthase kinase

GTPase: hydrolyze guanosine triphosphate hydrolase enzymes

H: histone

HAT: histone acetylase

HB-EGF: heparin-binding growth factor

HDAC: histone deacetylase

HER2: human epidermal receptor 2

HIF: hypoxia inducible factor

HIFU : high-intensity focused ultrasound

hK3: human kallikrein 3

HR : hinge region

HRE: hormone-responsive elements

HRPC: hormone-refractory PCa

Hsp: Heat Shock Protein

IGF: insulin-like growth factor

IL-: interleukine

JNK: Jun N-terminal kinases

kDa: kilo dalton

LBD: ligand-binding domain

LH: luteinizing hormone

LH-RH: luteinizing hormone releasing hormone

LNCaP: lymph node prostate adenocarcinoma cells

MAF: musculoaponeurotic fibrosarcoma

MAPK: mitogen-activated protein kinase

MED: mediator

MMTV-LTR: mouse mammary tumor virus-long terminal repeat

MR: mineral corticoid receptor

mTOR: mammalian target of rapamycin

N/C: amino/carboxy

NADPH: nicotinamide adenine dinucleotide phosphate

NCoR: nuclear receptor corepressor

NES: nuclear export signal

NFAT: nuclear factor of activated T-cell NF-κB: nuclear factor-κB

NH2: amine

NLS: nuclear localization signal

NRG: neuregulin

NTD: N-terminal domain

p27Kip1: cyclin-dependent kinase inhibitor 1B

OPG: osteoprotegerin

OS: overall survival

PC-3 : prostate cancer cell lines-3

PCA3: prostate specific gene 3

PCa : prostate cancer

PCAF: P300/cbp-associated Factor

PEP: parenteral polyestradiol phosphate

PDK1: pyruvate dehydrogenase kinase, isozyme 1

PEST: proline(P), glutamic acid (E), serine (S), and threonine (T)

PI 3- K: phosphatidylinositol 3-kinase

PIN: prostate intraepithelial neoplasia PIP(3): phosphatidylinositol 3,4,5-trisphosphate

PKA : Protein kinase A

PKC: Protein kinase C

PLC: phospholipase C

PMA: phorbol esters

PR: progesterone receptor

PRMTI: protein arginine methyltransferase

PSA: prostate-specific antigen

PSMA: Prostate specific membrane antigen

PTEN : phosphatase and TENsin homolog

PTM: post-translational modifications

RANKL : Receptor Activator for Nuclear Factor κ B Ligand

RGS: regulators of G-protein signaling

RHD: Rel homology domain

RNA: Ribonucleic acid

RP: radical prostatectomy

RT: radiotherapy

RT-PCR: Real-time PCR

RTK: receptor tyrosine kinases

SH2: Src homology 2

SMAD: combination of drosophila protein, mothers against decapentaplegic (MAD) and the C. elegans protein SMA

SMRT: silencing mediator of retinoic acid and thyroid hormone receptor

SRC-1: steroid-receptor coactivator

SSR: serine-rich region

STAT: signal transducer/activator of transcription

SWI/SNF: SWItch/Sucrose NonFermentable

SUMO: small ubiquitin-like modifiers TAD: transactivation domains

TAU-1: the transcriptional activation unit

TCF: transcription factors ternary complex

TF: transcription factor

TGF: transforming growth factor

Thr: threonine

TK: tyrosine kinase

TNF: tumour necrosis factor

TNM: tumour, node, metastasis

TRAIL: tumour necrosis factor-related apoptosis-inducing ligand

UGM: urogenital sinus mesenchime

UGS: urogenital sinus

uPAR: urokinase like plasminogen activator receptor

UTR: untranslated region

VEGF: vascular endothelial growth factor

Wnt: Wg (wingless) and

Prostate cancer

Epidemiology

Prostate cancer (PCa) is the most common diagnosed non-skin cancer among men in most western countries. In the United States PCa is the second leading cause of cancer death among men, accounting, in 2008, roughly 25% of all cancers. Many presumed risk factors, including androgens, diet, physical activity, sexual factors, inflammation and obesity could be implicated, but their roles in prostate cancer etiology remain unclear.(Crawford, 2009)

Risk factors

Demographics factors are age and racial/ethnic variations. Advanced age is considered as the primary risk factor for prostate cancer as about 80% of cases, and 90% of deaths, occur in men over the age of 65. It is estimated that the largest percentage of diagnosed cases is placed in men between 65 and 74 years, follows those 75~84 years old and in a less percentage those ≥85 years old.

In an age-adjusted study of the PCa incidence suggests that ethnicity and racial components are crucial: the Black men were the more exposed compared with the Hispanic and at the last position the Asian/ Pacific. Black men emerge to be majorly affected in an age-adjusted ratio of 1.73 to 1 as compared to Caucasian patients. This population presents a worse prognosis due to the aggressiveness of the disease and a lesser response to therapy, particularly in young patients.

Furthermore, the contribution of the positive family history (related to the number of affected male relatives, and their age at diagnosis) was proportionally related to an increased prostate cancer risk by several epidemiologic studies. The familial risk does not vary with ethnicity.

The basis of these disparities could be explained by environmental, detection, including socioeconomic issues, as well as genetic-background, or physiologic status differences.

Socioeconomics status and occupation were long studied but in these cases, the association with prostate cancer was weak, excepted for occupation related to long term exposure with heavy metals such as cadmium, which could interfere with the zinc metabolism.(Haas and Sakr, 1997)

Several genetic modifications are proposed as prostate cancer risk factors. Polymorphism in the CAG trinucleotide repeat (polyglutamine) of the androgen receptor is associated with an increased risk for developing prostate cancer. Indeed, the prevalence of shorter, high-risk alleles is in agreement with the risk of prostate cancer by ethnic group.

Sexually transmitted infections were considered risk factors as well as several types of viruses have been isolated from cancer cells, such as human papillomavirus, and herpes virus. There is, however no evidence for a direct impact in PCa development.(Crawford, 2009; Nieder et al., 2003)

Genes polymorphism has been studied for genes associated to prostate cancer such as 5α-reductase type 2 (SRD5A2), α-methylacyl-CoAracemase (AMACR), and HPC2/ELAC2 that have already been involved in familiar cases of PCa.(van der Poel, 2007)

Prostate

Function and architecture

As part of the male reproductive system, the prostate gland primary function is to secrete a slightly alkaline fluid that forms part of the seminal fluid, a fluid that carries sperm.
Human prostatic glands are composed of a simple stratified epithelium containing basal and luminal layers separated by a basement membrane from a well developed stromal compartment.

Prostate development

The prostate gland is about the size of a walnut that is situated at the neck of the man urinary bladder and surrounding the urethra - the tube that carries urine from the bladder. Prostate is enclosed in a thin fibrous capsule. The functional part of the gland, the prostatic parenchyma, consists of a numerous branching tubuloalveolar glands terminating in ducts that ultimately empty into the urethra. The prostate develops from the endodermal urogenital sinus (UGS) which is derived from the caudal terminus of the cloaca, an embryonic chamber which is split up into separate tracts during the development of the urinary and reproductive organs. The endodermal UGS has an ambisexual embryonic origin, which develops into the prostate, prostatic urethra, and bulbourethral glands in males, or into the lower vagina and urethra in females, and bladder in both sexes. The endodermal UGS is surrounded by embryonic connective tissue called urogenital sinus mesenchyme (UGM). In response to fetal testicular androgens, epithelial buds emerge from the wall of the UGS and grow into the surrounding UGM. Androgens masculinize the reproductive tract during the ambisexual stage of development and lead to the formation of the prostate. Androgens are also required to continue embryonic and neonatal prostatic growth, and subsequently to begin the secretory activity. Histologically, each glandular acinus is lined with secretory (cuboidal to columnar) luminal epithelial cells and a discontinous layer of basal cells and is embedded in a connective tissue, the fibromuscular stroma. Human prostate is subdivided in three zones: central, peripheral and transitional. Stromal tissue mediates the androgen-induced growth and development of embryonic epithelium into differentiated prostate. The formation of prostatic

buds results from epithelia-stroma interaction that requires the presence of a functional AR in the urogenital mesenchyme but not in the epithelia, suggesting that DHT- regulated growth factors are secreted by the mesenchyme and act upon the developing prostate epithelium. After the development of the prostate, androgens continue to function in promoting the survival of the secretory epithelium, which is the primary cell type thought to be transformed in PCa. During the development of PCa, androgens become growth factors and survival factors for tumour cells.(Cunha et al., 2004; Thomson, 2001)

Stroma/epithelium exchange in adult normal prostate toward PCa

The prostate is a tubulo-alveolar gland composed of epithelial tissues surrounded by stromal components. The stroma compartment includes cell types such as fibroblasts, myofibroblasts and smooth muscle cells. Stromal cells secrete growth factors, produce extracellular matrix (ECM), and express androgen receptor (AR), estrogen receptor (ER), adrenergic receptor and 5-α reductase. The homeostasis of a normal prostate is preserved by a variety of signals between the stroma and the epithelium(Fig.1).

In cancer conditions, the stroma compartment increases myofibroblasts and fibroblasts amount and loses the smooth muscle cells. The "overactive" stroma cells can release soluble factors, secrete solid matrix components and repress cell apoptosis. In these conditions, stromal cells supply cellular self-renewal.(Niu and Xia, 2009; Thomson, 2001)

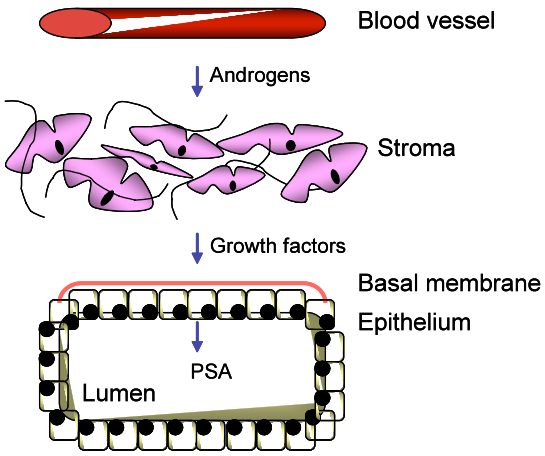


Fig.1: Representation of stroma/epithelium exchange in adult normal prostate.

Classification of prostate cancer

Established prognostic factors for prostatic carcinoma recommended for routine reporting are TNM (tumour, nodes, metastasis) stage, surgical margin status, serum prostate-specific antigen, and Gleason score.

The TNM System

The most widely used system worldwide for staging prostate cancer and others is called the TNM System, as a method to describe the extent of the primary tumour (T stage), the absence or presence of spread to nearby lymph nodes (N stage) and the absence or presence of distant spread, or metastasis (M stage).(Hoedemaeker et al., 2000)

Primary tumour (T)

TX: The primary tumour was not or could not be assessed.
T0: There is no evidence of a primary tumour.
T1: The tumour could not be found by examination or with the use of imaging (like ultrasound or an MRI scan), but was incidentally found during a biopsy or surgery.
T1a: The tumour is found in 5% or less of the tissue that was taken.
T1b: The tumour is found in more than 5% of tissue that was taken.
T1c: The tumour was found by needle biopsy.
T2: The tumour is found only within the prostate itself.
T2a: The tumour is found in 50% or less of one lobe.
T2b: The tumour is found in more than 50% of one lobe.
T2c: The tumour is found in both lobes.
T3: The tumour has extended through the capsule that surrounds the prostate.
T3a: The tumour has only gone through the capsule without invading the seminal vescicles.
T3b: The tumour has invaded the seminal vesicles.
T4: The tumour has invaded structures or tissues near the prostate other than the seminal vesicles. These include the bladder neck, the rectum, and the pelvic wall along with other structures.

Nodes (N)

NX: The lymph nodes were not or could not be assessed.

N0: The nodes do not show evidence of cancer.

N1: The nodes show evidence of cancer.

Metastasis (M)

MX: The presence of metastases was not or could not be assessed.

M0: There is no evidence of distant metastasis.

M1: There is evidence of distant metastasis.

M1a: Cancer has been found in lymph nodes far from the prostate.

M1b: Cancer has been found in the bone.

M1c: Cancer has been found in another area of the body.

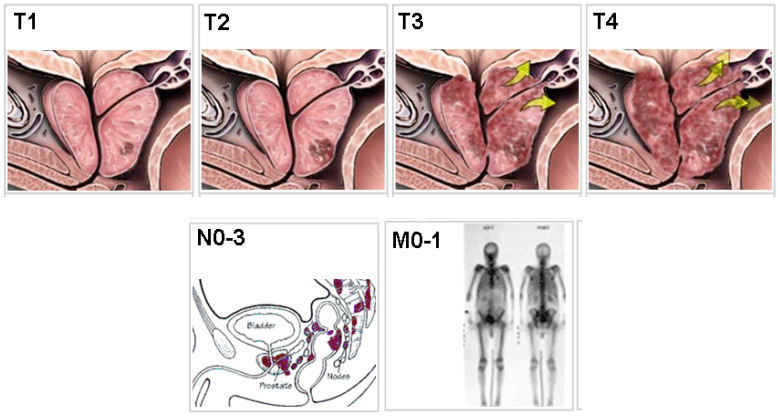


Fig.2: Representation of the TNM classification of malignant prostatic tumours.

The Gleason grading system

The Gleason grading system for prostate carcinoma was created by Donald F. Gleason in 1966, based on the architectural pattern of the tumour (Fig.3). It has been updated in 2005, with the purpose to obtain a good correlation between grade, stage, tumour extent and serum PSA, and to better characterize the difference between low and high grade malignancy of prostate carcinoma.

Nowadays, the system distinguishes five histological patterns of glandular growth and decreasing differentiation features, pattern 1 being the most differentiated and pattern 5 being the least differentiated.

The estimation of a score depends on all histological patterns observed in the tumour, the most prevalent and the second most prevalent pattern (if at least 5% of the tumour) being added together to obtain a Gleason score - e.g. Gleason grade 3+4=7.(Epstein et al., 2005)

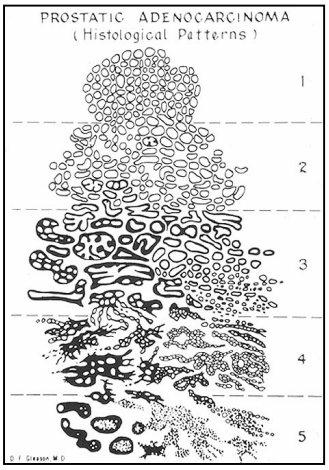


Fig.3: Schematic diagram of the architectural pattern of the tumour, developed by Gleason. The diagram does not present demarcations between the various Gleason patterns, showing the gradual transition from one pattern into the other. 1 small uniform gland; 2 more space between glands; 3 infiltration of cells from glands at margins; 4 irregular masses of cells with few glands; 5 lack of glands, sheets of cells.

Diagnosis

Digital rectal examination (DRE)

Most of the prostate cancers originate from the peripheral zones of the prostate. When the tumour volume is about 0.2ml or more a PCa can be detected by digital rectal examination. (http://www.uroweb.org/nc/professional-resources/guidelines/online/)

The serum Prostate-specific antigen evaluation

Prostate-specific antigen (PSA), also known as human kallikrein 3 (hK3), has the major relevance in oncology as a biomarker to contribute to PCa detection. PSA is an androgen-regulated serine protease, which is mostly confined within the prostate gland.
The single layer secretory epithelial cells in the acini and ducts of the prostate gland are responsible for the production of PSA that is secreted directly into the lumen, whereas only a small amount of PSAcan be found in blood.
PSA is the major protein that is present in the seminal fluid. Its concentration ranges from 0.5 to 2.0 mg/mL, where its function is to cleave semenogelins in the seminal coagulum. The rise of PSA levels in the blood do not solely reflect the presence of cancer, but may also be driven by certain nonmalignant causes such as prostatic inflammatory processes and nodular hyperplastic changes in the prostate gland. This apparent lack of specificity may limit the application of PSA for early PCa detection purposes.(http://www.uroweb.org/nc/professional-resources/guidelines/online/)

To enhance the specificity of the PSA in early detection of cancer, PSA mesure can be tweaked by:

- the PSA density, that is defined as the ratio of an individual serum PSA measurement evaluating its corresponding prostate volume, that value being assessed by transrectal ultrasonography;

- the PSA molecular precursor isoforms (pPSA), initially found in patients with benign prostatic hyperplasia (BPH) and thus designated as “benign” PSA (BPSA);

- the free/total PSA ratio (f/t PSA)

The free/total PSA ratio is the concept most extensively and most widely used in clinical practice to discriminate BPH from PCa. The f/t PSA test has no value as a first level screen but should be considered to investigate results that are in the 4.0 - 10.0 µg/L of protein range, and has been used to stratify the risk of PCa in men with total PSA levels between 4 and 10ng/mL and with a negative DRE. The human kallikrein 2 hK2 is responsible for the cleavage of the propeptide PSA to active mature PSA in the prostate;

- the PSA velocity (PSAV), PSA doubling time (PSADT)

In patients with surgical PCa, it might be useful to adopt the PSA velocity and the PSA doubling time derivatives systems, to mesure the velocity of PSA increases on an annual basis (the PSA velocity exceeding 0.75ng/ml/year was associated with a higher risk of PCa than was a slower rise in PSA over time), and to assess the exponential increase of serum PSA over time reflecting a relative change.
(http://www.uroweb.org/nc/professional-resources/guidelines/online/)

Prostate cancer gene (PCA3) marker

PCA3 is a prostate-specific gene upregulated in almost all PCa tissues. PCA3 mRNA is measured in urine sediment obtained after prostatic massage or digital rectal examination. The determination of this PCa-specific gene as a new marker for the diagnosis of the PCa is still experimental. The high specificity of PCA3 for PCa biopsy supports the use of urine PCA3 testing. (Shappell et al., 2009)

Prostate biopsies

The need for prostate biopsies should be determined on the basis of the PSA level and/or a suspicious DRE.

The patient's biological age, potential co-morbidities and the therapeutic consequences should also be considered.(http://www.uroweb.org/nc/professional-resources/guidelines/online/)

Other biomarkers for PCa

The concept of "age-specific serum PSA range" takes into account that the PSA level is highly related to the prevalence of BPH, which is increasing with age.
With regard to PCa aggressiveness, it was shown that within the 4–10ng/ml PSA range, within the human kallikrein family, others members are candidates as PCa markers such as human kallikrein 2 (hK2) and human kallikrein 4 (hK4) are primarily expressed in prostate tissue and are regulated by androgens.
For example, the human glandular kallikrein 2 (hK2) a serine protease, shares 80% sequence homology with PSA as well as the property of being mainly expressed in the prostate under androgen regulation. In the blood, hK2 is present at levels of 1–2% of the concentration compared with PSA, and the level of hK2 in prostate tissue, seminal plasma and blood is only 1% of the concentration of PSA.(Steuber et al., 2007)

- Prostate specific membrane antigen (PSMA). PSMA is a membrane glycoprotein that is expressed on the surface of prostate cancer cells, with an increased expression in high-grade cancers, metastatic disease and hormone-refractory prostate cancer. PSMA is also present at high levels neovasculature.(Olson et al., 2007)

- Urokinase like plasminogen activator receptor forms (uPAR). The plasminogen activation cascade participates in the degradation of the extracellular matrix during cancer progression. As a result, full-length intact uPAR and cleaved isoforms are liberated from the cell surface by several mechanisms.

- Early prostate cancer antigen (EPCA). Alterations in nuclear matrix proteins have been associated with carcinogenesis in multiple kinds of cancers; EPCA is a nuclear matrix protein, which is considered to be PCa associated.

- Prostate cancer specific autoantibodies. The existence of autoantibodies against PCa specific antigens have been reported and immunodetected in blood such as huntigton-interaction protein 1, prostasomes and α-Methylacyl-CoA-racemase (AMACR), an enzyme participating in the fat metabolism.

- Transforming growth factor beta1 (TGF-*β1*) and interleukin-6 (IL-6). Experimental PCa models have demonstrated the potential role of TGF-β*1* in the process of tumour cell progression. Subsequently, some studies have shown increasing tissue levels of TGF-β*1* with tumour grade, pathological stage and lymph node metastasis in PCa patients. TGF-β*1* can also be detected in the circulation by using a commercialized quantitative sandwich enzyme immunoassay, which does not cross-react with TGF-β*2* and TGF-β*3*. This assay is however not performed in routine practice.

The molecular detection of circulating tumour cells can be achieved by reverse transcription followed by polymerase chain reaction. This assay is not yet in routine practice. (http://www.uroweb.org/nc/professional-resources/guidelines/online/)(Damber and Aus, 2008; Gurel et al., 2008)

Treatments

Localized PCa treatment

Initial treatment for prostate cancer confined within the prostate capsule is usually prostatectomy or radiation to remove or destroy cancer cells.

Radical prostatectomy

Radical prostatectomy (RP) is the reference surgical treatment for localized PCa. RP consists in the removal of the entire prostate gland, including the resection of seminal vesicles. The laparoscopic, robot-assisted and perineal prostatectomies might induce lower morbidity than the standard retropubic operation, but randomized trials are unavailable.

Radiotherapy (RT)

Radiotherapy is the alternative to the surgical intervention for localized prostate cancer. Two possibilities are proposed: the external beam therapy or the brachytherapy that consist on the positioning of internal sources of radiation.(Garnick and Fair, 1996a, b)

Cryotherapy and high-intensity focused ultrasound (HIFU)

Cryosurgical ablation of the prostate (CSAP) and HIFU emerges as alternative therapeutic options to RP and RT in patients with clinically localised PCa. CSAP uses freezing techniques to induce cell death, while HIFU focused ultrasound waves emitted from a transducer to cause tissue damage heating malignant tissues above 65 °C and destroying them by coagulative necrosis.(http://www.uroweb.org/nc/professional-resources/guidelines/online/)

Advanced PCa Treatment

Endocrine treatment

The widespread use of the PSA assay led to earlier diagnosis and earlier detection of recurrent disease. The percentage of patients having bone metastases at the time of diagnosis of prostate carcinoma has decreased and patients with recurrent prostate carcinoma after the failure of local therapy are now diagnosed with recurrence on the basis of a rising PSA level. The overall survival (OS) of these patients is of 10-15 years versus 2 years for patients having metastatic disease. (Wilt and Thompson, 2006)

PCa endocrine therapy was proposed the first time by Huggins and Hodges in 1942 and is based on androgen deprivation from the tumour environment, through either the elimination of the testosterone production by testis or blocking the androgen receptors of the prostate. About 6% of all PCa are not responsive to the androgen deprivation.

Since 60 years, the endocrine treatment remains as a standard in patients with advanced prostate cancer. However, its proper use and scheduling of endocrine therapy remains controversial regarding adverse events carried after every type of endocrine treatment, that influence quality of life in different ways.(Tammela, 2004)

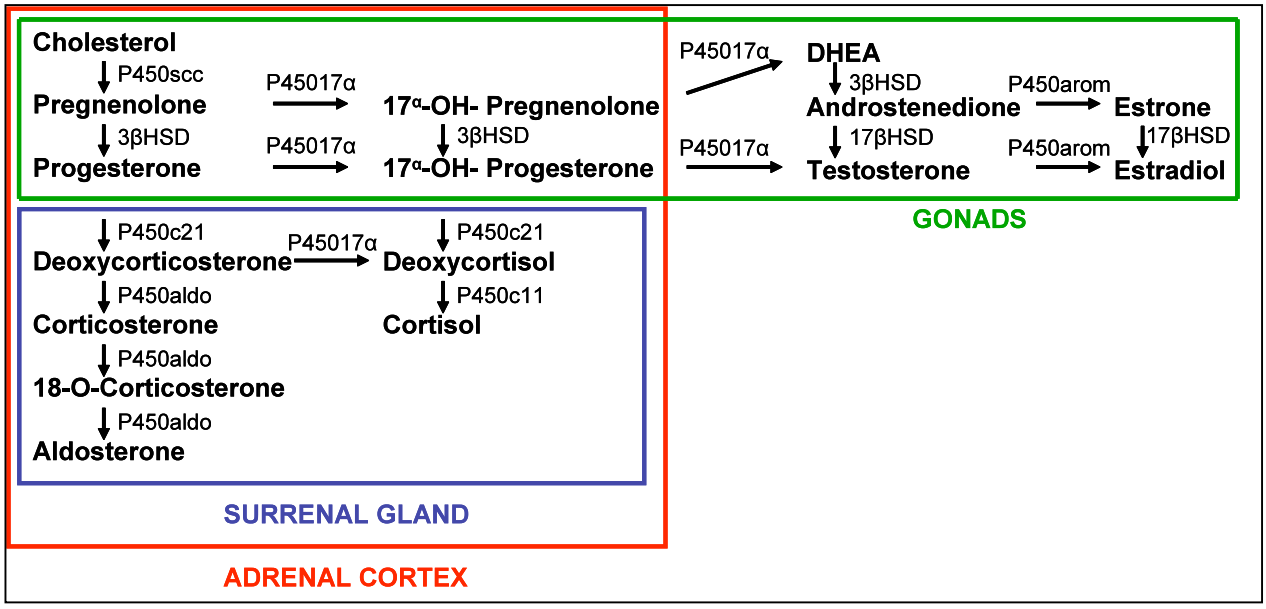


Fig.4: Androgen biosynthesis.

Androgen deprivation may be achieved through either surgical or medical approaches.

Hormonal agents

Two major classes of agents are approved and are currently in use in advanced PCa. These include LHRH analogues and the antiandrogens (Fig.5).

- Gonadotropin Releasing Hormone agonists

Gonadotropin Releasing Hormone is synthetized and released from neurons within the hypothalamus and secreted in a pulsatile manner directly into the hypophyseal-portal blood circulation. GnRH is responsible for the production and the release of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the adenohypophysis, to regulate the production of the testosterone.

Continuous stimulation of the pituitary gland leads to the desensitization of the receptors and is followed by receptors down-regulation, resulting in the inhibition of the LH release, which further inhibits the production of testosterone by the testicle (Leydig cells).

GnRH analogues are compounds with a prolonged half-life time and increased resistance to peptidase. These molecules are obtained by altering the basic amino acid structure of synthetically produced analogues. Only 5% of the patients treated with this therapy failed to achieve testosterone level <50ng/ml.

The use of all LHRH agonists is associated with the tumour flare side effect that is an initial transient rise in testosterone and consequently an increased tumour growth that may cause pain and spinal cord compression.

Some examples of GnRH agonists are goserelin, leuprorelin and tritorelin.

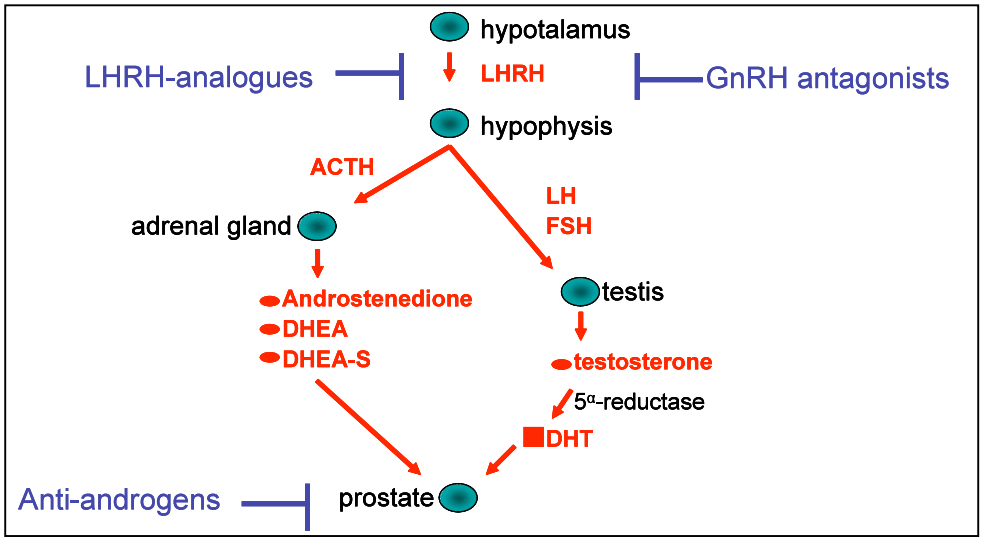


Fig.5: Androgen deprivation therapy. Treatments target the inhibition of the hypothalamus/hypophysis axis and the neutralization of the steroid precursors secreted from the adrenal gland.

Complete androgen blockade (CAB)

About 5% of serum testosterone concentration is under the GnRH-independent control of the adrenal gland. To address this issue, GnRH agonists may be used in combination with specific anti-androgen to produce a "complete androgen blockade (CAB)", blocking the effects of both adrenal and locally-produced androgens.

Antiandrogens

Two categories of antiandrogens (AA) are available: steroidal and non steroidal AAs.
Cyproterone acetate is a steroid competitor of DHT for binding with androgen receptor. Furthermore, cyproterone acetate binds to the progesterone receptor on pituitary gland also inhibiting the release of LH and production of testosterone by testicles. The treatment with GnRH antagonists and antiandrogens induces a rapid reduction in serum testosterone level and avoids the flare effect.
Bicalutamide, Flutamide and Nilutamide are non-steroid inhibitors, which raise LH production as well.(Tammela, 2004)

Inhibition of 5α-reductase

The conversion of circulating testosterone to dihydrotestosterone (DHT) synthesis is catalysed by two 5α-reductase isoenzymes (5αR1 and 5αR2). In the human prostate, both 5αRs are present in epithelial cells and stromal cells. The 5αR2 is considered as the main isoenzyme with clinical relevance in PCa because its high concentrations in prostatic tissue and its higher testosterone-binding affinity compared to the 5αR1 isoform. Dutasteride is an inhibitor of both 5αR1 and 5αR2 isozymes. Administration of a 5αR inhibitors prevents the development of spontaneous prostate cancer in animals and in humans. Finasteride is a potent inhibitor of human 5αR2, and is a less effective inhibitor of the human 5αR1.(Crawford, 2009; Tammela, 2004; Zhu and Imperato-McGinley, 2009)

Intermittent androgen deprivation: androgen deprivation treatment (ADT)

The proliferation of androgen-independent cancer clones limits the long-term efficacy of hormone suppressive therapy. Intermittent androgen blockade improves the outcome delaying the onset of hormonal resistance. Currently there is no evidence to this hypothesis but preliminary results suggest that intermittent androgen deprivation does not decline time to progression and improves quality of life.(Tammela, 2004)

Adjuvant ADT after local definitive therapy

Adjuvant androgen deprivation has been investigated. Although several non randomized data suggest that patients may benefit from ADT after RP. An adjuvant treatment after RP may be more relevant since 70% of cases of PSA recurrence are due to metastatic disease. It looks especially interesting in patients who undergo radical prostatectomy and have lymph node metastases, therefore being an increased risk.(Tammela, 2004)

Androgen deprivation and association with RT

ADT in association with RT is routinely recommended for patients with locally advanced PCa. ADT in association with may reduce the number of clonogens that the radiation is required to eradicate, furthermore it can inhibit angiogenesis reducing tumour bulk. It might

also remove tumour cells from the active phase of cell cycle turning them into the resting phase.

In addition, neo-adjuvant treatment, can reduce the volume of the prostate by 30-50%, allowing the reduction of field size and dose of radiotherapy, thus reducing radiation-related adverse effects.(Tammela, 2004)

Progression of prostate cancer to hormone-independence

Chemotherapy

The majority of prostate cancers (85%) show an initial favourable response to hormone therapy, unfortunately, over time, molecular and cellular changes occur and cancer recurs.(Small and Vogelzang, 1997)

After endocrine treatment failure, chemotherapy has become a standard. Chemotherapy leads to response in 50–75% of men with androgen-independent prostate cancer with an overall survival (OS) of 1–2 years from endocrine therapy failure.(Savarese et al., 2001; Tammela, 2004)

In the 1990s, preclinical studies demonstrated that PCa were especially sensitive to inhibitors such as paclitaxel and docetaxel. Docetaxel, a semisynthetic taxane, is the only cytotoxic agent which has demonstrated an overall survival improvement and has therefore become the new standard of care for first-line treatment in men with hormone refractory disease.(Pienta and Smith, 2005)

Molecular and cellular basis of androgen-independent PCa development

Prostate cancer growth depends on the ratio of cells proliferating to those dying.
Since androgens are the main regulators of this ratio by both stimulating and inhibiting apoptosis, the androgen ablation causes cancer regression.
There are five main causes for the androgen-independent PCa (AIPC) development as shown by the literature.(Feldman and Feldman, 2001; Heinlein and Chang, 2004; Suzuki et al., 2003)

The hypersensitive pathway

After androgen ablation, the tumour increases its own sensibility to low androgens levels, owing to several mechanisms.

- Amplification of the androgen receptor (AR), occurs in 30% of tumours and results in a stable or increased level of androgen/AR levels, even in the setting of reduced androgen concentration. Amplification of AR follows androgen ablation and may be the result of clone selection from cells able to proliferate despite very low levels of circulating androgens.(Visakorpi et al., 1995)

- Increased AR sensitivity, resulting in increased stability of androgen/AR complex, and enhanced nuclear location of AR in recurrent tumours cells.(Gregory et al., 2001)

- Increased androgen levels, may be due to superior 5α-reductase activity resulting into a higher rate of testosterone conversion to DHT. After androgen ablation therapy, serum testosterone levels decrease by 95%, but the concentration of DHT in prostate tissue is reduced by only 50%.(Heinlein and Chang, 2002)

Broadened specificity of the AR

The acquisition of genetic changes may lead to aberrant activation of the androgen signalling axis through several mechanisms.

- AR mutations. Mutations in different positions of the androgen receptor sequence may produce a gain of function leading to a growth advantage by the tumour.(Bergerat and Ceraline, 2009; Edwards and Bartlett, 2005; Taplin et al., 1999)

- Co-regulators alterations. Several proteins act together with steroid hormone receptors as co-activators and co-repressors of transcription. The modulation of these co-regulatory proteins and their function is likely to be another mechanism by which prostate cancer progresses to AIPC. The over-expression of AR co-activators such as SRC-1 (steroid-receptor coactivator) or ARA70 (nuclear receptor coactivator 4, NCOA 4) could facilitate AR transactivation and enhance responses to low levels of androgens.(Agoulnik and Weigel, 2006; Heinlein and Chang, 2002)

Outlaw pathway

Growth factors and cytokines signalling pathways can subvert the AR toward the independence from androgens. Steroid receptors activated by ligand-independent mechanisms have been referred to as 'outlaw' receptors.
Growth factors and cytokines can indirectly activate the AR and therefore induce AR target genes in the absence of androgen.(Wen et al., 2000; Zhu and Kyprianou, 2008) Indeed, the binding of growth factors and cytokines to membrane receptor-tyrosine-kinase triggers several kinase pathways that induce the phosphorylation of the AR, creating an androgen-independent outlaw receptor.(Ghosh et al., 2005; Liu et al., 2005)
The Akt pathway has an anti-apoptotic activity, phosphorylating and inactivating several anti-apoptotic proteins as BAD and procaspase-9. Akt can also phosphorylate the AR on the ser 213 and the ser 791, transforming it in an androgen-independent receptor.(Agoulnik and Weigel, 2006)

Parallel survival pathways

It is possible that alternative or complementary pathways are involved in the progression of prostate cancer, bypassing the AR axis. These pathways would be able to facilitate proliferation and to inhibit apoptosis, in the absence of androgens and AR. The anti-apoptotic

gene *BCL2* is a candidate for the avoid mechanism. Indeed, *BCL2* gene is often overexpressed in AIPC, where it can evade the signal for apoptosis that is normally triggered by androgen ablayion.(Feldman and Feldman, 2001; Franke, 2008; Pfeil et al., 2004)

Androgen-independent cancer cells

It is possible that androgen deprivation leads to AIPC, not only through additional mutations whitin PCa cells, but also by selecting AIPC cells that were initially present in the tumour.
Indeed, within the basal cells layer, there are epithelial stem cells that seem to be androgen independent based on their ability to proliferate or die independently to the androgen ablation. (Feldman and Feldman, 2001)

Taken together this data emphasize the key role of AR in mediating PCa progression and hormone response failure. Of note, it is likely that several sequential or concomitant events are required to turn hormone sensitive prostate cells to androgen-independent clones that will ultimately be responsible of the patient's death.
Results illustrated in the present study go into details of the role of AR mutations and their link with kinases pathways in AIPC.

Androgen receptor

The androgen receptor (AR) is a transcription factor that regulates the expression of genes required for the normal male sexual development and the maintenance of the accessory sexual organs function. The AR is a member of the nuclear receptor family of ligand-activated transcription factors, and belongs to the class I subfamily of steroid receptors that also include the glucocorticoid receptor (GR), the progesterone receptor (PR) and the mineral corticoid receptor (MR). All these receptors bind to their related DNA-response elements to activate the transcription of target genes.
The assumed molecular mass of the AR is 110KDa, with approximately 918 amino acids (a.a.). The single gene copy of the *AR* gene is located in the long arm of the X-chromosome.(Germain et al., 2006; Navarro et al., 2002)

AR target genes

Since the primary function of the prostate is to secrete a fluid that forms part of the seminal fluid, the AR target gene include different families of regulated genes that mostly encode for proteins involved in the secretory pathway, polyamine synthesis and lipogenesis.
In addition, the regulation of the expression on target genes involved in transcription, splicing, ribosomal biogenesis, mitogenesis, bioenergetics and redox processes by the androgen/AR pathway has been recently speculated in prostate cancer cells.(Kaarbo et al., 2007)

AR organisation

The AR is composed of few main domains (Fig.6-1).(Reid et al., 2003)

-An **N-terminal domain** (NTD) fundamental for transcription activation. AR makes direct and/or indirect interactions with the transcription machinery in order to regulate gene expression. The NTD contains two transactivation domains (TAD), the transcriptional activation unit 1 (TAU-1, a.a. 101-370) and the transcriptional activation unit 5 (TAU-5, a.a. 360-485) that altogether form the activation function 1 (AF-1, a.a. 142-485). The AF-1 is the

major transactivation domain. The AF-1 contains a polyglutamine repeat site (CAG) starting at position 57 (the number of the repetition can vary from 9 to 36, with a highest frequency of approximately 20), a polyglycine (GGN) and a polyproline repeats involved in the regulation of the AR transcriptional activity. The length of the polyglutamine tract has been associated with different levels of receptor activity. Epidemiological studies on PCa suggest that racial differences and shorter polyglutamine repeats are associated with an increased activity of the AR.(Claessens et al., 2008)

- The regulation of target genes expression requires the homodimerization and the recognizing of the promoter and/or the enhancer sequence of these genes by the AR. The identification and binding to the DNA is realized by the **DNA-binding domain** (DBD) (a.a. 550-624, approximately 80 amino acids long). The DBD of all nuclear receptors consists in well-conserved central two zinc finger-like modules organized in three α-helices (1, 2 and 3). Each zinc atoms coordinates four cysteines and determines the DNA sequences recognized by the receptors. Helices 1 and 3 are perpendicular to each other and form many hydrogen bonds. The N-terminus of the first helix contains the so-called P-box residues (5a.a. long) partially involved in DNA sequences recognition. The P-box fundamental residues found within the AR are Gly-575, Ser-576 and Val-579, which are identical to those found within the GR, MR and PR. During DNA-binding, this helix is inserted in the major groove of the response element sequence. The second zinc-module is involved in DNA-dependent dimerization via the so-called D-box.(Claessens et al., 2001)

-The DBD is followed by a **hinge region (HR),** located exactly between the last α-helix of the DBD and the first α-helix of the ligand-binding domain (a.a. 623-671). This region contains a nuclear location signal (NLS) that binds importin α and is responsible for the translocation of the AR from cytoplasm to the nucleus. Indeed, a mutation on the a.a. 650 reduces the nuclear export of the AR. That region contains a PEST sequence (rich in proline-, glutamine-, serine-, and treonine-residual), which is a degradation motif present in proteins with short half-lives and that are target for the ubiquitin-mediated proteasome system. (Haelens et al., 2007)

-A **C-terminal ligand-binding domain** (CTE-LBD) consists in 12 α-helix forming the hydrophobic ligand-binding pocket that contains a region termed Activation function 2 (AF-2), involved in the regulation of transcription, and which is a docking site for co-

activators. The CTE of the AR recruits a group of co-regulatory proteins, preferentially containing an FxxLF (where x is any amino acid) motif than LxxLL, in a hormone-dependent manner. The hormone binding causes the LBD structural re-arrangement.(Bevan and Parker, 1999)

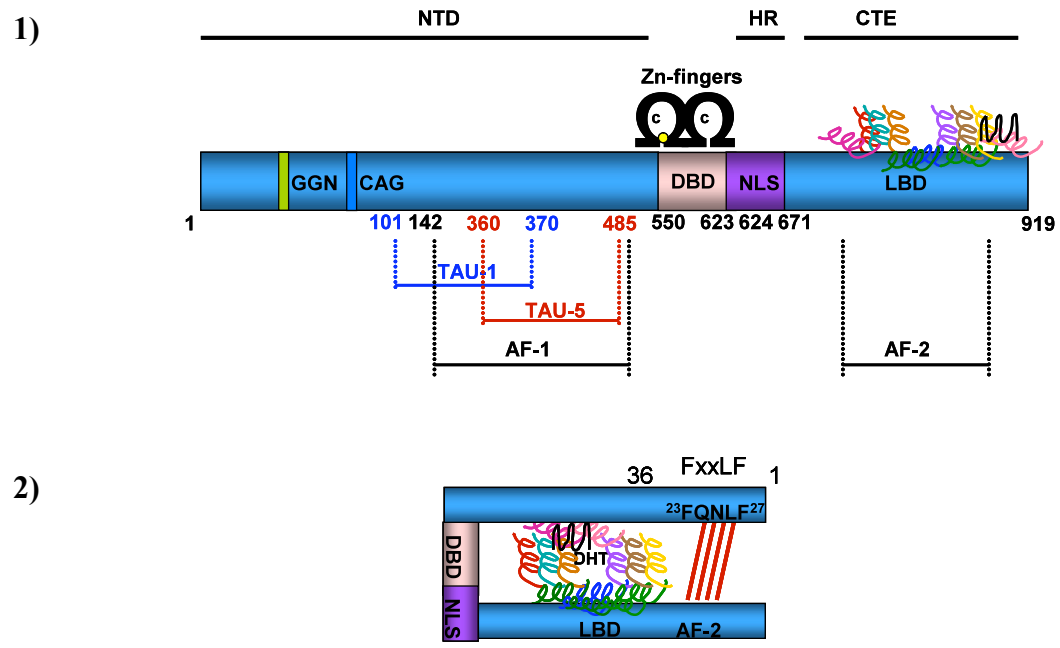


Fig.6: AR organisation. 1) AR is composed by 4 domains. The N-terminal end domain contains transcriptional activation unit 1 (TAU-1) and transcriptional activity transcriptional activation unit 5 (AF-5), which result in the activation function 1 (AF-1) complex that is required for the full transactivation of the receptor and is responsible for the constitutive activity of the receptor. The DNA binding domain (DBD). The hinge region connects the DBD with the LBD and include the ligand dependent nuclear location signal (NLS). The ligand binding domain (LBD) contains activation function 2 (AF-2) that is responsible for agonist induced activity. The CTE contains eleven α-helices and one β-sheet.
2) A conformational change occurs during the ligand binding, forming the ligand binding pocket within α/ β structures. The NTD contains a dimerization surface involving residues 1-36, which includes the FXXLF motif (F = phenylalanine, L = leucine, and X = any amino acid residue). This residual interacts with the AF-2 domain, induces a conformational change that allows the N/C interaction and the binding with cofactors containing the LXXLL or preferably FXXFL motifs.

General mechanism of AR action

Hormone binding and activation

In the inactive state the receptor is complexed with a number of molecular chaperones such as Hsps 90 and 70, p60 and p23 polypeptides as well as the immunophilin FKBP. The dissociation of these chaperones molecules occurs concomitant with the hormone binding and the LBD rearrangement, previously mentioned, leads to the receptor dimerization and the translocation of the activated receptor to the nucleus (Fig.7).(Reid et al., 2003)

Nuclear import

Androgen receptor nuclear transport is achieved from 15 to 60 min after hormone stimulation. The contact with Hsp90 masks the NLS site enabling the AR to bind other proteins in that site. The ligand binding induces a conformational change and allows the nuclear-protein import. The NLS motif is formed from either a single or bipartite cluster of Lys and Arg residues and is positively charged. In the cytoplasm, AR is recognized by importin-α, which is considered as an adaptor to the nuclear transport factor importin-β. AR binds to importin-α primarily through residues 629-634 a.a. (629-RKLKKL-634), but not to residues 617-618 a.a.that are integral components of the second zinc finger. The strongest interaction occurs through Lys630 that occupies the P2-binding pocket of importin-α. The complex translocates to the nucleus through the nuclear pore. The Ras family GTPase enzyme enhances the AR nuclear release, followed by the cytoplasm recycle of importins. Point mutations on the AR 629, 629 and 630 a.a. are found in PCa and decrease the binding affinity of the mutated receptors for importin-α.(Cutress et al., 2008)

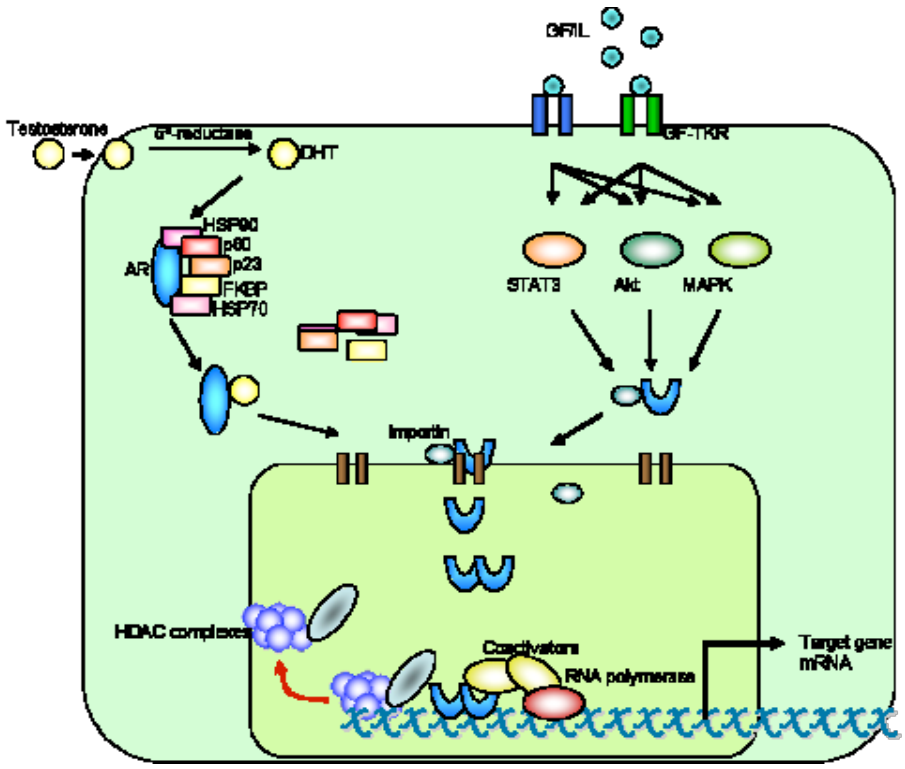


Fig.7: Activation mechanism of the AR. AR in the inactive state is situated in the cytoplasm compartment, complexed with more proteins including Hsps 90 and 70, p60 and p23 polypeptides and the immunophilin FKBP, in a structural conformation that avoid the exposition of the NLS domain. In prostate cells, the circulating testosterone is converted in DHT, a compound with a major affinity for the AR. The binding of the DHT to the AR produces the separation of the chaperones-complex and the activation of the protein. In the cytoplasm the activated AR is bound by importin-α that allows the nuclear translocation. Importin-α in the nucleus is separated from the AR. The dimerization is produced by interactions between LBDs of two ARs. In the nucleus the receptor homodimers bind the hormone responsive elements on the DNA sequence, recruit the transcription complex and stimulate the transcription of the target genes. AR transactivation can be regulated by androgeno-independent mechanisms. Once activated from extracellular signals such as growth factors (GF) or interleukines (ILs), STAT-3, MAPK and PI3K pathways have been involved in the phosphorylation of the AR for the negative and positive regulation of the AR activation.

Dimerization

Upon binding with the DNA, the AR undergoes DNA-dependent dimerization mediated by the DBD.

In the first zinc finger, the P-box directly interacts with the major groove of DNA, whereas the second zinc finger containing the D-box is involved in DNA-dependent dimerization between receptor monomers.

Interaction between P- and D-boxes is thus involved in steroid receptor DNA sequence recognition, binding and dimerization.

The residues 625 to 636 in the CTE provide an additional dimer interface for the AR-DBD and the DNA-binding. Following the model proposed for the DBD-mediated dimerization, the first zinc finger of each AR monomer binds the one half–site of the ARE whereas the second zinc finger binds the second monomer. The three nucleotides located between the half-sites play a key role in the spatial orientation. The AR binds to the DNA as a dimer. LBD dimerization occurs before DNA-binding and may influence DNA-dependent dimerization by restricting DBD-mediated interactions only to those receptors that are already in the dimers organization through their LBD. LBD dimerization occurs in a similar manner and through common motifs in helix 10 of steroid receptors.(Centenera et al., 2008)

Androgen receptor amino/carboxy terminal ends (N/C) interaction

The AF-2 motif in the CTE preferentially interacts with FxxLF-like motifs contained in the AR-NTD (^{23}FQNLF^{27} and ^{433}WHTLF^{437}) over LxxLL-like motifs in co-regulatory proteins, predominantly due to a charge clamp formed by the positively charged residues K715, K718 and R724 and the negatively charges residues E707, E891 and E895.

The direct model of N/C interaction is a rapid ligand-dependent intermolecular interaction between AF-2 in the AR-LBD and ^{23}FQNLF^{27} in the AR-NTD. The AF-2 can also interact with ^{433}WHTLF^{437}but with a much lower affinity than ^{23}FQNLF^{27} (Fig.6-2).

The activity of the ^{433}WHTLF^{437}motif is independent of ligand binding and recent studies suggest that the ^{433}WHTLF^{437} sequence influences AR signalling by acting as an autonomous activation domain.

The AR co-activators Src-2 enhance the N/C interaction 10-fold, and are able to restore N/C interaction to an AR-NTD fragment in which the FxxLF motif is mutated to FxxAA, providing evidence for the indirect bridging model of N/C interaction.

The N/C interaction enhances AR activity stabilizing and maintaining the receptor in an active state, by slowing the rate of ligand dissociation and preventing receptor degradation.

In vitro studies show that the AR N/C interaction may only be required for a subset of target genes (as activating chromatin integrated AR target genes). Inactivating mutation in AF-2 of the AR-LBD that disrupt the N/C interaction without affecting ligand binding affinity have been identified in AIS patients, indicating that N/C interaction is required for normal androgen signalling.

The residues important for this interaction include amino acid N-terminal of helix 12 in the AR LBD and the first 30 residues of the AR-NTD. N/C interaction preferentially occurs in the nucleoplasm, and occurs less frequently when the androgen receptor is bound to DNA. There is evidence that N/C interaction takes place both within one molecule (intramolecular) and between two ARs (intermolecular).(Centenera et al., 2008; Reid et al., 2003)
Loss of AR function might occur because of the presence of mutations that disrupt the N/C interaction although they do not change AR-binding affinity.(Langley et al., 1998; Thompson et al., 2001)

Transcription activation complex

In the nucleus, the receptor binds to specific DNA response elements and recruits a series of co-activator complexes that modify chromatin structure, recruit RNA polymerase II, and induce transcription. The cofactor-binding groove is lined by approximately 13 amino acid residues which reside in helices H3, H4, H5 and H12. The activated receptor could also: induce transcription by binding to other transcription factors, functioning as a co-activator; stimulate the activation of downstream kinases.(Lu et al., 2000; Unni et al., 2004)
The rate of transcriptional initiation is regulated by the AR through alterations with the transcription machinery and through alterations in the state of chromatin organization at the promoter of target genes. Studies on LNCaP cells demonstrate that the formation of an active co-activator complex for the transcription of AR target genes, involves the recruitment of AR to both the enhancer and promoter regions, and the recruitment of co-activators including histone acetylases and RNA polymerase II. Nuclear receptors activate gene transcription through the recruitment of co-activators that modify chromatin structure and interact with the basal transcriptional machinery. Co-activator complex formation initiated by agonist-bound AR involves coordination between both the promoter and enhancer, while co-repressor complex formation is initiated by antagonist-bound AR only involves the promoter. Both enhancer-bound AR and the promoter-bound AR interact with a common co-activator complex that contains p160 proteins, CBP, p300, and CAF. According to this model the local chromatin is modified and configured upon AR binding such that it brings the enhancer and promoter into close proximity, and the communication between the enhancer and promoter is mediated through the use of shared co-activators protein.(Shang et al., 2002)
Acetylation of histone extremity results in a more open chromatin structure favoring the transcriptional initiation.

Specific demethylase and proteins with histone tridemethylase activity promote androgen-dependent transcription of target genes by ligand-induced demethylation of mono-, di-, or tri-methylated histone H3, strongly supporting methylation modifications in the correct functioning of the AR.(Kaarbo et al., 2007)

DNA response elements

Most DNA response elements recognized by steroid receptors are organized as semi-palindromic hexamers, however the AR binds to an additional set of response elements that are organized as direct repeats, known as specific AREs. Subtile amino acid differences between the direct and the inverted repeats ARE prevent the remaining steroid receptors from binding to direct repeat elements, thus providing specificity for the AR. The need of at least four residues (625-TLGA-628) of the AR-LBD have been demonstrated as a requirement for a proper AR-DBD/DNA binding to nonselective AREs, whether a CTE of at least 12 residues (625-TLGARKLKKLGN-636) seems to be essential for binding to androgen-selective AREs.(Haelens et al., 2007; Verrijdt et al., 2003)

Nuclear Receptor Class	Members	P-box	Binding element	Dimerization	Remarks
I	AR, GR, PR, MR	GSCKV	5'-TGTTCT-3'	-3nt spaced palindromes. - Only homodimers	Strict spacing, strictly palindromic AR on direct repeats
II	ERs	EGCKA	5'-TGACCT-3'	-3nt spaced palindromes. - Homodimers, possibly monomeric	Strict spacing and strictly palindromic
III	RXR, RAR, TR, VDR, PPAR...	EGCKG	5'-TGACCT-3'	- 0-6 nt spaced direct or inverted repeats	Homo- or hetero-dimers with RXR Dimer-specific spacing
IV	NGFIb, ERR1,2	EGCKG, EACKA	5'-TGACCTTTT-3'	- Monomer	Sequence-specific inter-action of the CTE with 30 flanking DNA

Table 1: Nuclear receptors classification. The steroid hormone receptors belong form the classes I and II, which all form homodimers recognizing three-nucleotide spaced inverted repeats (IR3). The same core motif is bound by the class III and IV receptors that in contrast with the class I and II receptors can recognize both palindromic and direct repeat elements with variable spacer lengths, and are able to heterodimerize with the promiscuous dimer partner RXR. Depending on the identity of the receptor and its partner, a specific spacer length and repeat nature (direct or palindromic) is required for the binding of the receptor complex. The class IV receptors bind their recognition motifs predominantly as a monomer.

Regulation of transcription: cofactors

Co-activators

The transcriptional activity of the AR is controlled by the formation of a multiprotein complex of cofactors. The co-activators function is to facilitate the assembly of transcription factors into a stable pre-initiation complex, together with RNA polymerase II.

Coactivators can contribute to the development of AIPC increasing AR transcriptional activity in the presence of low ligand concentrations or altering the ligand specificity of the AR and allowing the AR translocation to the nucleus.

A conformational change in helix 12 of the AR is essential for appropriate ligand binding, making a ligand-binding pocket. The ligand-pocket is lined by approximatively 20 amino acid residues and creates on the surface of the AR a protein interaction interface called the co-factors binding groove.

The co-factors need to have an LxxLL-like or an FxxLF-like motif to interact with the AR. The co-factor-binding groove is lined by approximately 13 amino acids which reside in helices H3, H4, H5 and H12. These groups of molecules have histone acetylase (HAT) and deacetylase (HDAC) activity, could be co-repressors or co-activators, could interact with different sequences on the AR and can have different mechanisms for their activies.

- The p160 co-activators family for example has intrinsic **HAT** activity and recruits additional HATs inducing histone acetylation that changes the chromatin conformation making binding sites on the DNA more accessible. Furthermore, three members of this group, SRC-1 (steroid-receptor co-activator 1), SRC-2, and SRC-3 are overexpressed in PCa and often in refractory prostate cancer. HAT co-activators such as CBP/p300 and P/CAF have histone acetylase and deacetylase activity. Therefore, some stabilizing protein is required to maintain their stable transcriptional complex.

In addition to HAT activity, co-activators are important for other enzymatic activities implicated in transcription mechanisms such as like methyltransferase, CARM1 and PRMTI, and protein kinases that can modify histones by methylation or phosphorylation.

- Co-factors can have ubiquitin ligase activity. Enzymatic activities modify not only histones but also proteins including coregulators, general transcription factors, RNA polymerase II, and specific transcription factors.

- Co-factors such as SWI/SNF are part of a large ATP-dependent chromatin remodelling complexes. The SWI/SNF (complex composed of 11 polypeptides, including pleiotropic transactivators important for transcription of target genes, probably by a mechanism involving remodeling of chromatin) possesses an intrinsic DNA helicase activity.

- The mediator (MED) complexes, such TRAP/DRIP/ARC complexes act as a bridging complex between more classes of protein.(Trapman and Dubbink, 2007)

	N-Terminal end	DBD/Hinge	DBD/LBD	LBD	Multi-domain interactors	Domain unknown
Corepressors	AES	Calreticulin, Cyclin D1, PIASy, RelA, SRY, TGIF	HBO-1, PAK-6	F-SRC-1	ERM, TR4	p53
Coactivators/ coregulators	ARA24/RAN, BRCA1, Cyclin E, Raf, TFIIH CAK, RB	ANPK, ARIP3/PIASxα, ARIP4, c-jun, GT198, PIAS1, PDEF, SNURF, Ubc9		ARA54, ARA55, β-catenin, PNRC, RIP140, Tip60, Zac1b	ARA70, ARA160, ARA267-α, caveolin-1, CBP, FHL2, GRIP-1, SHP, SRC-1, TIF2, TRAM-1	E6-AP, HMG-1/2, P/CAF, PIAS3, PRMT/CARM 1, PGC-1, Smad3, SRA
Others	ARNIP, GAPDH, PQBP-1, TFIIF	ATF2, Filamen, GR, OCT-1/2, SF-1		Cdc37, Hsps, SP1, Ydj1p	COXVb, ER-α, supervillin	Akt, BAG-1L, PITALRE

Table 2: Regulation of transcription can take place at many different levels, including the expression level and properties of cofactors. Indeed, steroid receptors function is dependent by binding to specific sequences on target genes and recruitment of cofactors by protein–protein interaction. Cofactors involved in that regulation can be able to modify histones and other proteins or to modulate the chromatin structure and protein complexes that function as bridging factors between the multi-protein complexes. Proteins bind via FxxLF-like and LxxLL-like motifs to the cofactor-binding groove in the androgen receptor ligand-binding domain (LBD) or interact with the FxxLF region in the androgen receptor N-terminal domain (NTD).

Co-repressors
Co-repressors have five main mechanisms of action.

- Chromatin modification: by recruiting histone deacetylases to the AR complex, these proteins loose the DNA-binding capacity and need the presence of adaptation molecules to induce the enzymatic activity such as nuclear receptor co-repressor (NCoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT).

- Modulation of AR N/C interaction: by interaction through intrinsic motif that competes for binding (e.g. hRaD9); by binding directly to regions of AR that mediate N/C

interaction (e.g. large tumour suppressor 2, lats2); by directly binding the AR FxxLF motif (e.g. Cyclin D1).

-DNA binding/nuclear translocation: PTEN for example is able to sequester the AR in the cytoplasm.

- Co-activator competition, in the absence of ligand or in the presence of an antagonist ligand NCoR and SMRT might inhibit co-activator recruitment to the AR complex.

- Alternative mechanisms, as SMRT via a mechanism not yet understood.

In addition to HDACs, phosphatases and methyltransferases are involved in regulation of transcription silencing.(Trapman and Dubbink, 2007)

Post-translational modifications (PTM)

The transcriptional activity of steroid receptors is mainly governed by ligand-binding but several studies document the prominent role of post-translational modifications.
Phosphorylation, acetylation, ubiquitylation and sumoylation modify the androgen receptor function with regard to regulatory mechanisms for protein-protein interactions, subcellular location and stability. A regulatory cross-talk between some of these modifications has been evidenced.(Faus and Haendler, 2006)

Acetylation

A variety of transcription factors are known to be directly acetylated. The AR acetylation sites are clustered in the KxKK motif located in the hinge region at position 630-633. Proteins as Tip60, P/CAF or p300 are responsible for modifications at this site. Mutation of the lysine residues to alanine impairs AR function and promotes the recruitment of co-repressor molecules, suggesting that AR acetylation could be a peculiar modification modulating the recruitment of co-activators or co-repressors. A cross-talk with the phosphorylation has been evidenced, based on the fact that some mutations on the AR phosphorylation site were associated with decreased p300 stimulation.(Fu et al., 2004; Gaughan et al., 2002)

Ubiquitylation

The AR ubiquitylation results in the protein degradation by the proteasome complex. Ubiquitylation of proteins involves three families of members, the E1 ubiquitin-activating enzyme, the E2-conjugating enzyme and the E3 ubiquitin-ligase enzyme. Poly-ubiquitylation usually targets a protein for degradation via the proteasome pathway; mono- and bi-ubiquitylation may affect protein-protein interactions or subcellular location. In most cases, the exact site of modification is difficult to indentify, possibly due to the instability of polyubiquitylated proteins.

Anyhow, for most steroid receptors the ubiquitylation and the successive degradation are events closely related to the transcriptional activation of their target protein. After the ubiquitylation, the 26S proteasome undergoes the AR degradation.(Gaughan et al., 2005)

Sumoylation

Small ubiquitin-like modifiers (SUMO) are involved in pathways that are similar to ubiquitylation. The role of these proteins is not imperatively related to the proteasomic degradation, but rather may affect subcellular localization, DNA-binding, transcriptional potential, the regulation of chromatin structure, DNA metabolism or genome stability. Sumoylation involves the same family of proteins that are involved in ubiquitylation, but different members such as the E1 activating-enzyme (Aos/Uba2), the E2 conjugation enzyme (Ubc9) and the E3 ligase belonging to one of three classes. The covalent attachment of a SUMO chain onto lysine residues embeds the consensus ψKxE motif (ψ = large hydrophobic residue). Seven SUMO specific proteases have been described as able to revert the sumoylation.
On the AR sequence, K386 and K520 have been identified as sumoylation target residues in hormone-dependent manner. Mutations of the SUMO acceptor sites have been found to stimulate AR activity.(Kaikkonen et al., 2009)

A**ndrogen receptor phosphorylation**

Signal transduction pathways control the androgen receptor by post-translational modifications. In these conditions, AR is a part of multiple extracellular signals. Several data suggest that the AR is regulated more or less directly by phosphorylation, which could control AR function in part by activating the AR in the absence of ligand, and increasing the awareness of the AR for low ligand levels.
The major sites of AR phosphorylation have been identified using a combination of peptide mapping, Edman degradation, and mass spectrometry. The androgen receptor contains over 40 predicted phosphorylation sites. Seven phosphorylation sites (only on serine) as 16, 81, 94, 251, 308, 424, 650 residues were identified in vivo in cos-1 cells and confirmed in LNCaP cells.(Gioeli et al., 2002)

All the sites of serine phosphorylation seem to be regulated by androgen. Stress kinases as Akt and MAPK may be able to directly phosphorylate the AR.(Gioeli et al., 2002)

Exposure to androgens increases AR expression, probably due to the stabilization of the receptor, and increases overall phosphorylation excepted for **ser-94**, which is constitutively phosphorylated.

In vitro studies demonstrate that Akt (protein kinase B) phosphorylates AR at serine residues **213** and **791** resulting in modulation of AR transcriptional activity.(Yang et al., 2005)
Ser-213 is phosphorylated in vivo in a cell-type specific manner and in the presence of ligand agonists. (Taneja et al., 2005)

S**er-81** is phosphorylated in presence of androgen, but this phosphorylation is not involved in the regulation of the androgen receptor transcriptional activities. Ser-81 phosphorylation depends on the nature of the ligand. Indeed, DHT, cyproterone acetate and hydroxyflutamide induce phosphorylation at the ser-81 and bicalutamide and mifepristone antagonists avoid the phosphorylation of the ser-81.
Ser-81 is phosphorylated by cdk1 (cyclin-dependent kinase), this phosphorylation enhances the expression of the AR. The stabilization of the protein is not phospho-ser-81-dependent. Cdk1 is overexpressed in several tumours.(Chen et al., 2006)

The intracellular compartmentalisation of the AR influences its phosphorylation at several residuals. Indeed, serines 81, **256** and **308** are phosphorylated when the AR is nuclear.(Kesler et al., 2007)

Ser-515 is considered as a target of the MAPK pathway after EGF-treatment; whereas the **ser-578** is target of PKC phosphorylation. Furthermore, it is speculated that the phosphorylation at AR Ser-578 limits nuclear phosphorylation at Ser-515 and modulates AR nuclear-cytoplasm shuttling.(Ponguta et al., 2008)

Stress kinases are also able to regulate **ser-650** phosphorylation in AR sequence. Phosphorylation on this serine inhibits the transcriptional activity of AR controlling the nuclear/cytoplasmic shuttle. Phosphorylation at Ser 650 occurs by both hormone-dependent

and hormone-independent mechanisms. Indeed, various agonists including activators of protein kinase A (forskolin), EGF, and protein kinase C [phorbol-12-myristate-13-acetate (PMA)] increase phosphorylation at that residues. Experiments performed in the purpose to understand this mechanism highlighted the role of JNK and p38 signalling pathway on regulation of the phosphorylation of the ser 650, even in the absence of stimulation with an agonist such as PMA. (Gioeli et al., 2006)

AR mutations recently observed in PCa

A yeast functional assay has been developed in our laboratory to screen PCa samples for AR mutations, to analyse transcriptional activities of the mutant ARs in the presence of a panel of agonists or antagonists.

Briefly, total RNAs were extracted from PCa samples and a fragment encompassing the DBD, the LBD and the AF2 domains of AR was amplified by RT-PCR. During the last five years, in the frame of a national clinical study we have screened both localized and metastatic prostate cancer specimen for mutations in the androgen receptor (AR) gene, as these mutations are believed to sustain cancer cells growth and survival despite a low concentration of androgens by maintaining active the AR signalling pathway (Fig.8).(Bergerat and Ceraline, 2009; Ceraline et al., 2004; Ceraline et al., 2003)

Unfaithful AR mutant

The T877A mutation, extensively described in the literature, leads to promiscuous ligand activation of the AR. The replacement of the threonine in position 877 by an alanine enlarges the ligand-binding pocket allowing the binding of the antiandrogen, hydroxyflutamide, as an agonist. T877A mutation was discovered to affect the ligand-induced conformational change of the AR, and to considerably reduce the repressive action of the co-repressor NCoR. T575A/T877A AR variant was found in a hormone-refractory metastatic PCa, in which threonine 575 in the DNA binding domain, and threonine 877 in the ligand-binding domain, were both replaced by an alanine.

The T575A mutation is localized in the first zinc finger of the DNA-binding domain (DBD), just before the P-box, which is involved in ARE recognition. This mutation displays uncharacteristic transcriptional activities. Indeed, the T575A binds preferentially non-classic androgen responsive elements; while a low transcriptional activity is observed in the presence of specific ARE, thus caused by the weak recognition of these elements by the T575A. The two mutations T575A and T877A cooperate to confer new functional properties to the AR, and the mutant AR works simultaneously as a promiscuous AR due to the T877A mutation, and an unfaithful AR due to the T575A mutation. (Monge et al., 2006)

Exclusively cytoplasmic AR

The AR 23 splicing variant contains an insertion of 23 amino acids between the carboxyl-terminal part of the first zinc finger of the DBD and the second zinc finger.
The inserted sequence results from an aberrant splicing using a cryptic acceptor site in intron 2 and corresponds to the last 69 nucleotides of intron 2 p.[Glu588_Gly589ins-GluIleProGluAspSerGlyAsnSerLeuSerGlyLeuSerThrLeuVal- PheValLeuPro]. Transcripts of this AR variant are characterized by a 69-nucleotides insertion between exon 2 and exon 3. This AR has been identified in a hormone-refractory metastatic PCa.
The adding of 23 amino acids in this region of the DBD influences the subcellular compartmentalization of the AR preventing the nuclear translocation of the receptor. Indeed, AR23 variant is exclusively cytoplasmic. AR 23 forms aggregates under DHT stimulation. Furthermore, cytoplasmic actions of this AR affect the activity of transcription factors such as nuclear factor-κB (NF-κB) and activator protein- 1 (AP-1) suggesting the contribution of this AR variant to tumour progression by protecting PCa cells from apoptosis. In LNCaP cell line, the expression of the AR23 leads to an increase of transcriptional activities of two AR-responsive promoters, the MMTV-LTR and the human PSA gene promoters, after DHT stimulation. The AR23 has no effect on these two promoters in AR-negative COS-1 cells, highlighting the need of another receptor to trigger a signal to the nucleus and enhance transcription from androgen-responsive promoters.(Jagla et al., 2007)

Constitutive active AR

Various nonsense mutations that lead to a premature termination codon and consequently to a truncated AR protein devoid of its carboxy-terminal end (CTE) were found in 8 out of 44 (18%) localised tumour specimen analysed so far, and in 5 out of 6 (83 %) metastatic PCa specimen. These CTE-truncated AR lack the entire or a great part of the ligand binding domain and all the AF-2. We have recently demonstrated that these CTE-truncated AR form specific transcriptional complexes due to altered cofactors recruitment, and exhibit constitutive ligand-independent transcriptional activities. These AR, devoid of the ligand binding domain but displaying an autonomous transcriptional activity, represent the paradigm of a mutant AR able to guide PCa to a hormone-independence status. Also, we have associated their presence with a differential gene expression profile, the activation of AP-1

and NFAT signalling pathways, with the secretion of paracrine factors that activate the AR signalling pathway in neighbouring cells in the absence of androgens, and finally with an induction of osteoclastogenesis.(Lapouge et al., 2007; Lapouge et al., 2008)

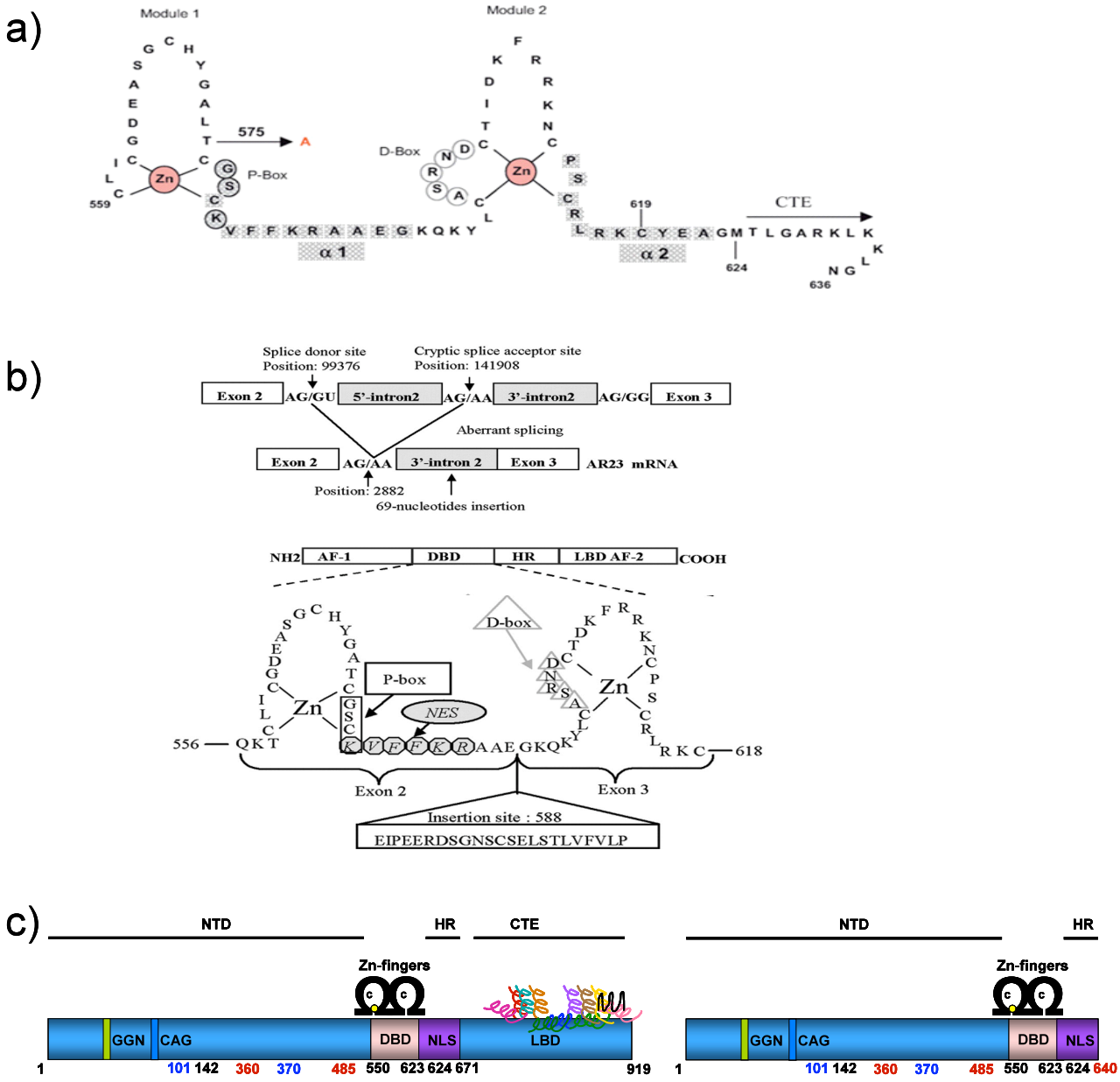


Fig.8: Androgen receptor mutations recently described. a) The T to A mutation is located at position 575 in the first zinc module, of the DNA-binding domain (DBD), just before the P-box, which intervenes in ARE recognition.T575A mutation affects AR transcriptional activities upon hormonal stimulation in a promoter-dependent manner. **b)** AR23, results from an aberrant splicing of intron 2, wherein the last 69 nucleotides of the intronic sequence are retained, leading to the insertion of 23 amino acids between the two zinc fingers in the DNA-binding domain, in position 588. **c)** The Q640Stop mutation leads to a truncated AR molecule downstream the DNA binding domain, changed glutamine at position 640 to a termination codon.

Growth factors in prostate cancer

The regulation of prostate growth depends on several factors such as cell growth, proliferation and apoptosis involved factors. The loss of apoptosis toward apoptotic evasion, the over-expression of factors promoting cell survival, uncontrolled proliferation and increased invasive potential are the physiological basis of the tumourigenesis and cancer. Since growth factors bind their cognate tyrosine kinase receptors, causing the activation of kinase phosphorylation cascades altering the ordinary balance between proliferation and apoptosis, they are involved in the progression of the prostate cancer.
Several studies offer widespread eventuality of the cross-talk between the androgen receptor and growth factors signalling and suggest that AR phosphorylation might promote development of hormone-refractory PCa (HRPC). It has also been reported that activation of phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway can induce expression of AR at the protein and mRNA level, again suggesting that this pathway may be involved in hormone-refractory disease.(Reynolds and Kyprianou, 2006)

Growth factors

Transforming growth factor beta (TGF-β)

TGF-β is a ubiquitous cytokine involved in various cellular mechanisms.
In the prostatic gland TGF-β is expressed by both epithelial and stromal compartments, acting as a tumour suppressor via the inhibition of the prostatic epithelial cell growth and the induction of apoptosis. Once released from prostatic stromal cells, TGF-β binds the surface serine/threonine kinase cell receptors TβRI and TβRII, and exerts its paracrine functions. The heterodimerization of TβRI and TβRII causes the activation of the intracellular phosphorylation cascade targeting the SMAD effectors. Within the effector molecules of the TGF-β, SMAD3 is responsible of the increase of the androgen receptor transactivation (Fig.9).
In PCa, the over-expression of TGF-β, mutations on the TβRs, alterations of the downstream signalling molecules are all responsible of implication in the tumour progression. In advanced

PCa, TGF-β is involved in the formation of the osteoblastic metastatic remodelling.(Reynolds and Kyprianou, 2006; Zhu and Kyprianou, 2008)

Insulin-like growth factor 1 (IGF-1)

IGF-1 is produced by prostatic stromal cells in response to androgen stimulation, and exerts mitogenic action on prostatic epithelial cells in a paracrine manner. The binding of IGF-1 ligand to its cognate receptor (IGFR) leads to the activation of signalling pathways involved in cell growth and proliferation (Fig.9).
In normal prostate conditions, the stimulation of prostatic epithelial cells by IGF-1 enhances proliferation and cell survival events. The activation of the IGFR transactivates the downstream cell kinase survival pathway PI3K/Akt, via the phosphorylation of phosphoinositol-3 kinase (PI3K). The activation of PI3K causes a rapid activation of Akt that phosphorylates proapoptotic Bad protein blocking apoptosis. PI3K activated by IGF1 inactivates FOXO1 (Forkhead box O1) interfering with AR/DNA binding, and suppresses the AR expression and transcriptional activity.(Zhu and Kyprianou, 2008)

Fibroblast growth factor (FGF)

The fibroblast growth factors family includes 22 members, which are heparin-binding proteins and conserve a high affinity for heparin analogues, in the ECM (extracellular matrix). Four FGF-receptors (FGFR) have been identified: FGFR1, FGFR2, FGFR3, and FGFR4. Each FGF can bind several FGF receptors and heparin-binding regions. The binding with heparin preserves FGF from degradation and allows proteins stores. FGF-2 and FGF-7 have been proposed as a contributor in the prostate cancer development mechanism toward hormone-independent tumour. The expression of more FGF in prostate is regulated by estrogen and androgen receptors; few members of the family positively regulate the activity of the AR (Fig.9).(Reynolds and Kyprianou, 2006; Zhu and Kyprianou, 2008)

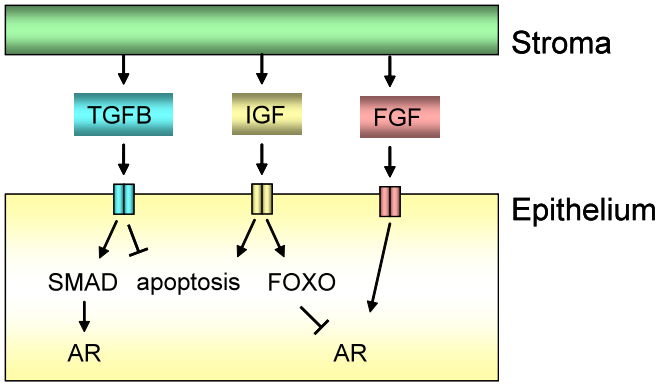


Fig.9: Schematic representation of TGFB, IGF and FGF in stroma/epithelium interaction.

Vascular endothelial growth factor (VEGF)

VEGF is a mitogen cytokine for endothelial cells, an activator of cellular division, proliferation and angiogenesis and a crosstalk molecule between the stromal and epithelial cells of the prostate (Fig.10).

In androgens ablation conditions, VEGF expression is temporally decreased, but the activation of RalA (a small GTPase) by the reactive oxygen species enhance the VEGF-C synthesis.

Released-VEGF starts the downstream pathway involved in the tumourigenesis binding to the tyrosine kinase receptors located on endothelial cell membranes, VEGFR-I and VEGFR-II. The activated VEGFR can trigger molecules such as the signal transducer/activator of transcription-3 (STAT3) and hypoxia inducible factor 1-a (HIF-1a), or induce cell survival and mitogenic pathways through the PI3K/Akt and the Ras-mediated MAP kinase pathways. VEGF-C also enhances the expression of coactivators supporting the androgen receptor transactivation.(Reynolds and Kyprianou, 2006; Zhu and Kyprianou, 2008)

Interleukine-6 (IL-6)

IL-6 is a cytokine that induce phosphorylation of the downstream signal transducers and activators of transcription STAT-3, MAPK, as well as phosphatidylinositol 3-kinase (PI3-K). AR transactivation is influenced by the availability of these three signal transducer (Fig.10).

IL-6 and its receptor are expressed in the majority of prostate tumours. Serum levels of IL-6 and its cognate receptor are elevated in men with hormone-refractory prostate cancer that display high levels of serum prostate specific antigen. IL-6 has been considered a positive growth factor in late-stage prostate cancer cells and a potential target for therapeutic interference.

Indeed, IL-6 and the related cytokine can induce activation of the androgen receptor (AR) in the absence of androgen.

Furthermore, it is known that the activation of STAT3 signalling pathways may enhance AR transactivation in an androgen-independent manner and is associated to a positive regulation of the proliferation.(Cavarretta et al., 2007; Culig, 2005)

Interleukine-8 (IL-8)

IL-8 is a CXC chemokine (chemoattractant cytokine with one amino acid between the first two cysteines) involved in events such as angiogenesis, tumourigenesis and lymph node metastasis. IL-8 contributes to the progression toward an androgen-independent state of the disease. Elevated serum of IL-8 and IL-8 G-protein coupled receptor (CXCR-1 and CXCR-2) have been reported in patients with localized disease and AIPC.

IL-8 increases the expression, alters the distribution and the transcriptional activation of the AR. Indeed, the level of mRNA transcripts encoding for the AR is deregulated in the presence of IL-8 in LNCaP and 22Rv1, human PCa and androgen-responsive human prostatic carcinoma cell lines respectively. The blockade of IL-8 signalling sensibilizes CaP cells to treatment with bicalutamide. Furthermore, the activation of p42/44 mitogen-activated protein kinase, phosphatidylinositol-3 kinase, STAT-3, and other co-activators of the AR, involved in signal transduction cascades in AIPC cells have been already established, subsequent to the IL-8 signaling induction (Fig.10).(Seaton et al., 2008)

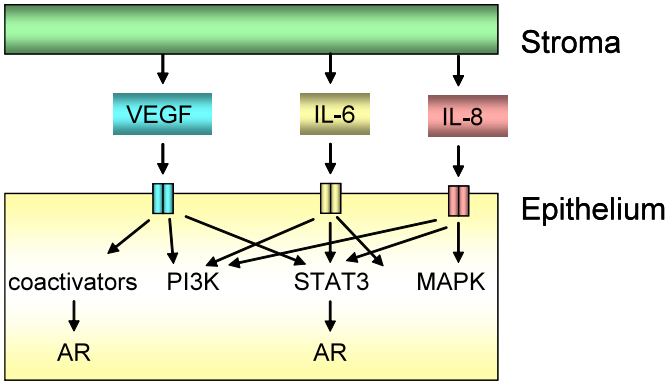


Fig.10: Schematic representation of VEGF, IL-6 and IL-8 in stroma/epithelium interaction.

The EGFR/downstream kinases pathways

Epidermal growth factor (EGF) and Transformed groth factor α (TGF- α)

Epidermal and transformed growth factors belong to the family of natural ligands of the epidermal growth factor receptor (EGFR).

Normal prostate or localized carcinomas express low level of EGF and TGF-α. In normal adult prostate, EGF is expressed by the luminal/apical region of prostate luminal epithelial cells and released into the lumen of the prostatic duct, whereas TGF-α is expressed and secreted by smooth muscle cells of the stroma.

In physiological conditions, the low concentrations of stromally produced TGF-α, but not the high levels of EGF produced by the apical region of the luminal cells, can reach the EGFR present on the basolateral membrane, under the tight junctions of the luminal cells. EGF secretion into the lumen avoids autocrine stimulation of EGF receptors located in the basolateral membranes.

In the neoplastic acini, the lack of this organization allows the exposition of the EGFR to EGF secreted from the apical membrane and creating an autocrine stimulating loop. Taking into consideration protein or RNA expression, epidermal growth factor seems to be the main EGFR ligand in early and localized prostate cancer.

TGF-α appears as a principal ligand during the progression of the disease in later step of the progression such as development of bone metastasis, suggesting a role in metastasis development, whereas the EGF level in this step is diminished.
The binding of TGF-α to the cognate receptor activates kinase pathways such as AKT and MAPK.
EGF could enhance the cytokine osteoprotegerin (OPG) releasing against the differentiation of the osteoclasts.(DeHaan et al., 2009; Mellinghoff et al., 2004; Montano and Djamgoz, 2004)

The epidermal growth factor receptor (EGFR)

Studies on the involvement of growth factors receptors show that nearly 30% of PCa patients express an elevated EGFR level, thus stimulating the interest in the knowledge of its working mechanism.
EGFR belongs to a family of transmembrane receptor tyrosine kinases that includes three other members of human epidermal growth factor receptor (HER) such as: erbB2/HER-2, erbB3/HER-3, and erbB4/HER-4. EGFR is normally involved in cell proliferation, survival, adhesion, migration and differentiation (Fig.11).
These receptors are anchored in the cytoplasmic membrane and share a similar structure that is composed of three major functional domains: an extracellular ligand-binding domain, a hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase domain. The extra-cellular domain is the less conserved among the four receptors, suggesting a different specificity in ligand binding.
In prostate cancer, EGFR becomes activated by receptor overexpression as well as ligand-dependent and ligand-independent mechanisms. EGFR over expression have been associated to progressive PCa but not with metastatic disease.
There are seven known ligands that bind to the ErbB receptors. The first group includes EGF, transforming growth factor α (TGF-α) and amphiregulin (AR), which bind specifically to the EGFR (HER1). The second group includes betacellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR), which show dual specificity by binding both EGFR and ErbB-4. The third group is composed of the neuregulins (NRGs) and covers two subgroups based upon their capacity to bind ErbB-3 and ErbB-4 (NRG-1 and NRG-2) or only HER-4 (NRG-3 and NRG-4).

Ligand binding to the receptor induces a conformational change of the receptor resulting in receptor dimerization and internalization. Activation of the receptor leads to the phosphorylation of tyrosine residues within its COOH-terminal portion. Phosphorylation and dephosphorylation of this part provides specific sites for cytoplasmic proteins containing a Src homology 2 and phosphotyrosine binding domains. These proteins bind to specific phosphotyrosine residues and initiate intracellular signalling via several pathways.

EGF binds to an EGF receptor to induce receptor dimerization and activate PI3K, while the receptor HER-2 has an intrinsic tyrosine kinase activity that activates the receptor-mediated signal transduction in the absence of ligand. In some tumours, constitutive ligand-independent receptor activation can occur in relation with some form of the EGFR and HER deleted of the extracellular domain. Ligand-independent receptor activation occurs as a result of cellular stresses, such as radiation, silencing phosphatases that antagonize the receptor kinase activity.(Harari, 2004; Lemmon, 2009; Navarro et al., 2002) ErbB receptor signal are involved in the optimal AR function at physiologically low androgen concentration conditions. HER2 enhances the AR binding to its cognate DNA-target regions and protects the AR from ubiquitin- mediated degradation.(Mellinghoff et al., 2004)

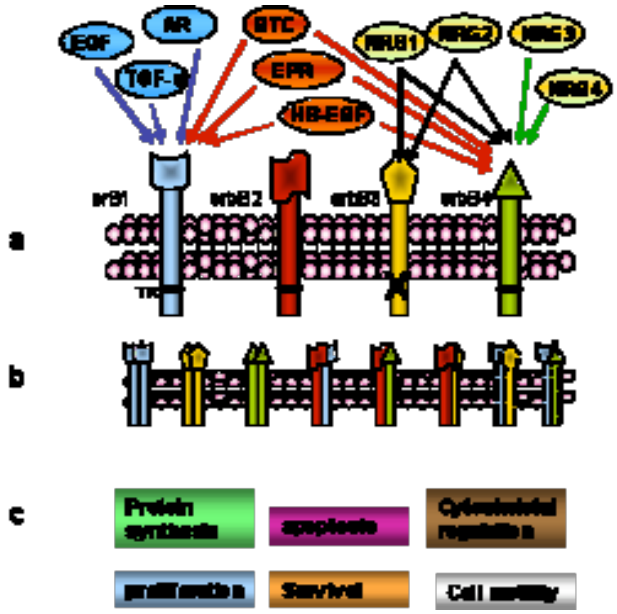


Fig.11: EGFR family. The HER receptors family members are anchored in the cytoplasmic membrane. They display an extracellular ligand-binding domain, a hydrophobic transmembrane region, and a cytoplasm tyrosine kinase domain. EGFR family consists of four receptors having different specificity in ligand binding (ErbB1-4).
The first group of EGFR ligand includes EGF, transforming growth factor α (TGF-α) and amphiregulin (AR) which bind specifically to the EGFR (erbB1)(a). The second group includes betacellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR), which are able to bind both EGFR and ErbB-4. The third group includes the neuregulins (NRGs), where NRG-1 and NRG-2 bind to bind ErbB-3 and ErbB-4, whereas NRG-3 and NRG-4 bind only ErbB-4. The bound receptor changes conformationally permitting dimerization and internalization of the homo/heterodimer (b). ERBB3 has impaired kinase activity and only acquires signalling potential when it is dimerized with another ERBB receptor.
The erbB2 has not ligand and display constitutively an open conformation that renders the receptor an optimal interaction protein for the formation of the heterodimer with the others member of the family. The activated receptors are phosphorylated in their C-terminal end and can interact with proteins containing Src homology 2 and phosphotyrosine binding domains, which enhance the activation of intracellular signalling pathways controlling several cellular mechanisms (c).

Growth factor downstream signals: kinase transduction molecules

Widespread downstream kinases including Shc and Grb2, leading to the Ras/MAPK or the regulatory domain of PI3K activation are activated by the tyrosine kinase receptors.(Reynolds and Kyprianou, 2006)

Guanine nucleotide-binding proteins (G-Protein)

G-proteins are activated by the exchange of a GDP molecule to a GTP molecule. The family of GTPase is divided in two main groups: the Ras small monomeric GTP-ase and the heterotrimeric G-protein.

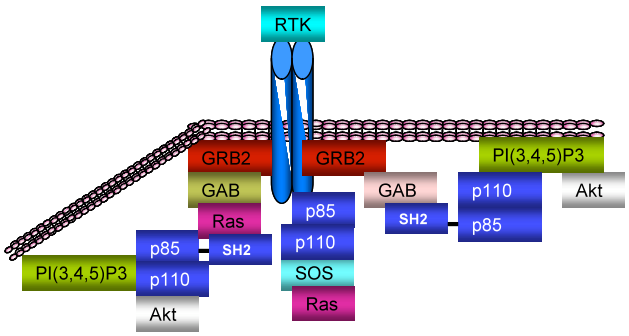
- H-, N-, K- are the three Ras isoforms, which share a well conserved G-domain responsible for the GTP-GDP exchange, a poorly conserved C-terminal region target of post-translational modifications, and a linker region. Cell membrane growth factor receptors trigger Ras isoforms activation. The serine-threonine kinases MAPK pathway is the common downstream Ras-effector, but the affinity for the downstream effectors changes following the Ras isoform.

- G-proteins are peripheral membrane proteins that have three subunits α-, β- and γ-Gs, which all can interact with the G-protein receptor and activate the heterotrimeric complex. The exchange GDP-GTP of the alpha subunit leads to the inactivation of the dimer, thus enabling interactions with effectors molecules.(Vogler et al., 2008)

Signalling pathways triggered by G protein-coupled receptors (GPCRs) promote PCa cells from an androgen dependent AR activation toward an androgen-independent activation state. In advanced PCas, GPCR system may be excessively activated by abnormally elevated ligands expression and/or over expression of GPCRs. G-protein signalling 2 (RGS2) inhibits androgen-independent AR activation by inactivating G-coupled signalling pathway in prostate cancer cells. The loss of this regulator expression contributes to the androgen-independent activation of AR in androgen-independent PCa. Through G-coupled receptors, the activated phospholipase C, enhances inositol-1,4,5-trisphosphate, diacylglycerol and activates ERK via both Ras-dependent and Ras-independent pathways. Activated-ERKs can directly phosphorylate AR in vitro.(Cao et al., 2006)

Phosphatidylinositol 3-kinase (PI3K)

Mammalian cells present three classes (I, II and III) of PI3K. The class I is subdivided into two subtypes: IA p110 (α/β/δ) and IB (p110γ). IA catalytic isoforms are associated to any of five distinct regulatory subunits (p85alpha, p85beta, p55gamma, p55alpha, and p50alpha) known as p85s, while IB p110γ is associated with p101/p84 regulatory subunit (Fig.12). The p85 and p110 subunits are present in equimolar amounts in mammalian cell lines and tissues.
The catalytic activity of p110 is inhibited by the p85 domain that stabilizes the labile p110 protein. The recruitment of the p85-p110 complex, via the p85 SH2 (Src homology 2) domains, to receptors and adaptor proteins activate the PI3K toward the production of PIP(3) (phosphatidylinositol 3,4,5-trisphosphate).
PI3K class IA p85α and p110β isoforms are essential for androgen-stimulated AR transactivation and cell proliferation. These two isoforms are highly expressed in malignant prostate tissues, and their expression levels significantly correlate with PCa disease progression.
The p110β enhances AR activity in the presence of castration level androgens or even in the absence of androgens. Knocking out p85α and p110β significantly suppressed the androgen-stimulated cell proliferation in LNCaP cells. Since PI3K activity is required for AR-DNA interaction and p110 β -derived signalling is essential for androgen induced AR-DNA binding, the PI3K signalling is probably involved in the AR-mediated transcription complex recruitment.(Zhu et al., 2008)



Class1	Subclass IA	Subclass IB
Catalytic	p110α p110B	p110γ
Regulatory	p85α p85β p55γ p55α P50α (p85s)	p101/p84

group	gene	protein
class 2	PIK3C2A	PI3K, class 2, alpha polypeptide PI3K-C2α
	PIK3C2B	PI3K, class 2, beta polypeptide PI3K-C2β
	PIK3C2G	PI3K, class 2, gamma polypeptide PI3K-C2γ
class 3	PIK3C3	PI3K, class 3 Vps34
catalytic	PIK3CA	PI3K, catalytic, alpha polypeptide p110-α
	PIK3CB	PI3K, catalytic, beta polypeptidep110-β
	PIK3CG	PI3K, catalytic, gamma polypeptidep110-γ
	PIK3CD	PI3K, catalytic, delta polypeptidep110-δ
regulatory	PIK3R1	PI3K, regulatory subunit 1 (alpha) p85-α
	PIK3R2	PI3K, regulatory subunit 2 (beta) p85-β
	PIK3R3	PI3K, regulatory subunit 3 (gamma) p55-γ
		PI3K, regulatory subunit 4 p150
	PIK3R5	PI3K, regulatory subunit 5 p101
		PI3K, regulatory subunit 6p87

Fig.12: PI-3K family members. Class I PI3Ks are responsible for the production of phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol (3,4)-biphosphate (PI(3,4)P2) and phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3. Phosphatidylinositol 3-kinase (PI3K) can become activated by the binding of ligand to receptor tyrosine kinases (RTKs) or by activated G-protein coupled receptors.
Class I PI3K is divided in two subclasses: IA and IB, which are composed of a regulatory and a catalytic subunit. Class I PI3K proteins are able to form heterodimer between the regulatory p85α, p55α, p50α, p85β or p55γ subunit and the catalytic subunits p110α, β or δ. The p85 subunits contain SH-domain and SH3 domain.
Class II includes three catalytic isoforms (C2α, C2β, and C2γ), which catalyse the production of PI(3)P and PI(3,4)P2 from PI. There is no evidence of regulatory proteins for this class.
Class III forms heterodimers as class I and their function is to produce only PI(3)P from PI.
Activated RTKs can bind Src homology 2 (SH2)-domain-containing molecules. Ras through the activation of SOS, and Ras activates p110 independently of p85. Ras-mediated PI3K activation might require the phosphorylation of YXXM motifs, and the activation of small GTPases.

Activated protein-serine-threonine kinase (Akt)

PI3K includes several substrates within the most relevant is Akt.

Akt, also known as protein kinase B (PKB), has been implicated in the stimulation of cell proliferation and survival (Fig.13). The activation of Akt requires an activating

phosphorylation at two sites, Ser-473 and Thr-308. Activated Akt promotes cell survival by phosphorylating and inactivating the pro-apoptotic proteins BAD and caspase-9.(Kandel and Hay, 1999)

The AFX/Forkhead family of transcription factors that are involved in cell-cycle progression, is negatively regulated by Akt. Indeed, Akt suppresses AFX-mediated transcription of target genes such as the cyclin dependent kinase inhibitor protein p27Kip1 (cyclin-dependent kinase inhibitor 1B).(Medema et al., 2000)

Increased activation of Akt and decreased activation of ERK have been proposed as predictif for poor clinical outcome in prostate cancer. The PI3K/Akt pathway mediates proliferation in androgen-dependent and -independent prostate tumour cell lines as well as in human prostate tumours in situ. Thus, in androgen-dependent cells, Akt acts on the AR transcriptional activity, whereas in androgen independent cells, Akt operates via the p70 S6 kinase.(Kreisberg et al., 2004) Indeed, Akt inactivates by phosphorylation the glycogen synthase kinase 3b (GSK3b), which in turn, promotes the downregulation of p27Kip1.

The induction of AR transcriptional activities may be the result of a direct phosphorylation of the AR by Akt. The expression of activated isoform of Akt, but not the total Akt, changes with tumour development and progression.(Ghosh et al., 2005; Lin et al., 2001; Sharma et al., 2002; Wen et al., 2000)

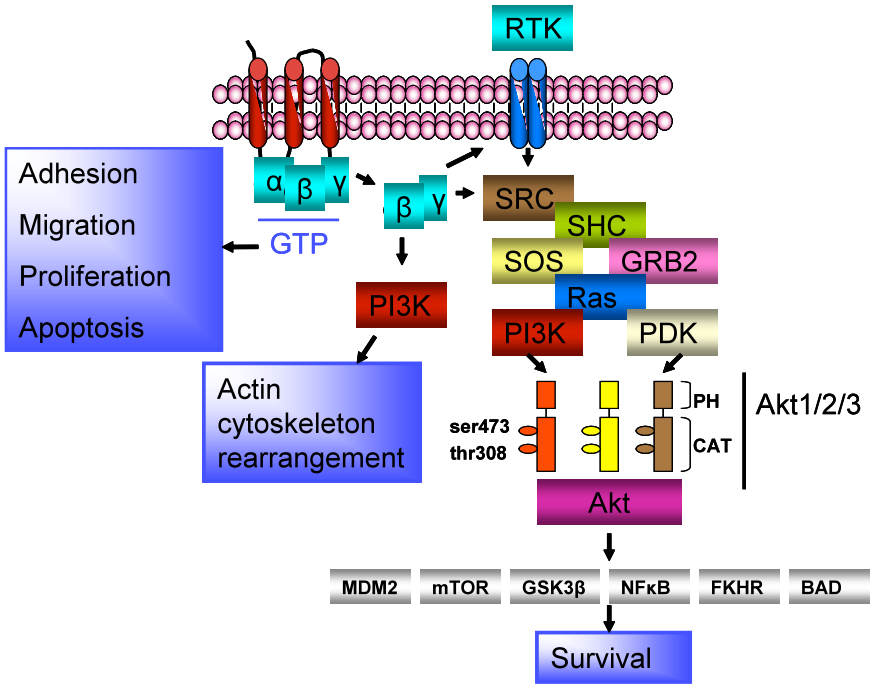


Fig.13: Akt activation pathways. Activation of AKT is initiated by membrane translocation, which occurs after cell stimulation and PtdIns (3,4,5)P3 (PIP3) production. Location of AKT to the plasma membrane is accomplished by an interaction between its pleckstrin-homology (PH) domain and PIP3. At the membrane, association with carboxy-terminal modulator protein (CTMP) prevents AKT from becoming phosphorylated and fully active. Phosphorylation of CTMP by an as yet unidentified kinase releases CTMP from AKT and allows AKT to be phosphorylated by PDK1 and PDK2 at Thr308 and Ser473, respectively. Phosphorylation at these two sites causes full activation of AKT.

Glycogen synthase kinase 3 (GSK-3)

GSK-3 activity is fundamental for androgen-stimulated gene expression. The GSK-3 activation is usually associated with phosphorylation of the tyrosine 216(Y216), whereas its inhibition is caused by the phosphorylation on serine 9 (S9) residual. Furthermore, GSK-3 can be activated by transient increase in intracellular calcium.

In response to the activating phosphorylation, GSK-3 is able to phosphorylate several downstream transcription factors as c-jun, c-myc, cAMP response element binding protein, heat shock factor-1, cytoskeletal proteins such as the microtubule-associated protein, the multifunctional protein-catenin and the glucocorticoid receptor.

PI3K/Akt and Wnt pathways negatively regulate GSK-3. PI3K pathway is involved in androgen-stimulated gene expression. Inhibitors of GSK-3 or PI3K blocked androgen-

stimulated gene expression in LNCaP and LAPC-4 cells. Furthermore, these cell lines express a PI3K-dependent pathway requirement for androgen-induced GSK-3 Y216 phosphorylation.
A study within several prostate cancer cells lines demonstrated that GSK-3 protein expression was higher in androgen-insensitive cells lines compared to androgen-sensitive cell lines. Moreover, androgens treatment rapidly increased GSK-3 Y216 phosphorylation level, without altering Akt S473 phosphorylation. No significant alteration on the S9 phosphorylation level was observed after androgen-induced GSK-3 Y216 phosphorylation.(Liao et al., 2004)

Protein kinase C (PKC)

Protein kinase C is involved in cell apoptosis and survival. PKC family includes at least ten serine-threonine kinases members based on their second messenger requirements as diacylglycerol (DAG). PKC activity is regulated by coupling-molecules and downstream target effectors, including the extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK), NF-kB, and Stat signaling pathways. Cytokines such as TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) and TNF-α induce cell death by PKC/p38MAPK and JNK cascades. Phorbol esters activators of PKC, PMA, enhance the autocrine secretion of death factors as TNF-α and TRAIL in prostate cancer cells. In the absence of androgen stimulation, PMA fails to release TNF-α and TRAIL, and consequently the overall apoptotic effect of the phorbol ester is impaired.
The human PKCd gene possesses several androgen responsive elements (AREs), thus making androgen regulators of PKC transcription. Indeed, androgen depletion induces a reduction in PKCd expression levels in prostate cancer cells. Moreover, androgens make LNCaP prostate cancer cells sensitive to the release of cytokines by PMA.(Xiao et al., 2009)

Mitogen-activated protein kinase (MAPK)

MAPK family regulates gene expression in response to extracellular stimuli. The activation of the MAPK pathways produces a cascade of kinases resulting in the activation of terminal kinases, such as p38, c-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERK). Several transcription factors are targets of activated p38, JNKs, and ERKs phosphorylation.

At least four classes of MAPKs have been identified including molecules such as extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38a/b/g/d) and ERK5, that are activated by specific MAPKKs: the MEK1/2 for ERK1/2, the MKK3/6 for the p38, the MKK4/7 (JNKK1/2) for the JNKs, and the MEK5 for ERK5.
The MAPKs signalling is transmitted in the presence of downstream molecules with serine or theonine followed by a proline sites.
Several MAPKK can generate the activating phosphorylation of each MAPK. MAPK signalling can be regulated by sequential interactions between members of a given cascade (JNK1/2 is bound by the N-terminal extension of MKK4 (JNKK1)), or following the ability of MAPKs to indirectly regulate the expression of both ligands and inhibitors for cell-surface receptors. Most transcription factors modulated by MAPKs are dimers, and structural analysis indicates that some MAPKs dimerize; MAPK dimerization may facilitate phosphorylation of dimeric transcription factors.(Chang and Karin, 2001)
ERK is involved in the stimulation of cell proliferation and cellular differentiation; its accumulation in the nucleus regulates transcription factors leading to DNA synthesis.(Pouyssegur et al., 2002)
An increase in ERK activation in primary prostate cancer, compared with normal prostate and benign prostatic hyperplasia and in PIN versus normal prostate ERK activation, was necessary for tumour initiation.(Royuela et al., 2002)

The cell survival function of the PI3K pathway

Induction of AR transcriptional activities by EGF

Akt activatin enhances the cell survival by blocking apoptosis via the inactivation of proapoptotic proteins. Activated-Akt phosphorylates specific targets such as Bad, pro-caspase-9, Bcl-2-related phosphoprotein and transcription factor FKHRL, by site-specific phosphorylation on the Akt consensus sequence R-X-R-X-X-S/T.
Since the androgen-independent activation of the AR signalling pathway may promote survival of prostate cancer cells during androgen deprivation, it has been lengthy investigated whether Akt is involved in the AR activation mechanism in androgen independent survival or growth of prostate cancer cells.(Lin et al., 2001; Wen et al., 2000)

PI3K is an essential mediator of cell survival in LNCaP cells; in this cell line the pharmacological attenuation of PI3K-signalling triggers a rapid and extensive apoptotic response. Apoptosis does not occur when cells are cultured in presence of a potent and specific inhibitor of the AR or when they were cultured in androgen-depleted medium.

The apoptotic response after the inhibition of PI3K is antagonized via the activation of the AR by the DHT and via EGFR receptor activation. In this case, EGFR ligands such as EGF, TGF-α, HB-EGF act as PCa survival factors in addition to their role as carcinoma and epithelial cell mitogens. (Lin et al., 1999)

Furthermore, Akt activates PSA transcription in the absence of androgen. In these conditions, Akt acts as an androgen-independent transactivation mediator, thus may enhancing prostate cancer cell survival during androgen deprivation. The use of dominant negative mutant for Akt or a PI3K inhibitor LY294002 suggested that activation of the AR pathway by HER-2 requires functional Akt. Indeed, the stimulatory effect of HER-2 on the activation of the AR is dramatically reduced by DN-Akt or LY294002. These results suggest that HER-2/neu promotes prostate cancer cell survival upon androgen withdrawal through the Akt pathway that restores the androgen receptor signalling during androgen ablation.(Wen et al., 2000)

EGF induces phosphorylation at one or more sites between AR residues 507 and 660. The phosphorylation of the AR in the P-box of the DNA-binding-domain first zinc is a crucial residual for AR transactivation in response to EGF signalling. In the CW22R-R1 prostate cancer cell line in the absence of androgen, EGF and DHT act synergistically to increase AR transcriptional activity.

Mutagenesis studies introducing single serine or threonine to alanine and tyrosine to phenylalanine in the AR sequence identified the EGF-dependent AR phosphorylation site(s): i) at the MAPkinase consensus phosphorylation sites S515 before and after treatment with EGF; ii) at the PKC consensus phosphorylation site at the Ser-578, when tests were assayed in COS and CWR-R1 cells. The AR phosphorylation and the increased AR transcriptional activity in response to EGF are associated with MAP kinase-dependent phosphorylation at AR Ser-515 and by phosphorylation at Ser-578 in the DNA binding domain.(Ponguta et al., 2008)

Akt suppresses androgen-induced apoptosis

Two Akt consensus sequences (RxRxxS/T) are located in the amino-terminal domain and the carboxy-terminal domain of the androgen receptor sequence. These sites may mediate HER-

2/neu/PI3K signalling. The AR may be substrate of Akt. Indeed, on LNCaP and DU-145 cells, the activated PI3K/Akt pathway can phosphorylate AR and inhibit AR target genes, such as p21, modulate androgen/AR-mediated apoptosis therefore expanding the classic role of AR in cell proliferation toward the Akt-mediated apoptotic pathway.(Lin et al., 2001)

The role of Her-2 activation of PI3K/Akt in the enhanced AR-mediated transcription was previously discussed, furthermore, evidences suggest that the opposite may occur and that androgen can activate PI3K via interaction of p85 and Src. Thus, AR could be able to directly activate the PI3K/Akt pathway and therefore avoiding the requirement for growth factor activation of cellular kinases to phosphorylate the AR. (Russell et al., 1998)

Transcription factor in prostate cancer

Nuclear factor of activated T-cell (NFAT)

The NFAT is a family of transcription factors (TF) involved in diverse cellular functions, such as immune response activation through mediators such as cytokines, neuronal development, adipocyte differentiation, angiogenesis, and cancer development.

Five members of the NFAT family have been identified in mammals, including four closely related members, NFAT1 (NFATc2/NFATp), NFAT2 (NFATc1/NFATc), NFAT3 (NFATc4), and NFAT4 (NFATc3/NFATx), and an NFAT like factor (NFAT5/NFATz/TonE-BP). Three members, NFAT1, NFAT2, and NFAT4 are mainly expressed in immune cells and participate in the activation of T and B cells, whereas NFAT3 is primarily expressed in nonimmune tissues, including heart, brain, and breast.

NFAT1/2/4 have a common architecture consisting of the NFAT homology domain and a DNA-binding domain. NFAT1 and NFAT4 are calcium-responsive transcription factors, regulated by the calcium/calcineurin signalling pathway. Within the NFAT family, the highly conserved DNA-binding domain family demonstrates similarity to the DNA-binding domains of Rel-family proteins.

The inactive form of NFAT is localized in the cytoplasmic compartment (Fig.14). The regulatory domain of NFAT (NFAT homology region, NHR) contains a series of serines, which are phosphorylated in resting cell conditions. Stimuli that trigger calcium mobilization result in rapid dephosphorylation of NFAT and its activation. Indeed, the dephosphorylation of NFAT by the calcium/calmodulin activated serine-threonine phosphatase occurs at a specific consensus site “PxIxIT”. This activating input produces nuclear translocation and DNA binding by NFAT, activating the NFAT-mediated gene transcription. In addition, activation of receptor tyrosine kinases such as EGFR, HER2 results in the generation of Inositol-(1,4,5) triphosphate (InsP3) and diacylglycerol (DAG) by phospholipase C. InsP3 mediates the release of calcium from internal stores, which in turn induces the opening of specific store-operated calcium channels. (Hogan et al., 2003; Medyouf and Ghysdael, 2008)

NFAT1 is considered as a pro-apoptotic protein since T lymphocytes lacking this protein have decreased expression of FasL and TNF-α and became resistant to apoptosis, suggesting its putative tumour suppressor role; while NFAT2/3/4 seem to induct cellular proliferation.

Indeed, NFAT can regulate cell cycle progression by modulating cyclin/CDK gene expression and can bind promoter regions of some effectors of apoptosis, such as TNF-α and FasL. NFAT family members are implicated in the regulation of vascular endothelial growth factor-mediated angiogenesis by dictating the expression of cyclooxygenase- 2, an enzyme involved in neovascularization.

In target genes, the NFAT consensus sequence is (A/T)GGAAA(A/N)(A/T/C)N. The NFAT-DNA complex stabilization is regulated by interactions with AP-1 proteins that influences the transactivation of NFAT and the DNA binding.(Macian, 2005; Viola et al., 2005)

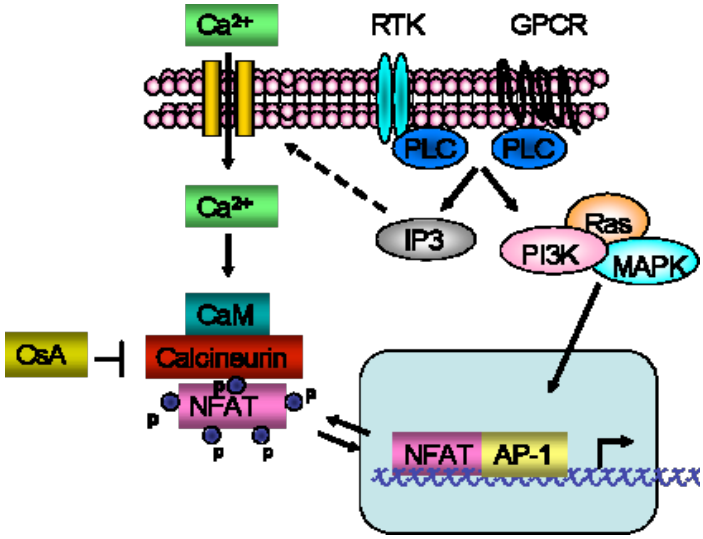


Fig.14: NFAT activation mechanism. The calmodulin (CaM), a calcium sensor protein, activates the serine/threonine phosphatase calcineurin, which in turn dephosphorylates the serine rich region (SRR) in the amino termini of NFAT proteins. Dephosphorylation of NFAT cause conformational modifications allowing the nuclear import of the transcription factor. Activation of receptor tyrosine kinases such as EGFR, HER2 results in the generation of Inositol-(1,4,5) triphosphate (InsP3) by phospholipase C (PLC). InsP3 mediates the release of calcium from internal stores, which in turn induces the opening of specific store-operated calcium channels. The nuclear import is balanced kinases in the cytoplasm and in the nucleus. Once in the nucleus, the DNA binding is allowed by the interaction with others transcription factors. A (CSA) blocks nuclear translocation of the transcription factor.

NFAT: phoshorylation

Several kinases can phosphorylate NFAT proteins and control their nuclear shuttling, including glycogen-synthase kinase 3(GSK), casein kinase 1(CK1), p38 and JUN N-terminal

kinase (JNK). NFAT activation is also mediated by cytokine signalling, regulating the binding of NFAT to severals promoters.(Macian, 2005; Viola et al., 2005)

The NFAT sequence includes at least 22 phosphorylation sites, among these 18 residues are located in the regulatory domain, 14 are conserved in the NFAT family and 13 are dephosphorylated upon stimulation.

The regulatory domain of NFAT includes the binding site of calcineurin, the serine-rich region (SSR), the serine-proline boxes, a nuclear export signal (NES) and a nuclear localization signal (NLS). In the cytoplasm, NFAT is activated by dephosphorylation at multiple sites in the SRR and the serine-proline (SP)-rich boxes. Once phosphorylated, the regulatory domain allows a conformational exposition of the NES region that favorizes the interaction with 14–3–3, thus resulting in the cytoplasmic location of NFAT. Stimuli that increase intracellular Ca2+ concentrations enhance the calcineurin bind to NFAT, causing the dephosphorylation on the TF. In the activated condition, the NLS is exposed and the NES is masked. Dephosphorylation of NFAT induces phosphorylation on the N-terminal transcriptional activation domain, which enhances its transactivation and mediates DNA-binding and transcriptional activities.

A persistent calcineurin/NFAT interaction undergoes rephosphorylation by several kinases, preventing a superfluous activity.

The interaction between NFAT and other transcription factors, such as AP-1, GATAs and MEF2, induces the transcription of target genes.

Few phosphorylation pathways may take part in the activation mechanism of members of the NFAT family. Indeed, GSK3, PKA, JNK, ERK, p38 and casein kinases phosphorylate *in vitro* and/or *in vivo* key serine residues in one or more members of the NFAT family.(Holmberg et al., 2002)

PKA opposes calcineurin-mediated dephosphorylation and nuclear accumulation of NF-ATc1 by direct phosphorylation at Ser-245, Ser-269, and Ser-294 in the conserved serine-proline repeat domain. PKA and GSK-3 cooperate to phosphorylate the single phosphate 2(SP2) and SP3 repeats of NF-ATc1. Since these three serines on NF-ATc1 are variably conserved throughout the NF-ATc family, they are candidates involved in differential regulation of the NF-ATc family members by PKA. GSK-3 decreases NF-ATc1-DNA binding affinity. PKA increases the association of several NF-ATc family members with 14-3-3 proteins, which are postulated to act as phosphoserine-dependent signalling modulators.(Sheridan et al., 2002)

Activator protein-1 (AP-1)

AP-1 transcription factors comprise a ubiquitously expressed family of proteins that include the Jun (e.g., cJun, JunB, JunD), the Fos (e.g., cFos, FosB, Fra-1, Fra-2), the ATF (activation transcription factor), and the MAF (musculoapneurotic fibrosarcoma) proto-oncoproteins (Fig.15). The AP-1–DNA-binding complex can be composed by different hetero- and homodimers as Jun–Jun or Jun–Fos dimers that bind to AP-1 consensus sequence TGA(C/G)TCA. Each different dimer triggers different target gene transcription causing widespread cellular responses. Indeed, AP-1 activity is implicated in many different cellular processes as cell proliferation, differentiation, apoptosis, and stress responses. The role of AP-1 in oncogenic events depends on the activity of different JUN proteins, on the tumour type and stage.

C-Jun plays a pivotal role in apoptosis, promoting cellular death in some tumours types and inducing survival in others. The c-JUN sub-unit, activated by JNK, induces the transcription of positive regulators of cell-cycle progression as cyclin D1, or represses negative regulators as p53. JUNB and JUND are negative regulators of cell proliferation. Furthermore, AP-1 regulates genes involved in tumour metastases, causing epithelial-mesenchymatous transition (EMT).

The AP-1 complex is stimulated by several external stimuli including growth factor and cytokines that trigger the activation of mitogen activated protein kinases and transcription factors ternary complex (TCF), and lead to the transcription of the AP-1 monomers.

The AP-1 complex can activate AP-1 target gene or can be the target of post-translational phosphorylations inducing the stability of the complex. The binding of the AR to androgen response elements can trigger cellular changes in reduction/oxidation (redox) state in androgen-responsive human prostate cancer cells. These androgen mediated changes may affect transcription factors that are sensitive to cellular redox status, such as the AP-1 proteins.(Church et al., 2005; Eferl and Wagner, 2003)

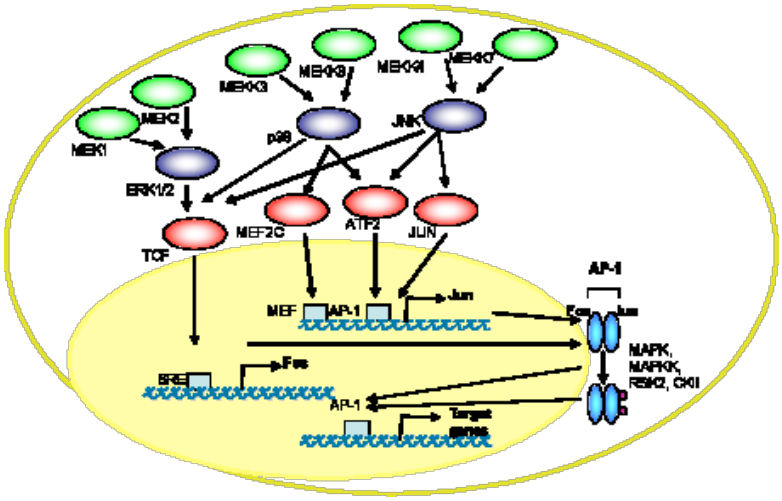


Fig.15: AP-1 activation complex. AP-1 comprises a dimer complex composed of proteins such as c-fos, c-jun, activating transcription factor and JDP families. AP-1 complex activation involves more mechanisms of post-transcriptional regulation. The regulation of the dimer formation is modulated by recruitment of Fos/Jun proteins in the complex that bind to DNA. AP-1 regulates transcription binding to the DNA sequences via a leucine zipper.

AP-1: phosphorylation

The activity of Jun/Fos and Jun/ATF complexes is directed by the synthesis and stability of the mRNAs, and regulation of protein stability via stimulus- dependent degradation via the ubiquitine pathway. In addition, the DNA-binding and transactivating capacities of AP-1 components are controlled through post-translational modification and protein/ protein interaction.

Jun proteins are phosphorylated by JNKs that stimulate the transcription without affecting DNA binding on the JNK docking region (DOCK) of c-Jun. Indeed, the Ser 63 and Ser 73 are preceeded by a short region that is recognized as an interaction surface on JNK, outside the catalytic pocket, which establishes the effectiveness of c-Jun phosphorylation and that directs toward the phosphoacceptor sites.

Fos genes are stimulated by several MAPKs, which phosphorylate Ets transcription factors that are directly involved in the regulation of Fos. The result of heterodimerization between Jun and fos forms activation protein 1 (AP-1) complexes.

ERKs can stimulate cell proliferation indirectly by enhancing AP-1 activity, resulting in cyclin D1 induction. In these conditions, the activation of ERK1/2 has been linked to cell

survival, whereas JNK and p38 are linked to induction of apoptosis. C-Jun and Fos(-related) proteins seem required for oncogenic transformations induced by constitutively active Ras, through the JNK/SAPK and ERK pathways. Jun : Fos and Jun : ATF dimers exhibit distinct functions in cell proliferation, differentiation, apoptosis and oncogenesis.(Chang and Karin, 2001; van Dam and Castellazzi, 2001)

Materials and Methods

Cell lines

LNCaP, Cv-1 and 22Rv1 cells have been adopted as cellular models of this study.
LNCaP cells derive from a lymph node metastasis of a human prostatic adenocarcinoma. This cell line expresses a mutated AR caused by an amino acid switch (threonine to alanine) at amino acid 877. This AR can bind others steroids different from testosterone and DHT. Moreover, the cells line has a mutant phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*). LNCaP cells are considered representatives of an early stage of human PCa cells with a minimal ability to produce metastatic lesions in mice.(DeHaan et al., 2009)
CV-1 cells are issued from kidney of a normal adult male African green monkey (*Cercopithecus aethiops*). CV-1 cells exhibit rapid growth and chromosome number shifts have been reported to occur at high passage levels. (Hronovsky et al., 1978)
Cv-1 cells are AR-negative.
The androgen refractory 22Rv1 PCa cell line, considered as a model for androgen-independent PCa, was derived from CWR22 PCa xenograft. CWR22 were originated from an androgen-dependent human prostatic carcinoma that maintains features expressing prostate specific antigen (PSA) at levels related to tumour burden, regresses after castration, and relapses 3-10 months after androgen ablation.(Sramkoski et al., 1999)

Cell culture reagents

The human androgen-sensitive prostate carcinoma cell line LNCaP clone FCG (European Collection of Cell Cultures, Salisbury, UK, ECACC) and the human prostate carcinoma 22Rv1 cells (ATCC, CRL-2505) were cultured in RPMI 1640 (Roswell Park Memorial Institute) medium supplemented with 10% fetal calf serum, 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 10mM HEPES pH 7.3 (Sigma Aldrich, St Quentin Fallavier, France) and 1mM sodium pyruvate (Invitrogen, Fisher Scientific, Illkirch France).

Cv-1 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FCS, 100U/ml penicillin, and 100µg/ml streptomycin.

Plasmid constructions

Luciferase reporter plasmids

The MMTV-LTR (Mouse mammary tumour virus-long terminal repeat) luciferase reporter plasmid containing glucocorticoid-responsive elements was a gift from Pr. P. Chambon [Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France].

For transcription factors AP-1 and NFAT luciferase reporter plasmid we used pTA- (minimal TA promoter, the TATAbox from the herpes simplex virus timidine kinase promoter) construct that was from BD Clontech. The AP-1 luciferase vector containing six copies of the AP-1 enhancer was fused to the TATA promoter.
The AP-1 responsive sequence (5'-CTGAGTCA-3') is not recognised by the androgen receptor. The NFAT-TA-luc plasmid contains three consensus site repetitions situed on the *IL-2* gene promoter. The NFAT responsive sequence (5′-GGAAA-3′) is not recognised by the androgen receptor Q640X.

The ARE (androgen responsive elements) luciferase reporter plasmids were from Dr. F. Claessens and Dr. G. Verrijdt (Faculty of Medicine, University of Leuven, Belgium). The ARE-PSA artificial promoter displays two copies of androgen responsive elements (AREI) present in the promoter of human *PSA* gene.
All constructs were checked by sequencing.

The pRenilla-luc was from Promega.

ARs expression plasmids: study of the Q640X AR properties

The pEGFP-C3 empty plasmid was purchased from BD Clontech.

The pEGFP-ARwt was constructed as follows. The full-length human AR cDNA was amplified from pSV-AR0 with the 5'-primer with XhoI linker, 5'-GCCAAGCTCGAGAGGATGGAAGT-3' and the 3'-primer with BamHI linker, 5'-TAGGGATCCAATGCTTCACTGGG-3'. The amplified XhoI-BamHI AR fragment was inserted into the corresponding cloning site in the pEGFP-C3 vector, yielding the pEGFP-ARwt.

The Q640X mutant AR cDNA was inserted between the XhoI and BamHI restriction sites in pEGFP-C3 plasmid (Clontech), in frame with the EGFP coding sequence to yield the pEGFP-ARQ640X expression plasmid.

The pEGFP-ARS213A plasmid and the pEGFP-ARS213A/Q640X plasmid were constructed by replacing the PstI-PstI (NM_000044, nt 1283-2101) of pEGFP-ARwt and pARQ640X by the corresponding fragment in pcDNA3-ARS213A that was kindly provided by Dr. S.K. Logan (NYU Langone Medical Center, New York).

Study of the 22Rv1 AR isoforms

ARs expression plasmids

The pEGFP-ARV3, ARV6, ARV7 variant plasmids were obtained from the pEGFP-ARwt replacing the HindIII-EcoRI fragment by the same fragment originating from the corresponding variant.

AR mutations detection

22Rv1 cells were screened for AR mutations using the yeast-based functional assay. Briefly, AR cDNA fragments amplified as described above were inserted into a yeast expression "gap repair" plasmid by homologous recombination.(Ceraline et al., 2003)
Transformed yeasts were plated on media depleted of adenine and containing the indicated concentration of steroid or non-steroid. In the same time, the experiment was performed with wild type AR cDNA fragments as a receptor activity control, whereas the negative control

plate was without hormone and the positive control plate was supplemented with adenine. Yeast colonies were incubated at 30°C during 72h. After incubation, the amount of colonies was scored by Gel Doc system (Biorad).
Results were determined from two independent RT products. From each RT product three independent PCR reactions were carried out. The reproduction of the results assured that variants obtained from the functional assay did not derive from PCR-induced mutations.

RNA isolation

Total RNAs were extracted from 10^6 22Rv1 cells with Trizol reagent (Invitrogen Life Technologies) following the manufactor's instruction for the homogenization, the phase separation, the RNA precipitation, the washing and the redissolving RNA steps.
RNAs were quantified by spectrophotometry (Amersham Pharmacia Biotech, Saclay France).

Reverse transcription

Single stranded AR DNAs were obtained by reverse transcription (RT) of 1µg of total RNA. The RT was realized with the Omniscript reverse transcription kit (Qiagen). After RT, samples were used for standard PCR.

Polymerase chain reaction

To obtain the amount of cDNA necessary for the following yeast functional assay, the RT products were amplified by polymerase chain reaction (PCR). The AR cDNA fragments along exons 1-8 (GenBank NM_000044.2, nt 2310-4101) were amplified with the forward (5'-TGCGGCGGCGCAGTGCCGCTAT-3') (nt 2310-2339) and reverse (5'-GGTGCCATGGGAGGGTTAGATAGGGAG-3') (nt 4075-4101) primers.

Yeast strain treatment conditions

Mutations on the AR sequence of the 22Rv1 cells were identified by the yeast functional assay, which was realized on the recombinant EJ250 yeast strain (MATa *ade2-101 his3-D200 leu2- D1 lys2-801 trp1- D1 ura3-52 URA3:ARE-ADE2* [*pRS/ARE-Ade2*]). In the EJ250 yeast

strain the expression of the *ADE2* gene, necessary for adenine biosynthesis, is under the control of an androgen-dependent promoter.

For the assay, yeasts were transformed with an AR expression plasmid. The yeast growth depends on the responsiveness of the expressed AR to the steroid or non-steroid conditions present in the culture medium.(Ceraline et al., 2003)

Standard yeast manipulations and selection were carried out as described in *Yeast Protocol Handbook* (Clontech Laboratories, CA).

Steroids, adenine and the anti-androgen Flutamide used for the yeast assay were from Sigma Aldrich (St Quentin Fallavier, France). Bicalutamide was kindly provided by Astra Zeneca.

Plasmid rescue

Transformed yeasts were cultured at 30°C.

The extraction of the AR expression plasmid was realized via lysis with 150-212mm acid-washed glass beads (Sigma Aldrich, St Quentin Fallavier, France). The purification of the plasmid DNA was performed with the Nucleospin plasmid kit (Macherey-Nagel, Hoerdt).

The purified plasmids or the pARwt were amplificated using the forward (5'-CCACCCCAGAAGACCTGCCTGATCTG-3'; nt 2778- 2803) and reverse (5'-AGTTGCGGAAGCCAGGCAAGGCCTTG-3'; nt 3275- 3300) primers, of a shorter fragment (flanking exons 2-4); (NM_000044.2; 522pb, nt 2778-3300).

After the PCR amplification, the size of AR variants cDNAs was measured. The PCR reaction was realized by 35 cycles of amplification (45s at 94°C, 45s at 57°C, 1.5min at 68°C) and a final cycle of 5min at 68°C.

Impact of the kinase pathway on the Q640X AR

Hormonal treatment

Dihydrotestosterone (DHT) is the natural ligand of the androgen receptor and is a biologically active metabolite of the hormone testosterone. DHT was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Gefitinib treatment

Gefitinib and prostate cancer

EGFR inhibition can be obtained by monoclonal antibody blocking the extracellular ligand-binding domain and targeting intracellular tyrosine kinase domain reversibly, enabling phosphorylation of the EGFR-TK and inducing the internalization and the degradation of the receptors, thus by the small-molecule inhibitors.(Rocha-Lima; 2007) Both ErbB1 (EGFR) and ErbB2 inhibitors can be involved in the therapeutic targeting of hormone-refractory prostate cancer. Indeed, upon long-term androgen ablation, cells develop the ability to use alternative transduction pathways. Since the overexpression of the erbB1 and erbB2 receptors are involved in tumour progression toward androgen independence in human PCa, the combination therapy of an EGFR tyrosine kinase (EGFR-TK) quinazoline inhibitor, gefitinib, with a nonsteroidal antiandrogen (androgen receptor antagonist), bicalutamide have been suggested as antagonists to tumour cell growth. Indeed, it is known that the treatment with EGF causes an increase in cell proliferation in a dose-dependent manner. This effect is comparable to that induced by DHT. Furthermore, in the absence of androgens, EGFR may increase the enzymatic activities of effectors including PI-3K and MAP kinase, involved in cell survival mecanisms. The blockade of ErbB1 signalling pathways by gefitinib was able to reduce cell proliferation, but the treatment with DHT could counterbalance the action of the inhibitor.(Gross et al., 2004)

Inhibition mecanism

Gefitinib inhibits the EGFR signal transduction competing with the binding of ATP to the tyrosine kinase domain of EGFR, thus inhibiting the autophosphorylation and activation of the receptor.

Gefitinib was kindly provided by AstraZeneca.

Trastuzumab treatment

Trastuzumab and prostate cancer

Her2 is a member of the growth factor receptors family. Her2 is implicated in the progression of breast cancer and can be over-expressed in prostate cancer. There is no evidence of the HER2 inhibitor benefit in PCa treatment. Instead of this, several studies encouraged the targeting of the ErbB signalling pathways against the PCa progression.(Gross et al., 2004)

Inhibition mechanism

The Trastuzumab is a monoclonal antibody that binds the extracellular side of the HER2-neu receptor, thus inducting the decrease of the proliferation.(Cho et al., 2003)

Trastuzumab (Herceptin) was from Roche.

LY294002 treatment

LY294002 and prostate cancer

Phosphatidylinositol 3-kinases (PI3K) belong to a family of lipid kinases that phosphorylate the 3-hydroxyl group of the inositol ring of phosphoinositides. Once phosphorylated the phosphatidylinositol 3,4,5-trisphosphate (PIP3) works as a second messenger involved in cellular responses such as proliferation, survival, motility and metabolism.

Combining PI3K inhibitors with radiotherapy was proposed as an effective approach to augment the therapeutic efficacy of radiotherapy and reduce side effects. Indeed, since evidence suggests that PI3K are involved in tumourigenesis, inhibitors targeting PI3K are considered to be potential drug candidates for cancer therapy.

LY294002 activity on cell growth inhibition was associated to dermal toxicity, poor solubility and low bioavailability, preventing its entry into clinical trials. Treatment with LY294002 and wortmannin had an impact on proteins involved in the DNA damage response and was reported as an enhancer on the radio sensitization of human tumour cells. Actually LY294002 and wortmannin inhibitors are used to study the exact function of PI3K and to test the interest of the combination treatment such as conventional cancer chemotherapy as well as radiotherapy and PI3K inhibitor to avoid the left side of treatments.(Kong and Yamori, 2008)

Inhibition mechanism

LY294002 is a selective phosphatidylinositol 3-kinase (PI3K) inhibitor that competitively inhibits ATP binding to the catalytic subunit of PI 3-kinases and does not inhibit PI 4-kinase, DAG-kinase, PKC, PKA, MAPK, S6 kinase, EGFR or c-src tyrosine kinases and rabbit kidney ATPase. LY294002 was purchased from LC Laboratories (Woburn, MA).

PI3K subunits inhibitors treatment

PI 3K IV, PI 3K-β (TGX-221) inhibitors were purchased from Calbichem. PI 3K IV binds to the ATP-binding site of Class I phosphatidylinositol 3-kinases (PI3K) and inhibits PI3K activity. PI 3K-β is a potent inhibitor of PI3K p100β.

UO126 treatment

UO126 and prostate cancer

The ERK pathway plays an important role in cell survival by inhibiting various steps of apoptotic signalling. Furthermore, ERK is activated in several tumours, including prostate cancer. UO126 is a selective inhibitor of the mitogen-activated protein kinase kinases, MEK-1 and MEK-2, but a weak inhibitor of PKC, Raf, ERK, JNK, MEKK, MKK-3, MKK-4/SEK, MKK-6, Abl, Cdk2 and Cdk4. U0126 is an inhibitor of AP-1 transactivation in cell-based reporter assays.

A combination of docetaxel and ERK/MAP kinase inhibitors such as PD98059 and U0126 was proposed to enhance the apoptosis of androgen-independent prostate cancer cells and to provide a novel strategy for the treatment of hormone-refractory prostate cancer. (Zelivianski et al., 2003)

Inhibition mechanism

U0126 inhibits MEK1-2 kinase activity (MAPKK) enabling MEK-1/2 to phosphorylate downstream ERK-1/2.(Favata et al., 1998)

Bisindolylmaleimide I, Hydrochloride treatment

The Bisindolylmaleimide I, Hydrochlorideis a kinase inhibitor with strong selectivity for PKC over several serine/threonine and tyrosine kinases.

U-73122 treatment

U-73122 and prostate cancer

The activation of PLC following the stimulus of the EGFR was involved within mecanisms required for enhanced cell motility in a PCa cell line. Pharmacological inhibition of the PLC signalling via the U-73122 reduced cell invasiveness both *in vitro* and *in vivo.*(Kassis et al., 1999)

NFAT increases the TNF production. Furthermore, the activation of the calcium receptors (CaR) increases the binding of NFAT to a consensus oligonucleotide for the transcription of target genes. The activation of NFAT and the stimulation of the TNF expression were blocked by U-73122.(Abdullah et al., 2006)

Inhibition mechanism

The PLC inhibitor U-73122 inhibits intracellular calcium mobilization in the presence and absence of extracellular calcium. Unfortunately, the mechanism of action this inhibitor remains unclear.(Smith et al., 1990)

U-73343 treatment

U-73343 is a very weak inhibitor of phospholipase C that is used as a negative control of the U-73122.
U-73343 and U-73122 were from Calbiochem.

EGF treatment

Epidermal growth factor (EGF) is the first component of the EGF family of growth factors and it bind the EGFR causing the activation of several kinase pathways.
EGF was Sigma-Aldrich.

Inhibitors and growth factors concentrations

The inhibitors were used at a concentration of: 10µM for Gefitinib, LY294002, UO126, U-73343 and U-73122; 100ng/ml for epidermal growth factor; 50µg/ml for Trastuzumab; 2µM Bisindolylmaleimide I, Hydrochloride.

Transfection reagent

JetPEI (PolyPlus Transfection) is a linear polyethylenimine (PEI) derivative used for transient transfection assays. JetPEI™ is a water-soluble polymer which forms stable aggregates with DNA. Lipid transfection reagents and calcium phosphate create precipitates with DNA in the cell-surface residues and enter the cell by endocytosis. In the endosome, jetPEI™ behaves as a proton sponge, buffering the pH. This mechanism leads to endosome swelling and rupture, permitting the release of the JetPEI™/DNA complexes into the cell cytoplasm.(Boussif et al., 1995)

Kinase inhibitors/ Luciferase reporter assays

Transient transfections were performed, using JetPEI transfection reagent according to the manufacturer's instructions.

Studies of: Q64OX AR activation on cv-1 cells; PI3K subunits involved on the Q640X AR transactivation in the LNCaP cell line; the S213A/Q640X AR properties, the impact of the presence of the Q640X AR on the AP-1 and NFAT activation in LNCaP cells were realized as follows.

LNCaP and cv-1 cells were seeded in 24-well plates in a confluence of $5x10^4$ and $3x10^4$ cells per well, respectively, in complete medium. The transfection was realized when cells growth reached around the 60% of confluence. LNCaP cells were transfected 48 hrs after seeding, whereas Cv-1 24 hrs after.

Cells were transfected with 0.5µg reporter plasmid pMMTV-LTR-luc or transcription factors-luc constructs in combination with 0.5µg of the EGFP empty plasmid, or the plasmid coding a receptor variant such as: wt AR, Q640X AR or S213A/Q640X AR. Twenty four hours after transfection, the growth medium was changed by phenol red and serum free medium, to minimize the presence of hormone thus simulating the hormone-deprivation therapy. The fresh medium was treated with 100 nM DHT, or the kinases inhibitors, or growth factors or vehicles.

-Luciferase expression measurement

Forty-eight hours after inhibitors treatment cells were lysed and analyzed following the manufacturer protocol of the luciferase assay kit (Promega). Twenty microliters of cell extract was assayed in one hundred microliters of luciferse kit substrate. The luciferase expression was measured with a TD-20/20 luminometer (Turner Designs). All samples were performed in triplicate. The induction of luciferase activity is indicated in arbitrary units (A.U.). Statistical analysis was performed with Graphpad Quickcalcs (Graphpad.com) using a paired t test from at least two independent experiments. A P value of less than 0.05 was considered significant ($p<0.001$=***, $p<0.01$=**, $p<0.05$=*).

Impact of kinases pathways on NFAT and AP-1 and the study of the impact of Gefitinib, LY29004, UO126, Herceptin and Bisindolylmaleimide I treatments on the Q640X activation in LNCaP cells were realized as follows.

LNCaP cells were seeded in 96-well plates in a confluence of $1x10^4$cells per well, in complete medium. The transfection was realized when cells growth reached around the 60% of confluence. LNCaP cells were transfected 48 hrs after seeding.

Cells were transfected with 150ng reporter plasmid coding for the Firefly luciferase in combination with 75ng of the EGFP empty plasmid, or the plasmid coding a receptor variant such as: wt AR, Q640X AR or S213A/Q640X AR, and with 25ng of a plasmid coding for the Renilla luciferase as a internal control. Twenty four hours after transfection, the growth medium was changed by phenol red and serum free medium, to minimize the presence of hormone thus simulating the hormone-deprivation therapy. The fresh medium was treated with 100 nM DHT, or the kinases inhibitors, or growth factors or vehicles.

-Luciferase expression measurement

Forty-eight hours after inhibitors treatment cells were analyzed following the manufacturer protocol of the Dual-Glo luciferase assay kit (Promega). Briefly, 75µl of Dual-Glo luciferase reagent were added to 75µl of medium left in each well. Cells were incubated during 10min. The luciferase expression was measured with the Glomax Multi detection system (Promega). After the measurement of the firefly luciferase activity, 75µl of Dual-Glo Stop&Glo reagent were added to the wells and plates were incubated during 10 min. The detection of the *Renilla* luciferase was obtained with the Glomax Multi detection system. Normalization was obtained via the ratio between firefly luciferase and Renilla luciferase.

Protein extraction for immunoblotting

LNCaP cells were plated at $1x10^6$ cells/10-cm dish. Forty-eight hours after seeding, medium was changed for phenol red and serum free medium and cells were transfected with 10 µg of the indicated pEGFP-AR or the control empty plasmid with the JetPEI reagent. Total proteins were extracted 48 h after transfection. Protein lysis was realized with lysis buffer containing 10 mM Tris-HCl pH=7, 140 mM NaCl, 3 mM, 0.5x MgCl2, 5 mM DTT, 1X phosphatases inhibitors cocktail and 1X proteases inhibitors cocktail.

Proteins concentration was quantified with the BCA™ Protein Assay Kit (Pierce), based on the colorimetric properties of the cell extract to complex with the bicinchoninic acid (BCA) following the manufactor intructions.

Immunoprecipitation

Immunoprecipitation was performed from an amount of 1000µg of total proteins with the µMACS GFP isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Each protein extract was mixed with 50µl of anti-GFP Microbeads and incubated over night at 4° C. The immunoprecipitation step was performed on MACS Separation Columns (Miltenyi Biotec). MACS Columns were placed in a a strong permanent magnet – the MACS Column matrix provides a magnetic field, strong enough to retain cells labeled with MACS separator reagents. The day after cell lysate was charged onto MACS Separation Columns. Proteins/microbeads compounds were washed and 70µl/ column of the pre-heated 95°C elution buffer were applied to columns. After a brief incubation the immunoprecipited compound was collected.

Immunoblotting

Protein extracts and immunoprecipitated samples were loaded on 10% SDS-PAGE (Bio-Rad, Marnes-la-Coquette, France).
Gels were run during 90 min at 100V. Proteins were blotted onto nitrocellulose membranes by electroblotting during 1 hour at 150V 250mA. Blotted membranes were blocked with 5% nonfat dry milk in TBS-T (tris buffered saline 0.1% Tween-20) at room temperature. After one hour membranes were incubated with a primary antibody in blocking buffer over night at 4°C. Membranes were incubated with a peroxidase-conjugated secondary antibody in blocking buffer 1hr at room temperature. Visualization was by chemiluminescence detection kit (GE Healthcare Life Sciences, Saclay, France). Signals were quantified using the Gel Doc 2000 imaging system and the QuantityOne software (Bio-Rad).

Antibodies

AR blots were probed with the mouse IgG2a monoclonal antibody G122-434 against human AR (BD Biosciences PharMingen) The EGFP tag was detected with rabbit IgG polyclonal anti-GFP (FL) antibody (sc8334; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and peroxidase-conjugated goat-antirabbit IgG (170-6515; Bio-Rad). Normalization of the blot was realized with β-actin mouse IgG1 monoclonal antibody C4 (Chemicon International, Hampshire, UK). Blots were incubated with peroxidase-conjugated secondary antibodies (goat antimouse IgG; BD Biosciences PharMingen). The p-AR (PhosphoSer-213) sc:52894, and Akt1/2/3 (H-136) sc:8312 were from Santa-Cruz Biotechnology. Phospho-Akt (Thr308) #9275, phospho-Akt (Ser473) #9271 were from Cell Signaling Technology and they were probed with anti-IgG HRP-linked antibody #7074 from Cell Signaling Tech.
For the immunofluorescence the p-AR (PhosphoSer-213) sc:52894 was visualized by goat anti-mouse IgG_1-FITC sc:2078 from Santa Cruz Biotechnology. $Cy^{TM}5$-conjugated goat anti-Rabbit IgG(H+L) #111-175-003 was from Jackson Immunoresearch laboratories. The anti-NFATc1was sc:13033 from Santa Cruz Biotechnology.

Immunofluorescence

Cells culture

LNCaP cells ($8x10^4$ cells) were seeded on Lab-Tek II chamber slides (Nalge Nunc Int. Corp.) in complete medium. Cells were transfected in complete medium using the JetPEI transfection reagent and 2μg of pEGFP-C3, pEGFP-ARwt, pEGFP-ARQ640X, pEGFPS213A/Q640X plasmids. Twenty-four hours after transfection, complete medium was replaced by fresh medium without FCS and containing 100 nm DHT or vehicle, and inhibitors or their vehicles. 24h after hormone stimulation, slides were rinsed with PBS and fixed in 2% paraformaldehyde solution.
-Immunofluorescence protocol
Slides were blocked for 1 hour with 500 μl of blocking solution (1% BSA/0.1%triton in PBS). After 1h, blocking solution was removed by vacuum aspiration and substitued by the primary antibody dilution in blocking solution over night at 4° C. Primary antibodies and isotypic

controls were used at a concentration of 2.5µg/ml. The day after, slides were whashed in PBS and incubated with the secondary antibody diluted in the blocking solution, during 1hour at room temperature lightless. Secondary antibodies were conjugated to fluorescent proteins, and were at a concentration of 5µg/ml. After whashing steps nucleus staining was performed incubating cells with 1 µg/ml Hoechst 33342 solution, during 5min at room temperature.

-Observation

Slides were visualized with a confocal microscope (Leica confocal SP2 UV inverted DMIRBE microscope) (IGBMC, Illkirch, France) using the x63 oil immersion objective.

Results I

New androgen receptor variants in the 22Rv1 cell line

Advanced prostate cancer (PCa) has emerged as a public health concern in the view of population ageing. Although androgen deprivation has proven efficacy in this condition, most advanced PCa patients will have to face failure to androgen deprivation. Mutations in the sequence of the AR are related with gain of function or unusual mechanism of activation of the AR. In hormone-refractory PCa, AR mutations occur probably as a result of adaptation against the treatment. Indeed, these mutations may be the fundamental modification driving the antagonist compounds toward an agonistic action and the disease progression. (Bergerat and Ceraline, 2009; Steinkamp et al., 2009)

In our laboratory, several AR variants have been identified in androgen independent prostate tumour samples. These AR variants may be the result of abnormal pre-mRNA splicing as in the case of the AR23, or the result of nonsense mutations.(Bergerat and Ceraline, 2009; Jagla et al., 2007; Lapouge et al., 2007)

22Rv1 cells are androgen-dependent and produce tumours in nude mice with similar morphology with the parental xenograft. This cell line secretes PSA.
22Rv1 cells are androgen-dependent (Sramkoski et al., 1999)
The 22Rv1 cell line represents a model of pre-clinic PCa progression, under hormone-therapy.(Tepper et al., 2002)
The CWR22 xenograft and the 22Rv1 cell line both present a point mutation in the AR sequence, the H874Y. Furthermore, more AR isoforms are expressed in 22Rv1 cells: a full-length version with duplicated exon 3 and the short AR truncated version, which lacks the COOH terminal domain (CTD. The duplication of exon 3 results in a larger AR protein with three zinc fingers in its DBD. This duplication of the exon 3 is absent in the parental CWR22 xenograft.

In the previous study, the short AR isoform on the 22Rv1 cell line have been proposed as a result of a proteolytic cleavage of the long form of the AR present in the cell line.(Tepper et al., 2002)

Our hypothesis was that this short forms of AR found in 22Rv1 cell line and in the PCa samples analysed in our laboratory may be caused by a clonal selection during the hormonal treatment toward the expression of AR forms able to stimulate the PCa progression in hormone free conditions.

In the present study, we analysed the AR forms expressed in the 22Rv1 cell line. Since the 22Rv1 cell line expresses CTE-truncated AR as well, the knowledge of the these variant could be helpful to elucidate the role of the CTE-truncated AR variants found in our laboratory.

Total RNAs were isolated from 22Rv1 cells and converted to cDNA. AR cDNAs were amplified by PCR and inserted into a yeast expression vector by homologous recombination.(Ceraline et al., 2003)
In yeast, responses to hormones were evaluated by scoring the number of yeast colonies obtained in the presence of different concentrations of hormones, antagonists or non-steroids molecules.
In this assay, abnormal yeast growth was observed compared to the wild type AR.
The profile observed displayed a decreased androgen dependence of the short ARs compared to the hormonal sensibitity of the wild type AR.
Interestingly the DNA sequencing of plasmid obtained from grown clones highlighted the presence of new AR variants not described before in this cell line and all AR mutants were the results of distinctive transcript.
In the following article we present the identification of these variants and we characterize their structures and properties.

Identification of Novel Truncated Androgen Receptor (AR) Mutants Including Unreported Pre-mRNA Splicing Variants in the 22Rv1 Hormone-Refractory Prostate Cancer (PCa) Cell Line

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Results II

Crosstalk between Q640X androgen receptor and kinase pathways

In physiological condition, binding of androgens to the AR produces a conformational change allowing AR phosphorylation and dimerization and nuclear internalization of the activated receptor. Once in the nucleus, the activated receptor can interact with coactivators and corepressors to regulate the assembling of the transcriptional complex.

Mutations could affect the promoter-selective cell-dependent transactivation, or the interaction with the ubiquitin ligase complex increasing protein stability, nuclear location in the absence of ligand and modified ligand specificity of the androgen receptor.(Steinkamp et al., 2009)

The huge amount of AR mutations identified in PCa suggests the heterogeneity of the tumour environment. Cellular clones selected after the hormonal treatment could express abnormal activation, DNA elements recognition and different activity because of different molecular composition and conformation.

CTE-truncated AR have been discovered in our laboratory using the yeast-based assay. These truncated AR variants are ligand-independent and may play a pivotal role in AIPC.(Bergerat and Ceraline, 2009; Ceraline et al., 2004; Lapouge et al., 2007; Lapouge et al., 2008)

The Q640X AR lacks the ligand binding domain, excluding any possible interaction with natural ligand of androgen receptor and cofactors via the carboxy-terminal end.

The loss of the CTE truncated implicates the loss of key regulatory elements for AR transcriptional activity.

Our hypothesis is that the CTE-truncated AR requires activating phosphorylation for its transcriptional activity, as we know that ser-81, ser-213, ser-515, ser-578 are important targets of phospohorylation in the AR sequence.

Since the androgen receptor is a phosphoprotein that presents several serines within the NTE, we want to focalize on the Q640X variant and on its putative phosphorylation sites.

Indeed, the location and the unexpected activities of this mutant AR may be regulated by phosphorylation.

Tyrosine kinase growth factor receptors, acting through the PI3K/Akt and MAPK/ERK kinase cascades activation, promotes AR phosphorylations, in androgen deprivation conditions.(Legrier et al., 2009)

Here, we investigate the impact of kinase cascade signalling on transcriptional activities of the Q640X using specific inhibitors of main kinases pathways known to be involved in the regulation of the wild type androgen receptor.

A mouse mammary tumour virus-long terminal repeat-hormone responsive elements (MMTV-LTR-HRE) luciferase reporter construct and an ARE-luc reporter plasmid were used to monitor AR transactivation. The MMTV-LTR-HRE is a progesterone and glucocorticoid responsive enhancer construction, which is also responsive to androgens.

To test whether Q640X AR transactivation from these promoters necessitates activating phosphorylation by kinase pathways, we used inhibitors such as: Gefitinib anti-EGFR-TK, LY294002 PI 3K IV and PI 3K-β anti-PI3K, the UO126 anti-MEK1/2, and the Bisindolylmaleimide I, Hydrochloride anti PKC (Fig.16).

We demonstrated that the Q640X CTE-truncated AR requires activating phosphorylation for full transcriptional activities from androgen-response promoters.

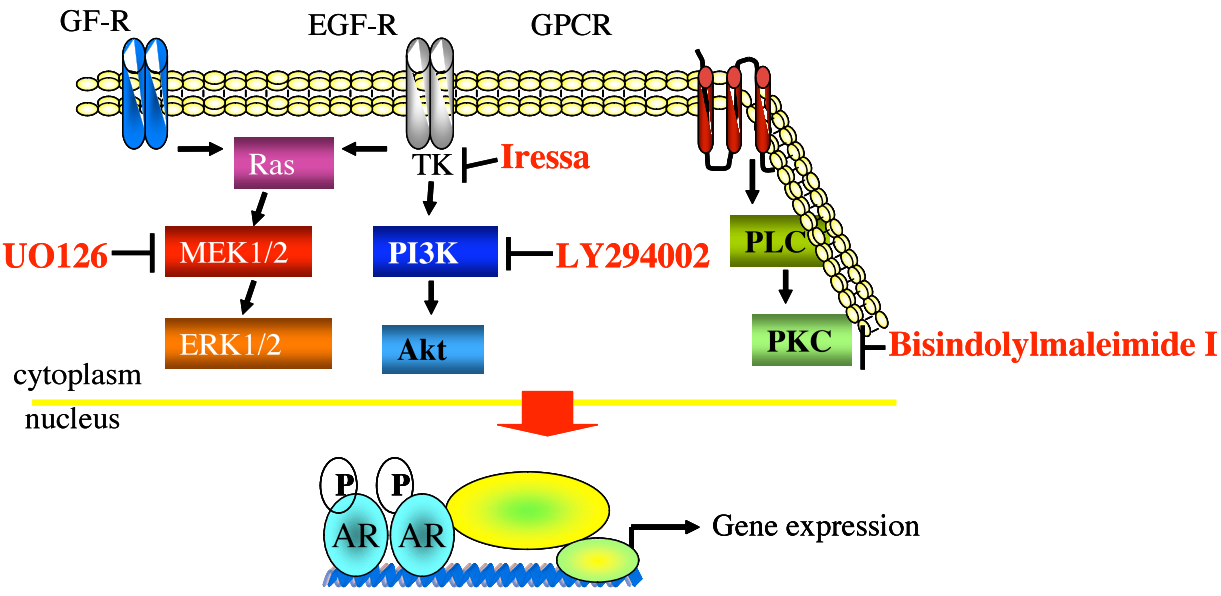

Fig.16: Schematic representation of the inhibitory mechanisms of the kinase inhibitors.

EGFR AND HER2 SIGNALLING IS REQUIRED FOR FULL TRANSCRIPTIONAL ACTIVITY OF CONSTITUTIVE ANDROGEN RECEPTOR IN PROSTATE CANCER.

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Running Title : Regulation of constitutive androgen receptor

Keywords: Androgen receptor, EGF, EGFR, PI3K, Akt, prostate cancer.

Abstract

Androgen deprivation therapy remains the standard treatment for advanced prostate cancer (PCa). However, most tumours eventually recur as hormone-refractory PCa (HRPC), in which the androgen receptor (AR) signalling remains active despite low levels of circulating androgens. Carboxy-terminal end (CTE)-truncated mutant ARs exhibiting constitutive transactivation are recurrent in HRPC. Here, we demonstrate that a crosstalk with the epidermal growth factor receptor (EGFR) and HER2 signalling pathways is required for full activity of the CTE-truncated Q640X AR in a PCa cell line. Targeting EGFR and HER2 with gefitinib and trastuzumab in LNCaP cells abrogated Q640X AR transactivation on androgen-dependent luciferase reporter constructs. Mechanistic dissection involves PI3K and ERK signalling pathways as downstream effectors. We further demonstrated that p110α catalytic subunit of PI3K, but not p110β, mainly contributed to this crosstalk with Q640X AR, and that downstream PI3K, PDK1/Akt but not PDK1/PKC is essential for CTE-truncated AR transactivation. Detailed phosphorylation studies indicated that Ser-213 was not the primary PI3K/Akt activating phosphorylation site in the context of the CTE-truncated AR. Confocal microscopy studies indicate that PI3K pathway is essential for a functional intranuclear distribution of the CTE-truncated AR. Together, our data indicate that targeting RTKs/PI3K/Akt and RTKs/MAPK/ERK pathways may be valuable to block CTE-truncated AR functions in HRPC.

Introduction

Androgen withdrawal remains the main treatment for local recurrence and androgen-dependent metastatic prostate cancer (PCa). However, after an initial response to androgen ablation therapy all tumours will recur ultimately as hormone-refractory PCa (HRPC). Taxane-based chemotherapy in HRPC provides further survival benefit and increases time to progression. However, this treatment is not curative. Several clinical trials evaluating new therapeutic approaches, including immunotherapeutic vaccines, platinum agents, microtubules stabilizing drugs, remain unsatisfactory. It is clear that deciphering molecular mechanisms of action that predominate in HRPC could result in novel effective therapies.

Crosstalk between the androgen receptor (AR) signalling pathway and chemokines, cytokines or growth factors pathways plays a pivotal role in the emergence and progression of HRPC (Culig, 2004; Wang et al., 2007; Zhu and Kyprianou, 2008; Lee et al., 2009; Malinowska et al., 2009; Yanase and Fan, 2009), and may represent a drugable target for treatment of HRPC. These interconnections have been reported to trigger ligand-independent AR phosphorylation resulting in AR inhibition, AR activation, or AR stabilization, depending on the model studied. For example, overexpression of the CXC chemokine interleukin-8 (IL-8) has been associated with PCa cells survival to androgen ablation by augmenting AR expression and activation (Seaton et al., 2008; Waugh and Wilson, 2008). IL-6 is a cytokine that leads to androgen-independent growth of AR-positive PCa in vitro and in vivo through AR activation (Malinowska et al., 2009). Insulin-like growth factor -1 (IGF-I), heregulin and epidermal growth factor (EGF) were found to increase transcriptional activities of AR, but these actions remain controversial (Wang et al., 2007; Yanase and Fan, 2009; Gregory et al., 2005; Léotoing et al., 2007; Recchia et al., 2009; Hakariya et al., 2006; Cai et al., 2009; Lin et al., 2001). Several lines of evidence indicate that the epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase, contributes to PCa progression by activating indirectly the

AR signalling pathway (Léotoing et al., 2007; Recchia et al., 2009; Hakariya et al., 2006; Cai et al., 2009; Lin et al., 2001; Ponguta et al., 2008). EGFR signals to the Ras/Raf/MEK/Erk pathway but also indirectly to phosphatidylinositol 3'(OH)-kinases (PI3Ks) (Wang et al., 2007). PI3Ks are a family of lipid kinases that generate lipid second messengers involved in regulation of a wide variety of cellular functions such as cell growth, differentiation, survival and migration (Vivanco and Sawyers, 2002; Shukla et al., 2007). Class IA PI3Ks are composed of a p110α, p110β, or p110δ catalytic subunit in complex with an Src homology 2 (SH2) domain-containing regulatory p85 subunit. Inhibition of PI3K signalling suppresses AR-mediated gene expression and tumour progression in PCa (Li et al., 2001; Nan et al., 2003; Zhu et al., 2008). Recently, p110β and p85α subunits have been found essential for androgen-stimulated AR transactivation in PCa cells (Zhu et al., 2008). Several studies provide evidence that upregulation of Akt/Protein kinase B, a downstream member of the PI3K cascade, is associated with the development of HRPC (Paliouras and Diamandis, 2008). A direct synergy between Akt and AR signalling to promote naïve prostatic epithelium to invasive carcinoma has been reported (Xin et al., 2006). PCa maintains an elevated Akt activity through the loss of phosphatase and tensin homolog (PTEN) function or autocrine signalling by growth factors and cytokines. Akt-mediated phosphorylation of AR is linked to AR inhibition or degradation in some studies, or to enhanced transcriptional activity in others (Hakariya et al., 2006; Lin et al., 2001; Paliouras and Diamandis, 2008; Taneja et al., 2005; Wen et al., 2000; Yeh et al., 1999).

Candidate phosphorylation sites on the AR are intensively analyzed, and it is now accepted that the AR can be phosphorylated at serine and tyrosine residues. Indeed, AR is phosphorylated at serine in position 16, 81, 256, 308, 424, and 650 upon ligand binding (Taneja et al., 2005; Gioeli et al., 2002; Ponguta et al., 2008). However, phosphorylation of Ser-650 is enhanced by treatment with forskolin, EGF, and phorbol-12-myristate-13-acetate.

In addition, EGF-induced increase in AR transcriptional activity is dependent on phosphorylation at Ser-515 and Ser-578 by MAP kinase and Protein Kinase C (PKC) respectively (Ponguta et al., 2008). Ser-515 and Ser-578 phosphorylation is associated with AR nuclear-cytoplasmic shuttling and transcriptional activity. EGF is associated with hormone-refractory PCa by inducing AR tyrosine phosphorylation at position 534 (Kraus et al., 2006; Guo et al., 2006). Tyr-534 phosphorylation is mediated by Receptor for Activated C Kinase 1 (RACK1) and Src kinase, and this phosphorylation is required for AR nuclear translocation and AR recruitment to the chromatin (Guo et al., 2006). Ser-213 and Ser-791 constitute putative phosphorylation sites for Akt, even though Ser-213 only is considered as an *in vivo* target for Akt (Taneja et al., 2005).

Excepted Ser-791, which is located in the ligand-binding domain, most of phosphorylation sites are located in AR NH_2-terminal domain and hinge region, two regions involved in AR nuclear-cytoplasmic shuttling, cofactors recruitment, transcriptional activity, and AR degradation. Probably due to the great importance of these two AR regions for AR activity, naturally occurring mutation at these potential phosphorylation sites are uncommon in PCa. However, nonsense mutations and aberrant AR pre-mRNA splicing that lead to carboxy-terminal end (CTE)-truncated AR have been characterized in PCa (Céraline et al., 2004; Lapouge et al., 2007; Lapouge et al., 2008; Bergerat and Céraline, 2009; Dehm et al., 2008; Hu et al., 2009). These AR variants are truncated within the hinge region downstream the first nuclear localization signal and lack key regulation sites including the ligand-binding domain (LBD), Ser-791 phosphorylation site, and sites for interaction with co-regulators. CTE-truncated ARs are functionally active and display constitutive transcriptional activity. However, molecular mechanisms involved in their ligand-independent transactivation are poorly understood.

In this study, we show that the EGFR and HER2 pathways are required for full transcriptional activity the Q640X CTE-truncated AR. We demonstrate that downstream of these two RTKs, MAPK and PI3K/PDK1/Akt pathways, but not PI3K/PDK1/PKC, regulate Q640X AR transcriptional activity. We further demonstrate that downstream to Akt, phosphorylation at Ser-213 is not a condition for Q640X AR activity. Together, these data bring new data concerning molecular mechanisms of androgen-independent activation of CTE-truncated ARs, and may guide to more efficient therapeutics for advanced PCa by targeting simultaneously AR and RTKs signalling pathways.

RESULTS

EGFR and HER2 signalling pathways are essential for CTE-truncated Q640X mutant AR transactivation

AR variants devoid of their carboxy-terminal end (CTE) display ligand-independent transactivation on androgen-dependent promoters (Céraline et al., 2004; Lapouge et al., 2007; Lapouge et al., 2008; Bergerat and Céraline, 2009; Dehm et al., 2008; Hu et al., 2009). It remains unclear how these CTE-truncated ARs become constitutively active and whether phosphorylation by independent signalling pathways is required for their transcriptional activities. Both EGFR and HER2 receptor tyrosine kinases (RTKs) are known to be involved in wild type AR transactivation in PCa. Here, we enquired whether interconnection with RTKs remains important for Q640X CTE-truncated AR activity. LNCaP cells were co-transfected with the MMTV-Luc reporter together with a plasmid encoding for the Q640X mutant AR or the empty plasmid as control. Cells were thereafter incubated for 48 hours in the presence of 10μM Gefitinib or 50μg/ml trastuzumab. The ligand-independent transactivation of the Q640X AR was diminished by over 20% after blockage of EGFR by gefitinib and by over 45% upon inhibition of the HER2 by trastuzumab (Fig.1A). Our data indicate that EGFR and HER2 pathways remain essential for Q640X AR activities in LNCaP cells.

EGFR and HER2 are widely known to initiate the activation of two major signalling pathways, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, and the phosphatidylinositide 3-OH kinase (PI3K)/Akt kinase cascade. We further demonstrated that blockage of MAPK/ERK in LNCaP cells resulted in a marked decrease of Q640X AR activity. Indeed, Q640X mutant AR transcriptional activity on MMTV-luc was inhibited by over 80% in the presence of UO126 (Fig. 1B). Blocking PI3K

with the LY294002 inhibitor resulted in a 90% decrease of Q640X transactivation from the MMTV-luc in LNCaP cells. Similar results were obtained when an artificial PSA promoter construct was used as reporter (data not shown). Together, our data indicate that Q640X AR require RTK signalling through both ERK and PI3K pathways for full transcriptional activities in LNCaP cells.

The class IA of PI3K isoforms includes p110α, p110β and p110δ associated with one of three regulatory subunits, namely p85α, p85β, or p55α. The catalytic subunits p110α and p110δ are supposed to signal downstream of receptor tyrosine kinases (RTKs), while p110β signals downstream of G protein-coupled receptors (GPCRs) (Guillermet-Guibert et al., 2008). We used PI3Kα inhibitor IV and TGX-221 as isoform-specific inhibitor for p110α and p110β respectively to demonstrate that PI3K-induced Q640X AR transactivation is related to RTKs, but not to a GPCR signalling. LNCaP cells were transfected with the AR expression plasmid together with the MMTV-luc plasmid. Cells were then incubated in serum-free medium containing or not 10μM inhibitor LY294002, PI3Kα inhibitor IV or TGX-221. In the presence of p110β inhibitor TGX-221, the Q640X transactivation was decreased by over 30%. This decrease was more pronounced (>75%) in the presence of PI3K IV, and was similar to the inhibitory effect of LY294002 (Fig. 1C). The results suggest that RTKs signal to Q640X AR mainly via the p110α catalytic subunit of PI3K.

Intranuclear redistribution of CTE-truncated Q640X AR upon inhibition of PI3K pathway

The lack of the ligand-binding domain leads to a constitutive nuclear compartmentalization of Q640X mutant AR (Céraline et al., 2004). We investigated whether the effect of EGFR and PI3K inhibitors on Q640X AR transcriptional activities could be explained by an altered intracellular localization of the mutant receptor. LNCaP cells were transiently transfected with

the plasmid encoding for the Q640X AR in fusion with the EGFP fluorescent protein and incubated in serum-free medium together with gefitinib or LY294002 or solvent as control. The ligand-independent nuclear localization of Q640X mutant AR was not affected when LNCaP cells were treated with gefitinib or LY294002 (Fig. 2). However, treatment with LY294002, but not with gefitinib, modified the intranuclear distribution of the CTE-truncated AR. Indeed, Q640X AR displayed a punctuated nuclear staining (Fig. 2, b and e), which was not observed with the DHT-stimulated wt AR (data not shown). The formation of these nuclear foci was maintained in the presence of gefitinib (Fig. 2, d-f). In contrast, a diffuse nuclear staining was observed in the presence of LY294002, indicating that the inhibition of PI3K pathway results in a redistribution of the Q640X AR within the nucleus (Fig. 2, g-i). As the formation of nuclear foci is known to be associated with transcriptional activity (Saitoh et al., 2002), we postulate that suppression of the AR Q640X transactivation by the PI3K pathway may involve an altered formation of active transcriptional complexes.

PI3K signals mainly to PDK1/Akt, but not to PDK1/PKC to enhance Q640X AR transactivation

PI3K generates 3'-phosphorylated phosphoinositides PIP3 and PIP2 at the plasma membrane, which bind with high affinity to the Pleckstrin Homology (PH) domain-containing AGC kinase, phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 activates protein kinase B/Akt and protein kinase C (PKC), two kinases known to regulate AR transcriptional activities by phosphorylation. Indeed, we investigated further the link between PI3K and Q640X AR using a specific inhibitor of PKC. LNCaP cells were co-transfected with Q640X AR expression plasmid and the MMTV-luc reporter construct, and incubated in the presence of 2μM bisindolylmaleimide I. Blocking of PKC resulted in a 60% decrease of Q640X AR transactivation from MMTV-luc (Fig. 4). Besides, PI3K blocking resulted in a more

pronounced effect. These results suggest that PI3K may signal mainly through PDK1/Akt pathway, but not through PDKI/PKC to enhance Q640X AR transactivation.

Serine 213 phosphorylation in Q640X CTE-truncated AR is PI3K-independent

The PI3K/Akt pathway modulates AR transactivation and stability by posttranslational modifications such as activating or repressing phosphorylation and phosphorylation-dependent ubiquitylation. Two Akt phosphorylation consensus sites (RXRXX(S/T) in wild type AR encompassing serine residues in position 213 and 791 have been reported (Taneja et al., 2005; Ghosh et al., 2005). Ser-791 being in the ligand-binding domain is absent in the context of the Q640X AR (Fig. 4A). Akt-induced Ser-213 phosphorylation requires a ligand–induced conformational change in AR amino-terminal region for kinase access. From these observations, we wonder whether Akt continues to mediate phosphorylation at Ser-213 in Q640X AR. LNCaP cells were transfected with pEGFP-ARQ640X plasmid and incubated for 48 hours in the presence of 10 μM of gefitinib or LY294002 or vehicle. The EGFP-AR Q640X fusion protein was thereafter immunoprecipitated with an EGFP antibody and immunoblotted. Besides, total Akt and phospho-Ser(473)-Akt were analyzed by western-blotting from total cell lysate. As visualized in Fig. 4B, Ser-213 phosphorylation was maintained in Q640X AR, and this phosphorylation was not affected by Akt inhibition. These data indicate that contrary to what is observed with the wild type AR, Ser-213 phosphorylation in Q640X AR is Akt-independent.

To further investigate the contribution of Ser-213 in RTK-induced Q640X AR transactivation, this phosphorylation site was mutagenized to alanine. LNCaP cells were transfected with Q640X AR or S213A/Q640X double mutant AR expression plasmid together with the MMTV-luc reporter. We demonstrated here that replacement of Ser-213 by alanine affects transcriptional activities of Q640X by over 55% (Fig. 4C). Moreover, residual activities could

be further inhibited by treatment with gefitinib, trastuzumab, LY294002 and UO126 (Fig. 4D). Together, these data point out that despite the absence of Ser-213, the transactivation of CTE-truncated AR remains sensitive to RTK signalling inhibitors, and suggest that additional phosphorylation sites contribute to the positive modulation of Q640X AR activity by EGFR and HER2 pathways.

Discussion

Mutations in the *AR* gene are a recurrent event in HRPC. CTE-truncated mutant ARs that display ligand-independent nuclear localization and constitutive transcriptional activities are potential candidates to support PCa progression on androgen ablation therapy. Up to now, molecular mechanisms underlying the mode of action of these CTE-truncated ARs remain uninvestigated. In particular their interconnection with cytokines and growth factors signalling pathways has not been studied so far. Previous reports indicate that EGFR and HER2 RTKs contribute to PCa progression by regulating AR transactivation. We sought to examine whether EGFR and HER2 pathways remain important for the regulation of a CTE-truncated AR transcriptional activity. We found that blocking endogenous EGFR and HER2 in LNCaP cells with gefitinib and trastuzumab respectively resulted in a clear down-regulation of Q640X AR transactivation. Our data indicate that EGFR and HER2 pathways continue to play a critical role in the regulation of transcriptional activity of a mutant CTE-depleted AR.

Multiple studies have focused on the functional link between the AR and PI3K signalling pathways with contradictory results. Overexpression of constitutively activated PI3K or Akt results in down-regulation of AR transactivation (Lin et al., 2001; Taneja et al., 2005). Besides, activation of PI3K by HER-2/neu promotes androgen-independent PCa cell survival by activating AR signalling (Gregory et al., 2005; Wen et al., 2000). We found that blocking PI3K pathway results in a down-regulation of CTE-truncated AR transactivation in LNCaP cells. Our experimental models, consisting in the use of specific inhibitors to target endogenous PI3K in our cell lines, could explain this discrepancy with data showing an inhibitory effect of PI3K on AR transactivation. However, our results are consistent with the recent report demonstrating that PI3K is essential for AR transactivation and progression to HRPC (Zhu et al., 2008).

Class IA PI3Ks are composed of a p110α, p110β, or p110δ catalytic subunit in complex with an Src homology 2 (SH2) domain-containing regulatory p85 subunit. Recently, the p110β catalytic and p85α regulatory subunits of PI3K have been described as critical for the interconnection between PI3K and AR pathways (Zhu et al., 2008). We provide evidence that p110α, but not the p110β catalytic subunit is mainly involved in the regulation of the CTE-truncated Q640X AR transactivation. The p110α isoform signals mainly downstream RTKs. Besides, the p110β isoform mediates GPCRs signalling in addition to signal downstream of RTKs. These observations suggest that CTE-truncated ARs could be interconnected to growth factors signalling pathways by a mechanism different to that of the wild type receptor. It is clear that a deeper analysis of the precise signalling by which growth factors are regulating Q640X AR transcriptional activities is required and will be of great help to understand CTE-truncated ARs activation in PCa.

Interestingly, EGFR and PI3K blocking affect differently Q640X AR transactivation. A clear difference in the intranuclear distribution of Q640X AR passing from a speckled to a more diffuse staining pattern could be observed in the presence of LY294002, but not in the presence of gefitinib. PI3K inhibitors are known to suppress AR-mediated gene expression without altering AR nuclear translocation (Liao et al., 2004). A recent report showed that PI3K is required for AR-DNA interaction or the dynamic assembly of AR transcription complex (Zhu et al., 2008). Then, we hypothesize that an impaired assembly of Q640X AR-mediated transcription complex or a defective AR-DNA interaction could explain the Q640X AR intranuclear redistribution in the presence of LY294002. As the speckled intranuclear distribution of Q640X AR was not altered in the presence of gefitinib, down-regulation of Q640X CTE-truncated AR after EGFR blocking may involve a different mechanism. A more

appropriate explanation could be an EGFR-mediated regulation of AR coactivation by phosphorylation of Steroid Receptor Coactivator-2 (SRC-2) (Gregory et al., 2004) or phosphorylation and ubiquitinylation of MAGE-11 (Bai and Wilson, 2008).

Ser-213 and Ser-791 are considered as putative phosphorylation sites for Akt, even though Ser-213 only is considered as an *in vivo* target for Akt (Taneja et al., 2005). As Ser-791 is absent in the CTE-truncated Q640X AR, it was interesting to determine whether Ser-213 plays a key role in Q640X AR transactivation. Western blot data indicate that Ser-213 phosphorylation status was not affected after PI3K blocking, but mutagenesis studies demonstrate that Ser-213 remains important for full activity of the CTE-truncated AR. We conclude that although Ser-213 is essential for full transcriptional activity, this phosphorylation does is probably not involved in the crosstalk between RTK/PI3K signalling and CTE-truncated AR. These data are in agreement with the observation that phosphorylation at Ser-213 and Ser-791 by Akt is not required for the synergy between Akt and AR to promote naïve prostatic epithelium to androgen-insensitive but AR-dependent carcinoma (Xin et al., 2006). Our finding further supports the hypothesis that it is likely that sites other than Ser-213 and Ser-791 may contribute to the modulation of AR activity by PI3K (Lin et al., 2001). As Ser-791 is absent in the CTE-truncated AR, our data indicate that these potential cryptic Akt phosphorylation sites should fit between amino acids 1-640. Tyrosine 534, a target for the cytoplasmic membrane anchored Src kinase, could not be one of these potential phosphorylation sites as Q640X AR exhibits an exclusively intranuclear localization. Interestingly, it has recently been shown that EGF-induced increase in AR transcriptional activity is dependent on phosphorylation at MAP kinase consensus site Ser-515 and at PKC consensus site Ser-578 (Ponguta et al., 2008). One possible scenario is that EGF-dependent phosphorylation at Ser-578 modulate AR association and dissociation from DNA and the

magnitude of the transcriptional activity through AR interaction with the Ku-70/80 regulatory subunits of DNA-dependent protein kinase (DNA-PK) (Ponguta et al., 2008). This observation is consistent with our data indicating that the Ras/Raf/MEK pathway signals to the Q640X AR. Further studies are required to demonstrate that in Q640X AR, Ser-515 is phosphorylated by MAPK. Moreover, PKC and Akt fall into the AGC (protein kinase A (PKA)/protein kinase G/PKC) class of protein kinases and require phosphorylation by phosphoinositide-dependent protein kinase-1 (PDK1) for activation. We have demonstrated that RTKs signal to the Q640X AR mainly by the PI3K/PDK1/Akt pathway. However, our data indicate that PI3K/PDK1/PKC signalling could be partly involved by phosphorylating Q640X AR at Ser-578.

Since the last five years, the presence of CTE-truncated ARs in PCa becomes a new research axis on the molecular mechanisms that contributes to PCa progression upon ADT (Céraline et al., 2004; Lapouge et al., 2007; Lapouge et al., 2008; Bergerat and Céraline, 2009; Dehm et al., 2008; Hu et al., 2009; Libertini et al., 2007; Taplin, 2008; Pignon et al., 2009). In this report, we show that interconnections with EGFR, HER2 and PI3K are required for full activity of one of these CTE-truncated ARs. This study brings new data concerning molecular mechanisms of androgen-independent activation of CTE-truncated ARs, and may guide to more efficient therapeutics for advanced PCa by targeting simultaneously AR and RTKs signalling pathways.

Materials and Methods

Materials

Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). LY294002 was purchased from LC Laboratories (Woburn, MA, USA). Gefitinib was kindly provided by AstraZeneca. PI3Kα inhibitor IV and PI3Kβ inhibitor VI (TGX-221) were purchased from Calbiochem (VWR, France). Bisindolylmaleimide I, hydrochloride was from Merck Chemicals (VWR, France). Trastuzumab was from Roche (Roche, Neuilly-sur-Seine, France).

Cell lines and cell cultures

The human androgen-sensitive prostate cancer cell line LNCaP clone FCG (European Collection of Cell Cultures, Salisbury, UK) (ECACC 89110211, passage number 08-11) was cultured in phenol red-free RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were cultured in a humidified atmosphere enriched in 5% CO2 at 37˚C.

Plasmids

The pEGFP-C3 plasmid was purchased from BD Clontech (Ozyme, France). pEGFP-ARwt and pEGFP-ARQ640X expression plasmids encoding for the EGFP-tag AR wild type or Q640X have been previously described (30, 31). The MMTV-Luc reporter plasmid containing steroid hormone responsive elements was a gift from Prof. P. Chambon (IGBMC, Illkirch, France). The pGL4.70[hRLuc] was purchased from Promega (Promega, Charbonnières, France). pEGFP-ARS213A and pEGFP-ARS213A/Q640X expression plasmids were

constructed by replacing the PstI-PstI fragment (Genbank NM_000044-2, nt 1284-2107) in pEGFP-ARwt and pARQ640X by the corresponding fragment from pcDNA3-ARS213A, kindly provided by Dr. S.K. Logan (NYU Langone Medical Center, New York).

Transfection and Luciferase reporter assay

Transient transfection was performed in both cell lines by using the JetPEI reagent (PolyPlus Transfection, Polyplus, France) according to instructions provided by the manufacturer. LNCaP cells were seeded in 24-well plates (5x 10^4 cells/well) and transfected for 48 hours later with 0.5 μg of the indicated pEGFP plasmid plus 0.5 μg MMTV-luc. Twenty-four hours later, cells were switched to phenol red- and serum-free medium containing the indicated inhibitor or vehicle. Luciferase activity was measured 48 hours after with the Luciferase reporter assay kit (Promega, Charbonnières, France). For the treatment with PI3Kα inhibitor IV and TGX-221, cells were transfected in phenol red-free medium 5% FCS and treated with inhibitors 24 hours after transfection in a phenol red- and serum-free medium during 6 hours. The induction of luciferase activity is indicated in arbitrary units (AU). Statistical analysis was performed with Graphpad Quickcalcs (Graphpad.com) using a paired t test from at least two independent experiments. A *P* value of less than 0.05 was considered significant.

Western blot and Immunoprecipitation

LNCaP cells were plated at 1x10^6 cells/10-cm dish and transfected at ~ 60% confluence with 10 μg of the plasmid encoding for the Q640X mutant AR with the JetPEI reagent. Cells were incubated in serum-free medium containing 10 μM gefitinib or LY294002 for 48 hours. Total proteins were extracted with lysis buffer containing 10 mM Tris-HCl pH=7, 140 mM NaCl, 3 mM, 0.5x MgCl2, 5 mM DTT, 1X phosphatases inhibitors cocktail and 1X proteases inhibitors cocktail. Cell lysates were subjected to immunoblotting with anti-Akt and anti-

phospho(Ser473)-Akt. To analyze the phosphorylation status of serine 213 in Q640X AR, immunoprecipitation was performed from an amount of 800 µg of total proteins with the µMACS GFP isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Immunoprecipitated samples were loaded on 10% Tris-HCl SDS-PAGE (Bio-Rad, Marnes-la-Coquette, France). After electroblotting, membranes were revealed as previously described (30) with the mouse monoclonal antibody p-AR (156C135.2) (sc-52894, Santa-Cruz Biotechnology, Santa Cruz, CA) for detection of Ser-213 phosphorylated AR and the mouse IgG2a anti-AR monoclonal antibody (G122-434, BD Biosciences, Le-Pont-de-Claix, France). Signals were quantified with the Gel Doc 2000 imaging system and the QuantityOne software (Bio-Rad, Marnes-la-Coquette, France) and normalized with the signal corresponding to GFP.

Immunofluorescence – Confocal microscopy

LNCaP cells (8 x 10^4 cells) were seeded on Labteck slides (Nalge Nunc, Rochester, NY), transfected at ~ 60% confluence with the plasmid encoding for the Q640X or S213A/Q640X mutant. Twenty-four hours later, cells were switched to serum-free and phenol red free medium containing 10 µM of Gefitinib or LY294002 or vehicle. Slides were fixed in 2% paraformaldehyde, and nuclei were stained with 1 µg/ml Hoechst 33342 solution. Slides were visualized with a confocal microscope (Leica confocal SP2 UV inverted DMIRBE microscope) using the x63 oil immersion objective. The *Volocity LE* High performance 3D imaging software (Improvision, Coventry, England) was used to visualize collected images.

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Figures legends

Fig.1. EGFR, HER2 and PI3K signalling pathways are essential for CTE-truncated Q640X mutant AR transcriptional activity. ***A,*** LNCaP cells were transiently transfected with plasmids encoding for the Q640X mutant AR together with the pMMTV-Luc reporter plasmid. Twenty-four hours after cells were incubated in serum-free medium in the presence of 10 μM Gefitinib or 50 μg/ml trastuzumab to block EGFR and HER2 respectively. ***B,*** LNCaP cells were transfected as indicated above and treated with 10 μM gefitinib or UO126 to block PI3K and MEK1/2 respectively. ***C,*** Transfected LNCaP cells were incubated in the presence of 10 μM PI3-Kα IV, PI3-Kβ VI (TGX-221) or LY294002 to block respectively p110α and p110β catalytic subunit, or total PI3K. Relative luciferase activity was measured 48 h after. All determinations were done in triplicate. Histograms represent percent of activity from a representative experiment are shown (bars) ± SEM.

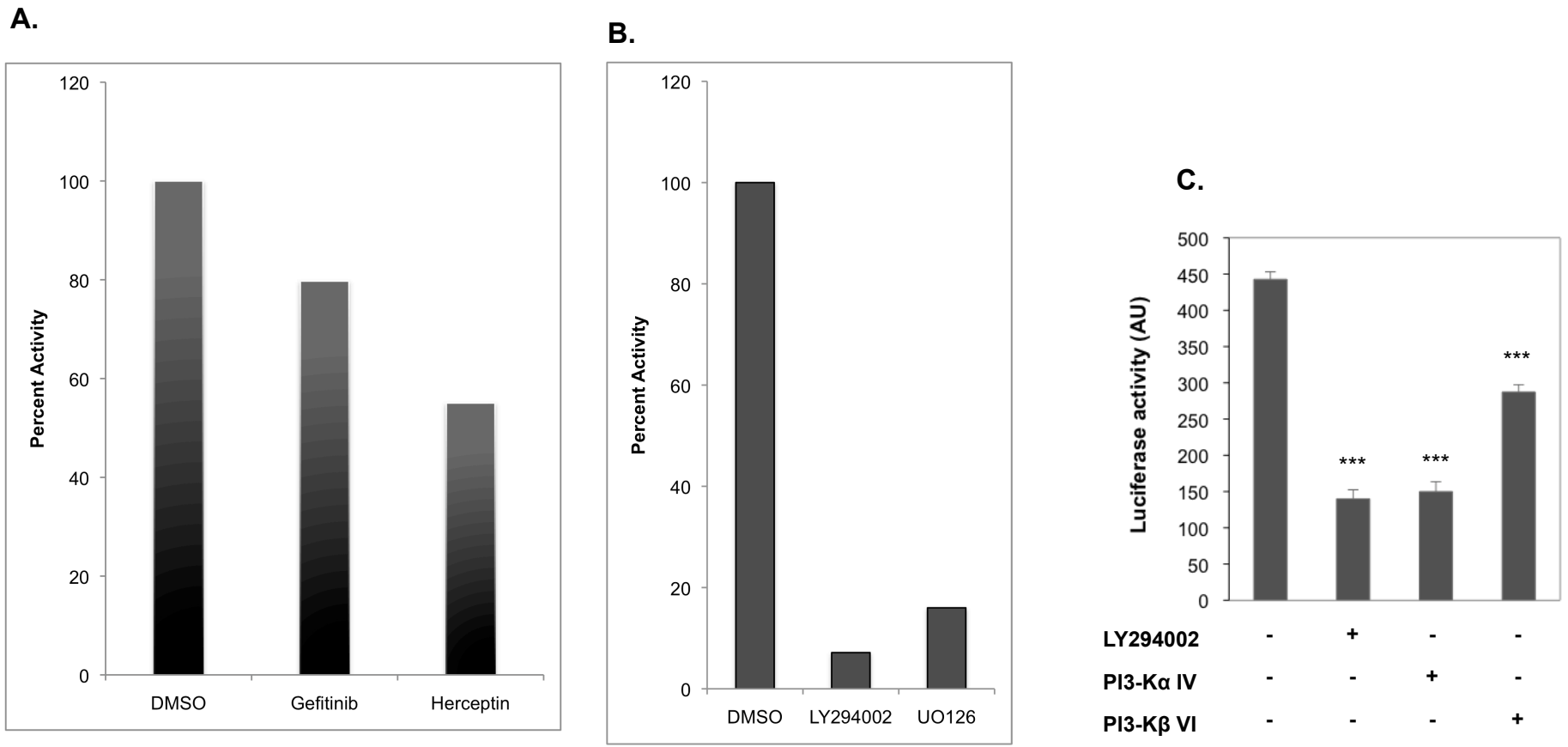
Fig.2. Inhibition of the PI3K pathway alters the intranuclear distribution of the CTE-truncated Q640X AR. LNCaP cells were transiently transfected with the plasmid encoding for the Q640X mutant AR in fusion with the green fluorescent protein EGFP and incubated for 48 h in serum-free medium containing 10 μM Gefitinib or LY294002. Cells were co-stained with DAPI and fluorescence was analysed with an immunofluorescence confocal microscopy (scale bar, 10 μm).

Fig.3. The PI3K/PDK1/Akt plays an important role in RTK signalling to Q640X AR. LNCaP cells were transiently transfected with plasmids encoding for the Q640X mutant AR together with the pMMTV-Luc reporter plasmid. Twenty-four hours after cells were incubated in serum-free medium in the presence of 10 μM LY294002 or 2 μM Bisindolylmaleimide I to

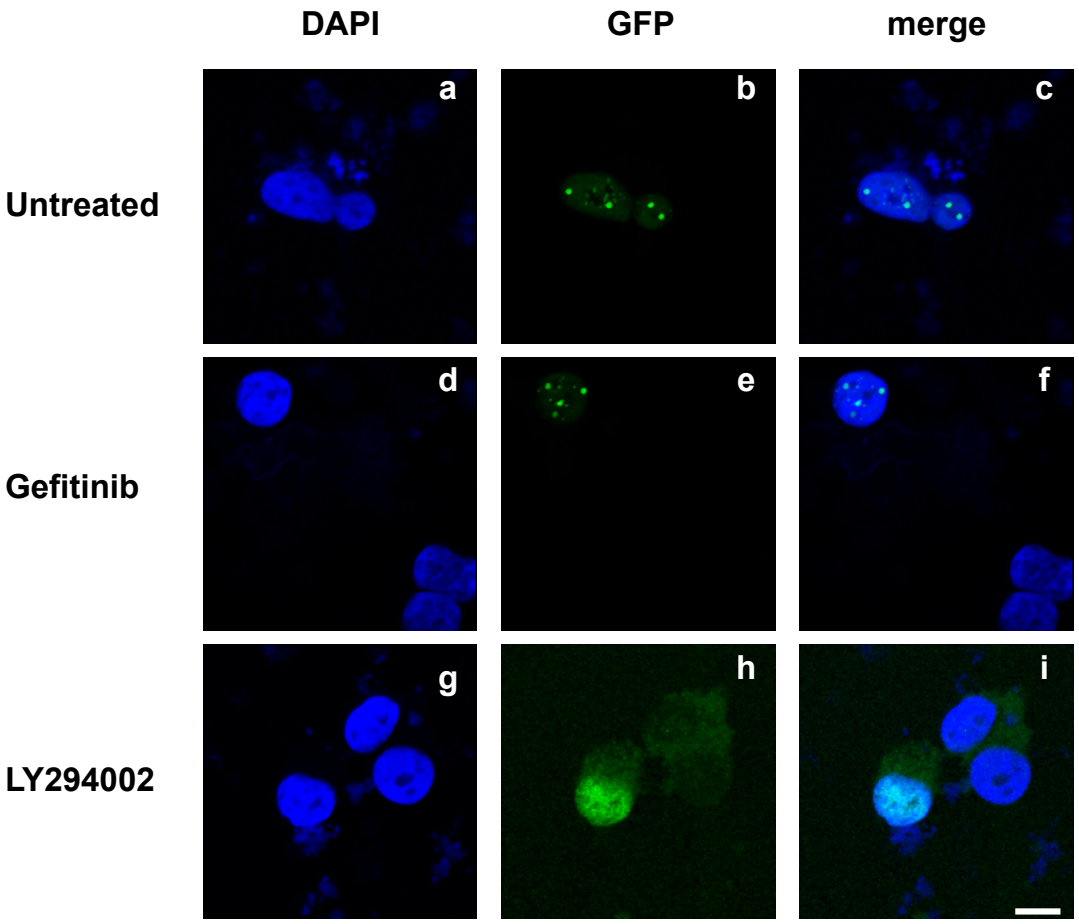
block PI3K and PKC respectively. Relative luciferase activity was measured 48 h after. All determinations were done in triplicate. Histograms represent percent of activity from a representative experiment are shown (bars) ± SEM.

Fig.4. Serine 213 is important for full transcriptional activity of Q640X AR. ***A,*** Representation of potential phosphorylation sites conserved in the CTE-truncated Q640X mutant AR. *NTD*, Amino-terminal domain; *DBD*, DNA binding domain; *HR*, Hinge region. Arrows indicate known amino acid targets for kinases. ***B*,** Effect of EGFR and PI3K blocking on serine 213 phosphorylation. LNCaP cells were transfected with the plasmid encoding for the Q640X mutant AR and incubated 24 h after in serum-free medium containing 10 μM Gefitinib or LY294002. Cells lysates were immunoblotted with anti-Akt, anti-phospho(Ser473)-Akt, anti-phospho(Ser213)-AR and anti-AR antibodies. ***C,*** Ser-213 was replaced by alanine in Q640X AR. LNCaP cells were transfected with the S213A/Q640X double mutant AR together with the MMTV-luc. Relative luciferase activity was measured 48 h after. D, Transfected cells were incubated in the presence of 10 μM gefitinib, LY294002 or UO126, or 50 μg/ml trastuzumab, and relative luciferase activity was measured 48 h after. Histograms represent percent of activity from a representative experiment are shown (bars) ± SEM.

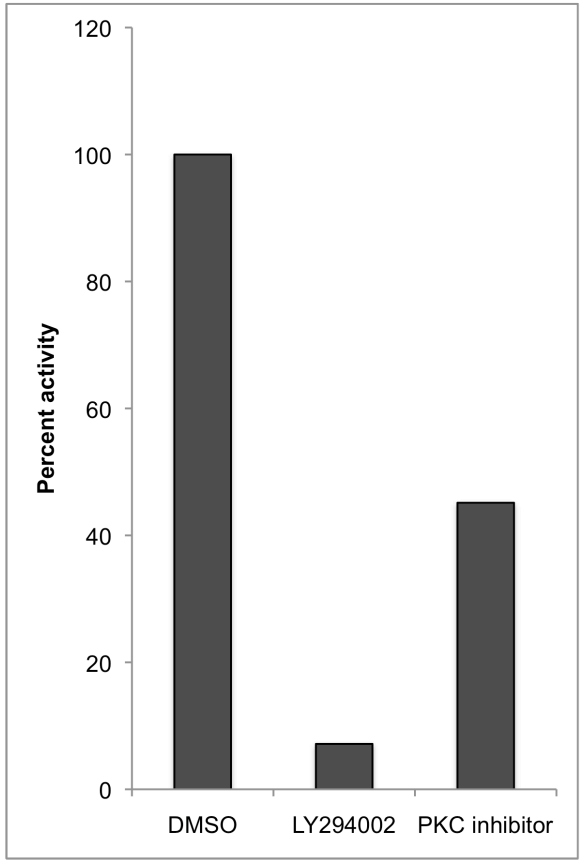
Marcias et al. Figure 1A; 1B; 1C



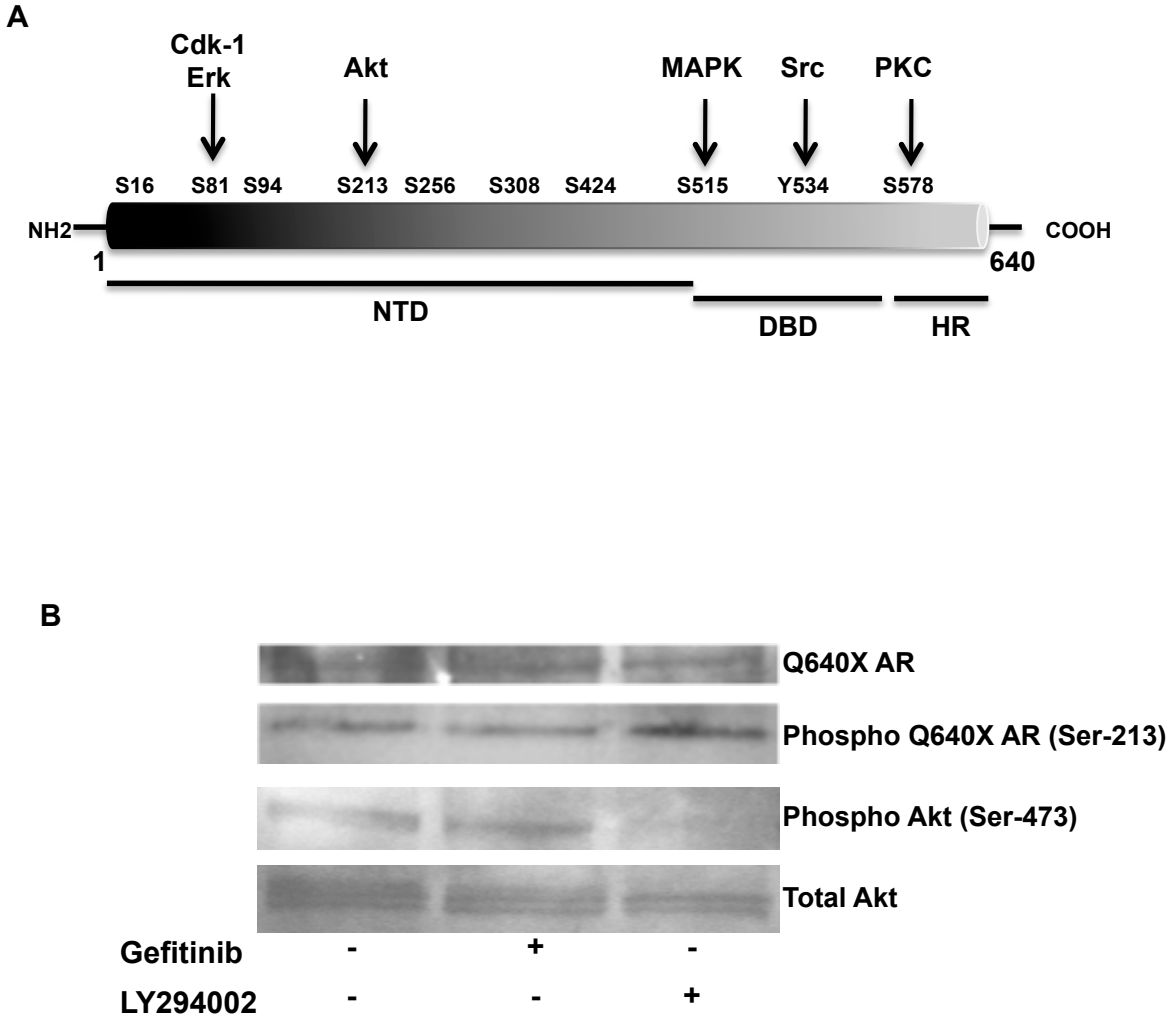
Marcias et al. Figure 2



Marcias et al. Figure 3

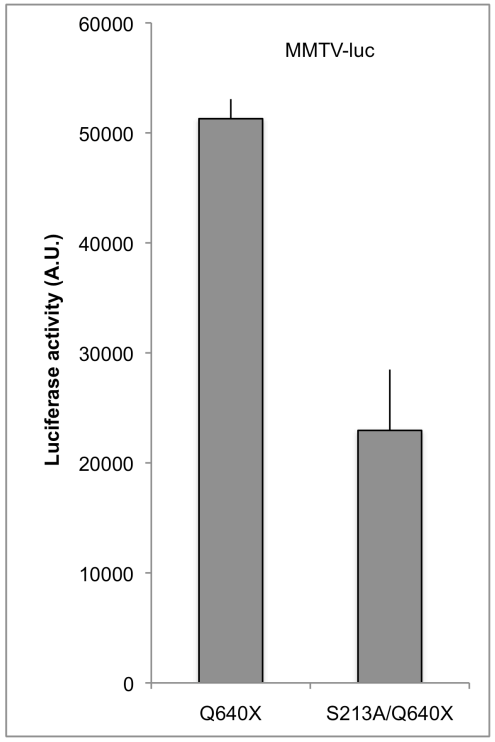


Marcias et al. Figure 4A; 4B

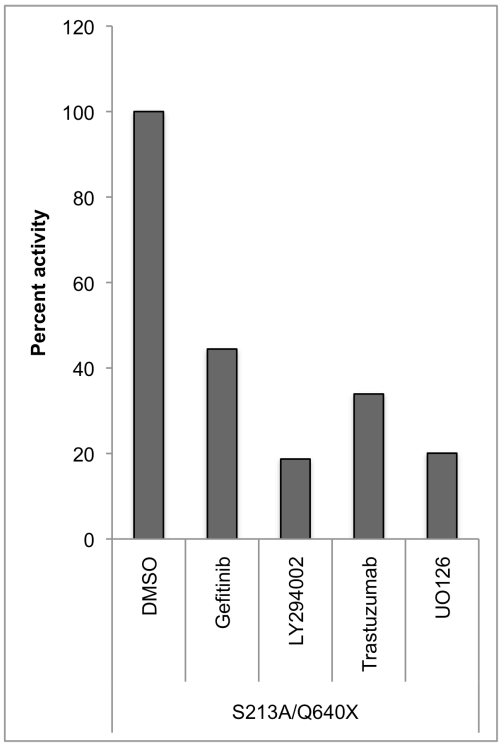


Marcias et al. Figure 4C; 4D

C.



D.



Results III

Impact of the Q640X AR on transcription factors transactivation

Posttranslational modifications such as phosphorylation and acetylation control positively and negatively transcription factors (TFs) activities. Indeed, kinases or phosphatases regulate the phosphorylation status of TFs serines, threonines, and tyrosines residues. Phosphorylation influences TFs activities by inducing allosteric conformational changes that result in repulsive or attracting forces. Those conformational changes are associated with TFs stabilisation by protecting from or facilitating the degradation and with their cellular location that determines the duration of nuclear location. Also, these conformational changes allow protein–protein interactions, DNA-binding, and the integration of multiple phosphorylation signals.(Macian, 2005; Viola et al., 2005)

Activator Protein-1 (AP-1) transcription-factor is a dimeric complex that comprises members of the JUN, FOS, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families.(Eferl and Wagner, 2003) AP-1 interferes with the AR ligand-dependent transactivation. Reciprocally, liganded AR interferes with AP-1 activity and cAMP response element Binding protein Protein (CBP) mediates this crosstalk.(Fronsdal et al., 1998)

The CBP protein takes parts as a co-integrator in several signalling pathways including AP-1, NFκB and the AR pathways. A competition between between AP-1 and AR for CBP recruitment may explain the mutual antagonism between these two TFs.(Aarnisalo et al., 1998) CBP could play a central role in the crosstalk between AR and Nuclear Factor kappa B (NFκB).(McKay and Cidlowski, 1998) Indded, NFκB TF is a heterodimeric protein that comprises the p50 and p65 (Rel A) subunits, and the mutual repression between AR and Rel A could be due to formation of AR-Rel A complexes that are held together by another partner or to competition for a common activator.(Palvimo et al., 1996)

Previous studies in our laboratory demonstrated that the Q640X AR is a ligand-independent TF that recruits CBP and TBP in LNCaP cellular model. Furthermore, transcriptional activities of TFs such as AP-1 (Fig.17-a), NFAT (Nuclear Factor of Activated T-cell) (Fig.17-

b) and NFκB are strongly augmented in the presence of the Q640X AR in LNCaP cell

a) .(Lapouge et al., 2008)(Marcias and Lapou; b) progress)

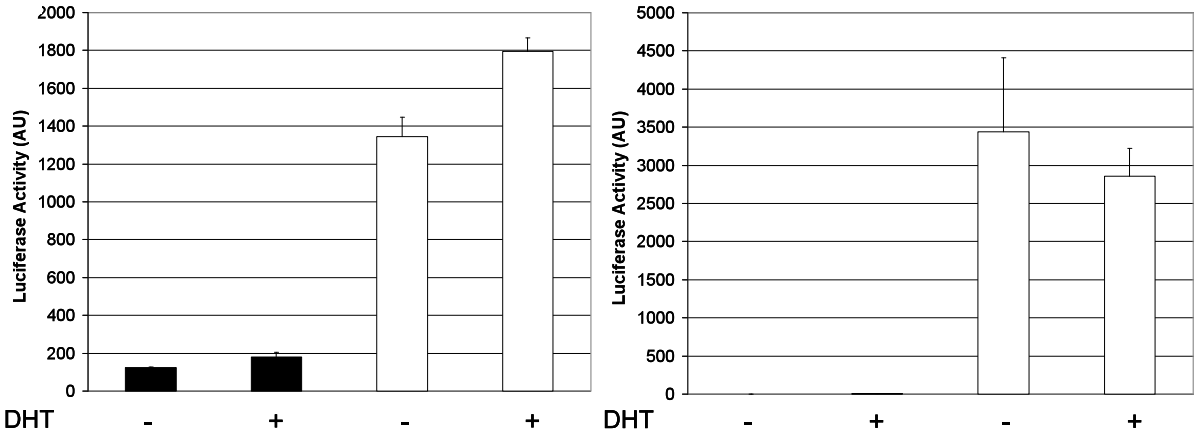


Fig.17: Transcriptional activities of AP-1 and NFAT are strongly augmented in the presence of the Q640X AR in LNCaP cell line. LNCaP cells were cotransfected with the pEGFP-Q640X AR expression plasmid (white bar) or the pEGFP-C3 empty plasmid (black bar) and the AP-1-luc (a) or the NFAT-luc (b) reporter plasmids. 24 hours after cells were treated with 100nM DHT or vector.

The increase of transcriptional activities of these TF in the presence of Q640X AR suggests a partnership instead of a competition between these TFs. As a consequence, the Q640X could directly cooperate with differentiation, proliferation and cellular survival pathways.

Furthermore, NFAT induces the differentiation of pre-osteaclasts to osteoclats, which are responsible for bone remodelling and are involved in metastatic bone lesions. Interconnections between NFAT and Q640X AR in PCa emphasize the potential role of the truncated AR as a key protein in PCa progression.

The objective of the present study was to elucidate the molecular mechanisms that connect Q640X AR to NFAT and AP-1 activities.

We investigated the following hypotheses.

- Q640X AR physically contributes to the activation complexes formed around NFAT or AP-1.
- Q640X AR activates upstream pathways leading to activation of NFAT abd AP-1 TFs.

Colocation between NFAT and Q640X AR was analysed by confocal microscopy to decipher any direct implication of Q640X in activation complexes formed by NFAT and AP-1.

For the second hypothesis, we adopted luciferase reporter assay performed in LNCaP cells in the presence of specific cell signalling inhibitors to identify how Q640X AR is connected to NFAT and AP-1 activities.

1- NFAT2 location in the presence of Q640X

NFAT proteins share a regulatory conserved N-terminal region called the NFAT homology region (NHR). This region is highly phosphorylated in resting cells, keeping NFAT in an inactive state and restricted to the cytoplasm. An increase in intracellular calcium activates calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase that directly dephosphorylates the NHR (NFAT homology region), allowing NFAT to translocate to the nucleus and to bind to DNA at specific promoters sites.

We studied the intracellular location of NFATc1 in LNCaP cells transfected with the plasmid encoding for Q640X or wt ARs in fusion with EGFP. We focused on NFATc1 (NFAT2), identified as the major NFAT protein participating in a biological response. NFAT2 is activated by increased intracellular calcium signalling and is involved in cellular proliferation mechanisms.(Hogan et al., 2003)

Twenty-four hours after transfection LNCaP cells were treated with 100nM DHT, and fixed 48 hours after. Cells were incubated with an antibody against the amino acid 1-110 of NFATc1 and then with a Cy5-coupled secondary antibody (Fig.18).

In non transfected LNCaP cells (Fig.18, white arrow), NFAT c1 was homogenously distributed in cytoplasmic and nuclear compartment. In the presence of DHT-activated wt AR, NFATc1 was homogenously distributed in both cytoplasmic and nuclear compartment (Fig.18, yellow arrow). We can observe the absence of colocation between nuclear activated NFAT and wt AR.

Similarly, in LNCaP cells transfected with the EGFP-Q640X AR expression plasmid, NFATc1 was in the nucleus and in the cytoplasm. We could not demonstrate a colocation between the two proteins in these studies. We conclude that the Q640X does not directly take part to the transcriptional complex of NFAT2

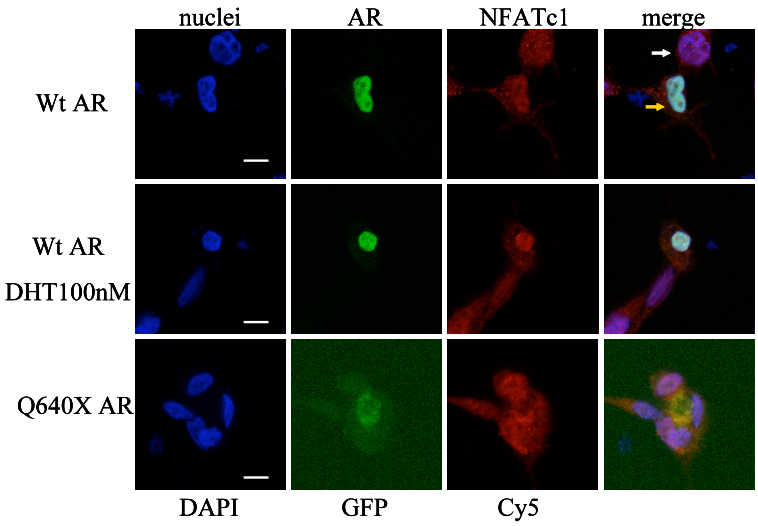


Fig.18: NFAT location in the presence of the wt or the Q640X ARs. LNCaP cells were transfected with the EGFP-Q640X AR or the EGFP-wt AR expression plasmids and treated with 100nM DHT or vector.

2- Signalling pathways involved in Q640X AR-induced NFAT activities

a) Role of the Phospholipase C

The calcium/calmodulin/calcineurin is the main pathway that leads to activating dephosphorylation of NFAT. In this pathway, activation of receptor tyrosine kinases such as EGFR, HER2 results in the generation of Inositol-(1,4,5) triphosphate (InsP3) and diacylglycerol (DAG) by phospholipase C. InsP3 mediates the release of calcium from internal stores, which in turn induces the opening of specific store-operated calcium channels. (Hogan et al., 2003; Medyouf and Ghysdael, 2008)

Since PLC is suspected as effector for extracellular stimuli in the stimulation of the NFAT signalling, we tested the impact of two inhibitors such as: U-73122 anti-phospholipase C and U-73343 a very weak inhibitor of phospholipase C that was used as a negative control.

LNCaP cells were co-transfected with the NFAT-luciferase reporter construct and the plasmid encoding for the CTE-truncated Q640X AR, or the empty plasmid as control.

After transfection, LNCaP cells were treated with 10µM U-73122 or U-73343.

Treatment with U-73343 treatment did not affect Q640X AR induced-NFAT activity, while treatment with U-73122 resulted in a strong decrease of NFAT activity (Fig.19).

These results indicate that the induction of NFAT activity by Q640X AR can be blocked by a PLC inhibitor. Thus, our data suggest that Q640X AR expression in LNCaP cells results in the activation of signalling pathways connected to PLC.

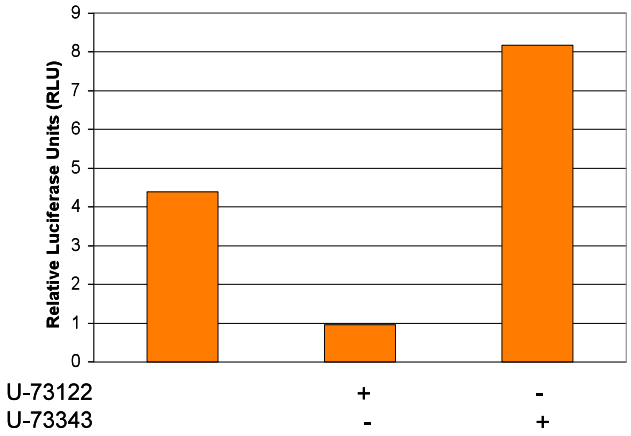


Fig.19 : Induction of NFAT activity by Q640X AR is blocked by inhibitor of PLC. LNCaP cells co-transfected with pEGFP-Q640X expression plasmid and NFAT-luc reporter construct, were treated with 10µM U-73122 a strong phospholipase C inhibitor or 10µM U-73343.

b) Role of EGFR in Q640X AR induced-NFAT activities

We have postulated that Q640X may lead to the upstream activation of signalling pathways leading to PLC activation. According to these results, the EGFR signalling pathway may contribute to Q640X AR action on NFAT activity.

This was investigated in the next experiment, in which NFAT activity was evaluated in LNCaP cells transfected with the wt or Q640X AR, after EGFR blocking or stimulation.

LNCaP cells were co-transfected with the NFAT-luciferase reporter plasmid and the plasmid encoding for the CTE-truncated Q640X AR, or the empty plasmid as control. Medium was replaced by fresh serum-free medium containing or not 10μM gefitinib, an inhibitor of the EGFR tyrosine kinase activity and NFAT luciferase activity was assayed 48 hours later.

The expected increase of NFAT activity in the presence of Q640X AR could be observed, and this activity was slightly augmented in the presence of gefitinib. However, the difference was not significative. These data indicate that in our experimental conditions blocking EGFR does not impair Q640X AR induced-NFAT activity.

We further investigated the impact of EGFR stimulation on Q640X AR-induced NFAT activity. In a similar experiment, transfected LNCaP cells were incubated in serum-free medium containing 100ng/ml EGF. In the presence of Q640X AR, EGFR stimulation led to a marked increase of NFAT activity by over 80%. These data suggest that EGF cooperates with Q640X AR to increase NFAT activity in our model.

Additional data obtained with transfected cells incubated with EGF and gefitinib showed that EGF action was mediated by EGFR. Indeed, a clear decrease of NFAT activity was observed when Q640X AR transfected cells were incubated with EGF and Gefitinib (Fig.20-a).

In controls, the stimulation of EGFR did not affect NFAT activity which remained at the background value (Fig.20-b).

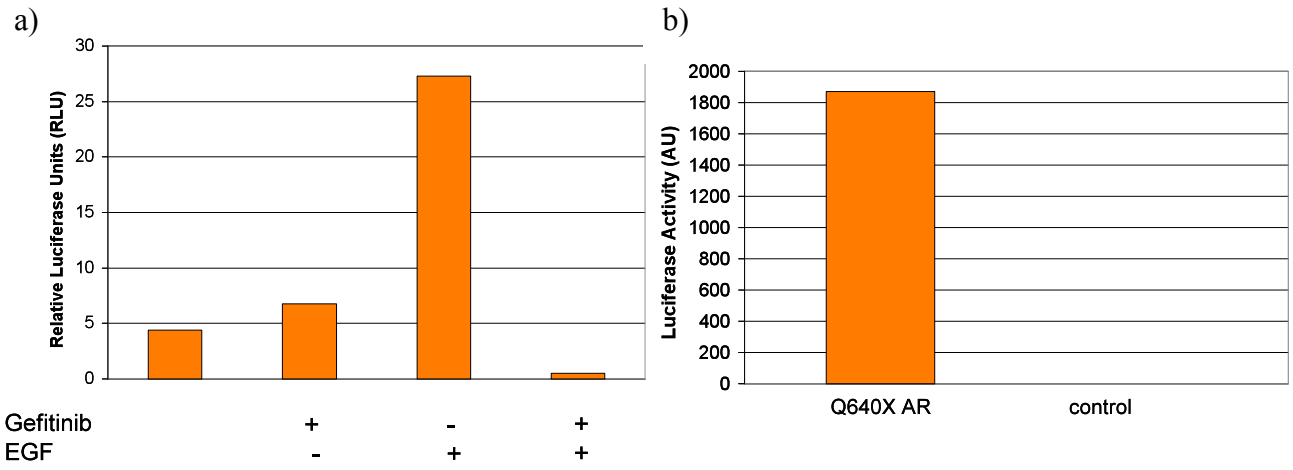


Fig.20 : EGF cooperates with Q640X AR to increase NFAT activity. a) LNCaP cells co-transfected with pEGFP-Q640X expression plasmid and NFAT-luc reporter constructwere, were treated with 10μM Gefitinib or 100ng/ml EGF or in combination with gefitinib and EGF. b) In LNCaP cells transfected with the empty plasmid pEGFP-C3 the stimulation of EGFR did not affect NFAT activity which remained at the background value

c) Intracellular effectors associated with Q640X AR induced-NFAT activity

We next studied the effect of inhibitors of intracellular effectors of RTKs. LNCaP cells were co-transfected with pNFAT-luc and pEGFP-Q640X AR and were treated 24hours after with 10μM UO126 or LY294002, or 2μM Bisindolylmaleimide I, to block MEK1/2, PI3K and PKC respectively.

Inhibition of PI3K strongly reduced Q640X AR induced-NFAT activity by over the 90% in LNCaP cells. Blocking MEK1/2 and PKC resulted also in a marked decrease (by over the 73 %) of Q640X AR induced-NFAT activity (Fig.21).

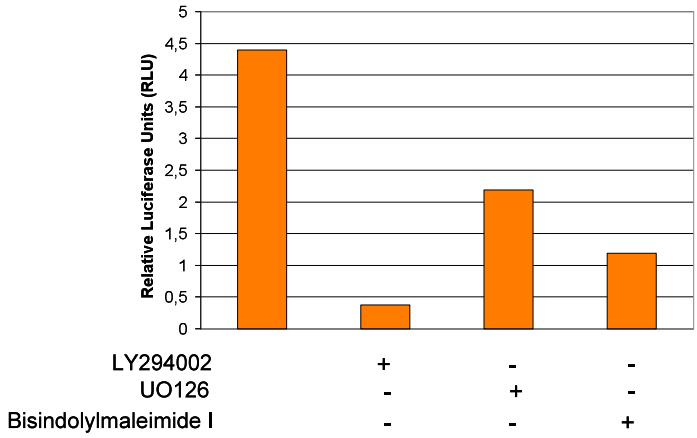


Fig.21 : Intracellular effectors associated with Q640X AR induced-NFAT activity. LNCaP cells co-transfected with pEGFP-Q640X expression plasmid and NFAT-luc reporter construct, were treated with 10µM UO126 or LY294002, or 2µM Bisindolylmaleimide I.

3- Signalling pathways involved in Q640X AR induced-AP-1 activity

AP-1 activity is controlled at transcriptional and post-transcriptional levels. AP-1 activity is stimulated by a complex network of signalling pathways that involves external signals as growth factors, stress and cytokines as well as mitogen-activated protein kinases (MAPKs) of the extracellular-signal-regulated kinase (ERK), p38 and JUN amino-terminal kinase (JNK) families. These intracellular signalling pathways entrance expression of *FOS* and *JUN* genes, but also allow activating phosphorylation of AP-1 complex. (Eferl and Wagner, 2003)

We have previously demonstrated that growth factors signalling pathways and activation of PLC are involved in Q640X AR induced NFAT activity. We postulated that these same pathways could be involved in the augmentation of AP-1 activity observed in the presence of Q640X AR. Moreover, AP-1 acts synergistically with NFAT on composite DNA response elements which contain flanking NFAT and AP-1 binding sites, where they form highly stable ternary complexes to regulate the expression of diverse inducible genes. Concomitant induction of NFAT and AP-1 requires concerted activation of two different signalling pathways. The calcium/calcineurin pathway regulates NFAT activity, and the protein kinase C/Ras pathway promotes the synthesis, phosphorylation and activation of members of the FOS and JUN families of TFs.

In these following experiments, we investigate the signalling pathways associated with Q640X AR induced AP-1 activity.

a) Role of RTKs in Q640X AR induced AP-1 activity

We studied the impact of gefitinib as inhibitor of EGFR and trastuzumab as an anti-HER2 on the transcriptional activities of AP-1 in LNCaP cells transfected with Q640X AR.

LNCaP cells were co-transfected with the pAP-1 luciferase reporter plasmid and the pEGFP-Q640X expression plasmid, and were treated 24 hours with 10µM Gefitinib or vehicle, or 50ng/ml trastuzumab. Inhibition of EGFR did not affect Q640X AR induced AP-1 activity (Fig.22-a). Besides, blocking HER2 by trastuzumab resulted in a 25% decrease of luciferase activity compared with the control. These data indicate that HER2, but not EGF, seems play a role in Q640X AR induced NFAT activity.

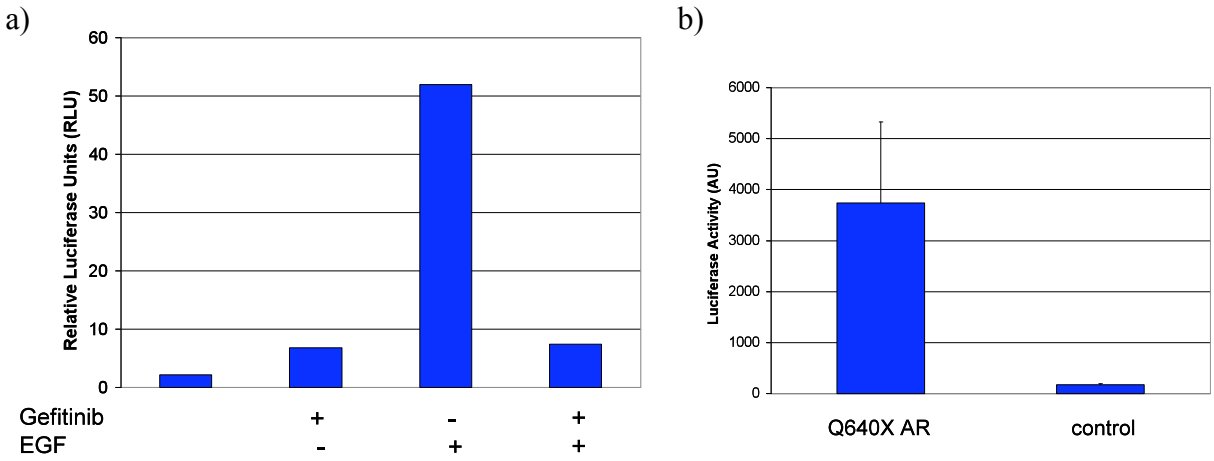


Fig.22 : EGF cooperates with Q640X AR to increase AP-1 activity. a) LNCaP cells co-transfected with pEGFP-Q640X expression plasmid and NFAT-luc reporter construct were treated with 10µM Gefitinib or 100ng/ml EGF or in combination with gefitinib and EGF. b) 100ng/ml EGF treatment in LNCaP cells co-transfected with the AP-1 luc reporter plasmid and the pEGFP-C3 empty plasmid did not stimulate the luciferase expression.

b) Kinases pathways involved in Q640X AR induced AP-1 transactivation

Signals mediated by the RTKs/Ras/Raf/MEK1/2/ERK1/2, RTKs/PI3K/PKC/ERK1/2 or by the G Protein coupled membrane receptors/PLC/PKC/ERK1/2 pathways lead to AP-1 activation.

In these experiments, we targeted MEK1/2 with UO126, PI3K with LY294002, PLC with U-73343, and PKC with Bisindolylmaleimide I, and analyzed the impact of these different inhibitions on Q640X AR induced AP-1 transactivation.

LNCaP cells were co-transfected with the AP-1-luciferase reporter plasmid and the plasmid encoding for the CTE-truncated Q640X AR, or the empty plasmid as control. Medium was replaced by fresh serum-free medium containing or not the indicated inhibitor, and luciferase activity was assayed 48 hours later.

Blocking PI3K with LY294002 resulted in a decrease of AP-1 transcriptional activity by 60% in the presence of Q640X AR.
When MEK1/2 was targeted with 10µM UO126, Q640X AR induced AP-1 transactivation was also decreased by 30%.
We next evaluated the role of PKC on Q640X AR induced AP-1 transactivation. Inhibition of PKC by 2µM Bisindolylmaleimide I did not affect the AP-1 activity in the presence of Q640X AR (Fig.23).

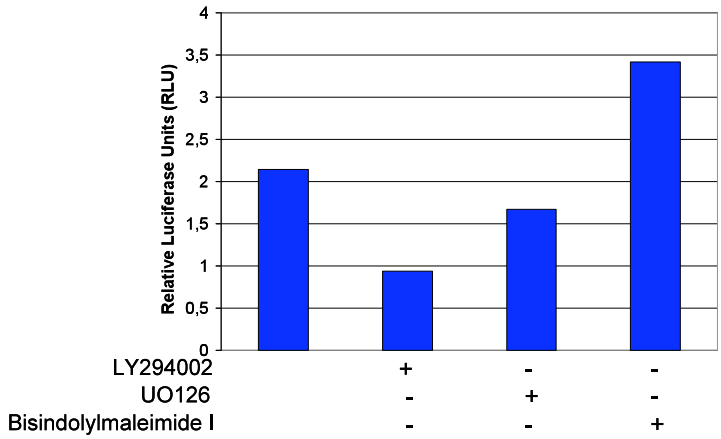


Fig.23 : Intracellular effectors associated with Q640X AR induced-NFAT activity. LNCaP cells co-transfected with pEGFP-Q640X expression plasmid and NFAT-luc reporter construct, were treated with 10µM UO126 or LY294002, or 2µM Bisindolylmaleimide I.

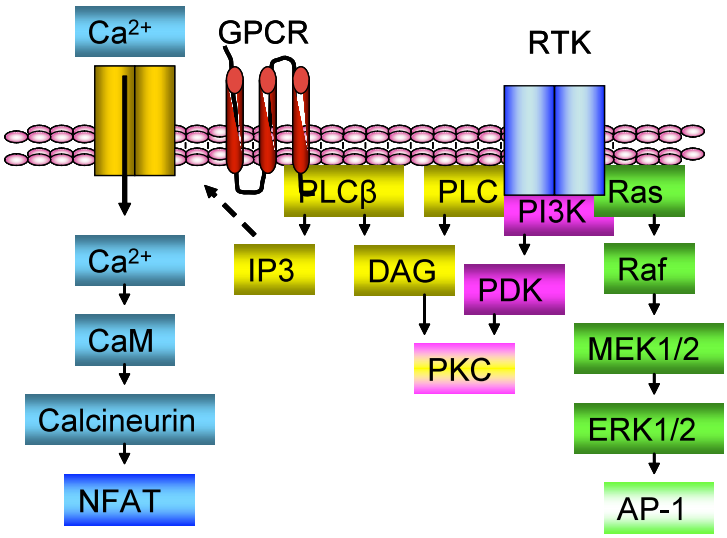


Fig.24: Kinases pathways activate in the presence of the Q640X AR.

Since the Q640X has been identified in recurring PCa, we speculate that this truncated AR could take part cell cycle progression, differentiation, apoptosis and angiogenesis mechanisms, regulating TF such as NFAT, AP-1, as a mediator of PCa progression.

Here, we demonstrate the relevance of phosphorylation pathways activating proteins such as PI3K, MAPK, PLC and PKC (Fig.24).

The Q640X AR could express the uncharacteristic property on the transactivation of NFAT and AP-1 by the stimulation of the expression of one or more ligand able to stimulate membrane receptors; the increase of the expression intracellular effectors or the stimulation of circulating intracellular calcium; or the increase of membrane receptors expression.

Conclusions

Prostate cancer (PCa) is the primary cause of mortality in over 50 years men and the second leading cause of cancer mortality in men. Lately, several new therapies for advanced PCas have been proposed. Unfortunately, results are disappointing and clinic use of molecules such as satraplatin, atrasentan, or the use of vaccinations fails to improve advanced PCa care and highlights the importance of the identification of molecular mechanisms implicated in PCa progression.

The main goal of my thesis work was to elucidate functional properties of CTE-truncated AR variants frequently observed in metastatic and hormone-resistant PCas.

The endocrine treatment persists as a cure suited for patients with advanced PCa. After an initial favourable response to hormone therapy, the majority of PCa displays over time molecular and cellular changes toward cancer progression.

Escape from androgen deprivation may be due to selection of cellular clones expressing mutated androgen receptors (ARs).

Indeed, several mutations on the AR sequence have been observed in androgen-independent PCas. (Bergerat and Ceraline, 2009; Steinkamp et al., 2009; Taplin et al., 1999)

CTE-truncated AR variants are frequently found in metastatic and hormone-resistant PCa. These variants arise from nonsense mutations, but also from aberrant splicing resulting in shorter mRNAs transcripts.(Ceraline et al., 2004; Dehm et al., 2008; Hu et al., 2009)

The 22Rv1 cell line can be considered as a preclinical model for hormone-resistant PCa. These cells express a full length AR together with a short form. In a previous study, the short AR variant in the 22Rv1 was interpreted as a result of AR protein degradation.(Tepper et al., 2002) Recently, two reports and our work demonstrated that AR variants presents in 22Rv1 cell line are the result of aberrant pre-mRNA splicing or nonsense mutations in the *AR* gene.(Marcias et al., 2009)

The study of the 22Rv1 cell line highlighted the prominent place of this cell model in the study of the PCa progression. Indeed, the co-expression of more AR mutants into the same model gets probably closer to the reality of a heterogeneous tumour withdrawal environment, which characterizes the aggressiveness of cancer. This model system therefore provides an excellent opportunity to study AR isoforms that either contain or lack the AR CTD as well.

Moreover, this study emphasizes the role of AR variant in the progression of the disease and gives an emerging role truncated ARs as a mechanism to avoid escape from hormone

therapies. The way these new AR variants are activated, their mechanisms of action, including identification of their target genes, their transcriptional partners, posttranslational modifications require further investigation.

We then carried out the study of the truncated AR in the other selected prostatic cancer model: the LNCaP cell line, which expresses a full length AR with a punctual mutation responsible of the enlargement of the affinity for hormonal stimulation. This model avoids the presence of others truncated receptors and seems interesting for the study of one particular AR variant: the Q640X AR.

Indeed, LNCaP cell line allowed to observe the activity of the exogenous receptor of our interest, and in the same time offered the possibility to observe the behaviour of the exogenous AR in the presence of a full length endougenous AR androgen-sensible.

CTE-truncated AR variants are constitutively localized in the nucleus.

The mechanism by which the wild-type AR is retained in the cytoplasm, and enters to the nucleus following activation is well described. An important point about most of CTE truncated AR found in PCa is the maintain of the localization signal (NLS1) in the hinge region. Moreover, several phosphorylation sites are still present in the CTE-truncated AR.

It is well known that AR activity is regulated at several levels, including stabilization, ubiquitylation, nuclear import by phosphorylation. Furthermore, the CTE region plays an important role by allowing ligand-dependent AR modifications and consequently a ligand-dependent transcriptional activity.

We postulated that phosphorylation of CTE-truncated ARs continue to be essential for full transcriptional activity.

As several reports underline the effect of growth factors or cytokines signalling on the AR phosphorylation status, we evaluated the effect of several kinases inhibitors on Q640X activation. We demonstrated the dependence of the Q640X AR for stimuli coming from the extracellular compartment and transmitted by membrane receptor tyrosine kinases such as EGFR and HER2.

We further demonstrated that PI3K and MEK1/2 pathways were mainly involved in the activation of Q640X transcriptional activity. Inhibition of PI3K modified the particular nuclear distribution reported in a previous study and reflecting the transcriptional active form of the receptor.(Lapouge et al., 2008)

The ser-213 is a putative phosphorylation target after the stimulation of the PI3K effector, Akt. We confirmed that the mutated AR needs activating phosphorylations and the ser 213 is

involved in this activation mechanism. We know that MEK1/2 plays also an important role in the Q640X activity. Ser-81 and Ser-515 could represent potential target phosphorylation sites for ERK as demonstrated for the wt AR.(Ponguta et al., 2008) Furthermore, we demonstrate that PI3K/PDK1/PKC pathway contributes weakly to Q640X AR activity. Consequently, phosphorylation at Ser-578 by PKC associated with AR nuclear-cytoplasmic shuttling and transcription activity could also be relevant for Q640X AR transactivation. The impact of the phosphorylation on the activation of this AR variant probably involves supplementary phosphorylation sites.

A larger screening of Q640X AR sequence for phosphorylated serine, threonine and tyrosine residues is imperative. A collaborative work with the Inserm U682/CNRS UMR 7178 team is in progress. The knowledge of sites modified by this posttranslational modification will be helpful to identify kinase pathways involved in the activation and/or the activity of the Q640X AR. In this purpose, we are performing a proteomic study of phosphorylated residues in the Q640X AR with the collaboration of the Dr. François Delalande.

The impact of the Q640X AR on AP-1 and NFAT transcriptional activities is enhanced by EGF stimulation in an LNCaP model. These data suggest that upon transfection with a Q640X AR plasmid, one or several signalling pathways are switched on, resulting in AP-1 and NFAT activation. The Q640X was able to stimulate the transcription of NFAT and AP-1 transcription factors.(Lapouge et al., 2007) These factors are implicated in the lost of apoptosis and in general into mechanisms enhancing the cellular proliferation.

The existence of a link between these factors and the truncated AR strenghthened the emergency to understand the function of the family of CTE truncated androgen receptors.

In LNCaP cell line the stimulation of both NFAT and AP-1 factors was dependent on the presence of the Q640X AR and on kinase pathways activation.

Alltogether these results confirm the unusual role of these novel variants AR in the progression to hormone-refractory PCa. Indeed, in one side the Q640X AR, and we speculate similar properties for the others members of this new CTE-truncated AR family, can be activated from non-hormonal signalling that avoid the effect of an androgen deprivation therapy. In the other side, the activation of this mutant AR implicates important consequences on the positive regulation of factor involved in the loss of apoptosis, cellular proliferation, cellular survival and in the formation of bone metastasis.

Targeting the RTK/PI3K/Akt or the RTK/Ras/Raf/MEK pathways may be a useful strategy for treating patients with tumours resistant to hormone therapy.

Functions of the CTE-truncated Q640X AR seem to be associated with apoptosis, cell proliferation and survival pathways.
To identify genes involved in the Q640X activity, we realized a study of the transcript in LNCaP cells transfected with the Q640X, the wt AR or the control empty plasmid.
This approach was possible thanks to the Microarrays platform of the IGBMC, Strasbourg.
The comparative analysis was driven within LNCaP cells not transfected or transfected with the wild type AR expression plasmid or the Q640X expression plasmid, each condition was performed in triplicate and in the presence of DHT 100nM or in the presence of the vector.
The analysis of first results is still in progress.

We speculated that the identification of new functional properties of the CTE-truncated ARs, such as the ability to crosstalk with growth factors signalling pathways and the capacity to influence transcription factors activities, may provide new therapeutic options.
These CTE-truncated ARs may evade the androgen ablation therapy and via their constitutive activity they may stimulate the production of factors, which can enhance proliferation and survival mechanisms. In this case, we speculate that it would be interesting to identify a group of truncated-AR positive PCa patients. Indeed, this new group of patient could benefit to adapted treatment against for example the EGFR or the HER2 activation.

Future aims

Short term

Molecular level: identification of posttranslational modifications targeting CTE-truncated ARs

- Mass spectrometry
- Site-directed mutagenesis

Cellular level: identification of signalling pathways responsible for the ligand-independent activity of truncated ARs

- siRNA
- kinome study

Tumor level: identification of CTE-ARs target genes

- At the transcriptional level : Quantitative RT-PCR
- At the protein level : Multiplex, TMA

Long term

- Role of CTE-truncated ARs as pronostic and/or predictif factors for PCa
- Identification of a subset of patients expressing truncated ARs
- Target treatment for exemple against membrane growth factors receptors

Publications

Marcias G, Erdmann E, Lapouge G, Siebert C, Barthélémy P, Keller L, Kolli K, Duclos B, Bergerat J-P, Céraline J, Kurtz J-E (2009): Identification of a novel truncated androgen receptor mutants including unreported pre-mRNA splicing variants in the 22Rv1 hormone-refractory prostate cancer cell line. Human Mutation *(in press)*.

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Lapouge G, Erdmann E, **Marcias G**, Jagla M, Monge A, Kessler P, Serra S, Lang H, Jacqmin D, Bergerat J-P, Céraline J (2007): Unexpected paracrine action of prostate cancer cells harboring a new class of androgen receptor mutation--a new paradigm for cooperation among prostate tumor cells. International Journal of Cancer. Journal International Du Cancer 121:1238-1244.

Lapouge G, **Marcias G**, Erdmann E, Kessler P, Cruchant M, Serra S, Bergerat J-P, Céraline J (2008): Specific properties of a C-terminal truncated androgen receptor detected in hormone refractory prostate cancer. Advances in Experimental Medicine and Biology 617:529-534.

Recently submitted

Marcias G, Lapouge G, Erdmann E, Kurtz J-E, Duclos B, Bergerat J-P : Epidermal growth factor and phosphatidylinositol 3-kinase pathways are necessary for carboxy-terminal end truncated androgen receptor activity in prostate cancer. Submitted to Journal of Cell Science.

In progress

***Marcias G**, *Lapouge G, Erdmann E, Kurtz J-E, Duclos B, Bergerat J-P (2009c): Constitutively active androgen receptor in prostate cancer: interconnection with transcription factors.

Communications

Marcias G, Lapouge G, Szatkowski C, Erdmann E, Ceraline J, and J-P Bergerat: Truncated androgen receptor devoid of the carboxy-terminal end region display constitutive transcriptional activities and lead to unexpected specific paracrine activities in prostate cancer cells. 13th International Congress on Hormonal Steroids and Hormones & Cancer, 2008, Quebec City.

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RESUME DE LA THESE DE DOCTORAT

Récepteurs des androgènes constitutivement actifs dans le cancer de la prostate: interconnections avec les voies de signalisation.

Constitutively active androgen receptor in prostate cancer: interconnection with kinases pathways.

Introduction

Le cancer de la prostate (CaP) est la deuxième cause de mortalité par cancer chez l'homme. Plus de 62000 nouveaux cas et 9000 décès sont comptés par an en France. Les thérapeutiques adaptées montrent une bonne efficacité pour les CaP dépistés très tôt, mais la prise en charge de formes avancées du CaP reste problématique en dépit des nombreux essais cliniques développés ces dernières années.

Depuis les travaux de Huggins en 1954, l'hormonothérapie du CaP avancé est basée sur la dépendance des cellules prostatiques normales et cancéreuses vis-à-vis des androgènes pour leur prolifération et survie. Malheureusement, l'échappement à la thérapie hormonale reste un problème récurrent. Ainsi, l'identification de nouvelles bases moléculaires de l'échappement hormonal est nécessaire afin de développer des thérapeutiques mieux ciblées et plus efficaces.

Un des mécanismes de résistance du CaP à l'hormonothérapie est une altération de la voie de signalisation du récepteur des androgènes (RA). Le RA est constitué par i) un domaine amino-terminal abritant une région activatrice de la transcription ligand-indépendante (AF1), ii) un domaine de liaison à l'ADN, iii) un domaine charnière avec un signal de localisation nucléaire, et iv) un domaine carboxy-terminal (CTE) abritant le domaine de liaison du ligand et une région activatrice de la transcription ligand-dépendante AF-2.[Feldman and Feldman, 2001]

Ainsi, la sensibilité du RA vis-à-vis des androgènes peut-être augmentée suite à une surexpression du récepteur résultant d'une amplification génique ou d'une production accrue d'hormones stéroïdiennes. Des mutations au niveau du gène du RA peuvent conduire à un RA sensible à des hormones autres que les androgènes. De même, des mutations non sens ou des épissages aberrants peuvent conduire à la perte partielle ou totale de la région CTE du RA. Ces RA mutés exhibant des activités transcriptionnelles ligand-indépendantes sont principalement retrouvées dans les CaPs métastatiques et en échappement hormonal suggérant qu'ils y jouent un rôle important.[Céraline et al., 2004; Lapouge et al., 2007; Dehm et al., 2008]

Projet de thèse

Mes travaux de thèse ont visé :

- à caractériser de nouvelles formes tronquées du RA dans une lignée de CaP hormono-résistante,
- à comprendre les mécanismes d'activation de ces RA tronqués en recherchant les interconnexions avec les voies de signalisation des facteurs de croissance et de cytokines,
- à identifier les sites de phosphorylation importants pour les fonctions des RA tronqués, et en conséquence les kinases responsables de leur phosphorylation,
- à élucider les mécanismes d'action d'un RA tronqué sur les activités de facteurs de transcription impliqués dans la prolifération, l'apoptose et la survie cellulaire.

Identification des formes tronquées du récepteur des androgènes dans un modèle préclinique de cancer de la prostate

Les cellules 22Rv1, issues d'une xénogreffe de cancer de la prostate, représentent un modèle d'étude préclinique du cancer de la prostate en échappement hormonal. L'origine des formes tronquées du RA précédemment décrites dans cette lignée a été recherchée grâce au test fonctionnel du RA chez la levure mis au point au laboratoire. Nous avons ainsi mis en évidence plusieurs transcrits du RA non encore décrits dans cette lignée et conduisant à des formes tronquées du RA.[Marcias et al., 2009a] L'originalité de ces résultats repose sur la meilleure compréhension des mécanismes moléculaires responsables de l'expression de formes tronquées du RA au sein du cancer de la prostate en échappement à l'hormonothérapie. En effet, nos résultats montrent la coexistence de transcrits caractérisés par des mutations non sens et de transcrits issus d'un épissage aberrant. Ces derniers, plus longs de 98 et 280 nucléotides que les transcrits normaux, sont issus d'un épissage aberrant de l'intron 2, et sont différents des variants d'épissage du RA conduisant à des transcrits plus courts récemment décrits dans cette même lignée 22Rv1.[Dehm et al., 2008] L'ensemble de ces résultats montre toutefois la très grande complexité et hétérogénéité des mécanismes moléculaires qui pourraient conduire à l'échappement à l'hormonothérapie.

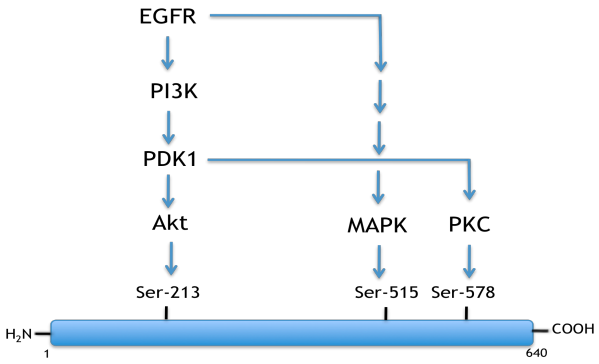
Mécanismes d'activation des formes tronquées du récepteur des androgènes dans les cellules cancéreuses prostatiques

Nous savons que la région CTE est responsable de l'activation ligand-dépendante du RA. Une fois activée par la liaison de son ligand, le RA subit de nombreuses phosphorylations activatrices sur des résidus sérine ou tyrosine. Notre hypothèse de travail est que malgré l'absence de la région CTE, des phosphorylations activatrices sont aussi requises pour une activité transcriptionnelle complète des RA tronqués. Ainsi, nous nous sommes focalisés sur

les voies de signalisation des facteurs de croissance et cytokines impliquées dans la progression du cancer de la prostate et menant à la phosphorylation du RA.

Nous avons ainsi étudié l'impact de l'inhibition des voies de signalisation des récepteurs à activité tyrosine kinase (RTK) EGFR et HER2 et de leurs effecteurs intracellulaires phosphatidylinositol-3(OH) kinase/Akt, MEK1/2 et la protéine kinase C (PKC) sur les propriétés de transactivation des RA tronqués. Des tests luciférase ont été menées dans les cellules LNCaP, une lignée de CaP hormono-sensible, et dans les cellules CV-1 de rein de singe vert qui n'expriment pas le RA. Les cellules sont transfectées en présence du plasmide d'expression d'un RA tronqué (Q640X) et d'un plasmide rapporteur contenant le gène *luciférase* sous contrôle d'un promoteur androgéno-dépendent. Les cellules ont ensuite été incubées en présence de gefitinib (anti-EGFR), herceptin (anti-HER2), LY294002, PI3Kα-IV et PI3Kβ-VI (anti-PI3K), UO126 (anti-MEK1/2) et de calphostin C (anti-PKC).

La transactivation du RA Q640X a été fortement affectée en présence de gefitinib et de LY294002 dans les lignée CV-1 et LNCaP, indiquant l'implication de la voie EGFR/PI3K dans la régulation positive du RA tronqué Q640X. De plus, des expériences de microscopie confocale réalisées sur les cellules LNCaP exprimant le RA Q640X couplé à la protéine fluorescente GFP, montrent que l'inhibition de PI3K entraine une redistribution intranucléaire du récepteur muté, se manifestant par un marquage diffus au lieu de la distribution en agrégat propre au RA Q640X actif. Par ailleurs, nous avons montré que la sous-unité p110α de la protéine PI3K, mais non la sous-unité p110β, est responsable de la signalisation de PI3K vers le RA Q640X. Ces résultats montrent la dépendance de ces RA tronqués vis-à-vis des cascades de signalisation des RTK. La voie de l'EGFR semble jouer un important rôle, cependant nous ne pouvons pas exclure la contribution d'autres RTKs.



Cibles potentielles de phosphorylation activatrice du RA Q640X.

La protéine kinase Akt, effectrice de la voie de signalisation de l'EGFR, conduit à la phosphorylation des sérines en position 213 et 791 au niveau du RA.[Ponguta et al., 2008] Du fait de la perte de la région CTE, le RA Q640X est dépourvu du site de phosphorylation en position 791. L'étude des activités transcriptionnelles du RA double mutant S213A/Q640X montre que la Ser-213 n'est pas un site essentiel à la transduction du signal par l'EGFR/PI3K. Cependant nos résultats indiquent que des sites de phosphorylation autres que la Ser-213, comme la Ser-515 et Ser-578, sites de phosphorylation par MAPK et PKC, sont impliqués dans l'activation du RA tronqué par la voie EGFR/PI3K.[Marcias et al., 2009b]

Mécanismes d'action des formes tronquées du récepteur des androgènes dans les cellules cancéreuses prostatiques

- Impact sur les activités transcriptionnelles de facteurs de transcription NFAT et AP-1

Le second volet de mes travaux de recherche a donc porté sur les mécanismes d'action des RA tronqués dans les cellules cancéreuses. Des résultats preliminaires ont montré que le RA Q640X pourrait soutenir la proliferation et la survie cellulaire en stimulant les activités transcriptionnelles des facteurs de transcription NFAT et AP-1. Une telle propriété n'est pas retrouvée avec le RA sauvage.[Lapouge et al., 2007; Lapouge et al., 2008] Nous avons développée un axe de recherche visant à élucider les mécanismes moléculaires de l'interconnexion entre le RA Q640X et les trois facteurs de transcription NFAT et AP-1.

Nous avons étudié dans un premier temps l'impact d'inhibiteurs de voies de signalisation intracellulaire sur cette propriété du RA tronqué Q640X. Les cellules LNCaP ont été transfectées avec le plasmide d'expression du RA Q640X ou le plasmide contrôle et un plasmide rapporteur pNFAT-luc ou pAP-1-luc. Les cellules sont ensuite incubées en présence gefitinib, herceptin, LY294002, UO126 ou de calphostin C.

En présence de LY294002, une diminution de 90 % des activités transcriptionnelles de NFAT induites en présence de RA Q640X est observée. Le blocage de EGFR et de HER2 ainsi que l'inhibition de MEK1/2 n'affectent pas l'effet du RA Q640X sur les activités transcriptionnelles de NFAT. Pour le facteur de transcription AP-1, nous retrouvons l'effet du blocage de la voie PI3K. En effet, les activités transcriptionnelles AP-1 induites en présence du RA Q640X sont diminuées d'un facteur 5 par le LY294002.

Ces données suggèrent un rôle fondamental de la voie PI3K dans le mécanisme d'action du RA tronqué Q640X sur NFAT et AP-1 dans les cellules cancéreuses prostatiques.

Dans un second temps, nous avons tenté de comprendre les mécanismes par lesquels le RA tronqué pourrait affecter les activités transcriptionnelles de NFAT et de AP-1. L'impact du RA Q640X sur la redistribution intracellulaire de ces facteurs de transcription, ainsi qu'une éventuelle co-localisation intranucléaire entre le RA muté et ces facteurs de transcription ont

été étudiés par microscopie confocale. Les résultats obtenus pour NFAT suggèrent que le RA Q640X conduit bien à la translocation nucléaire de ce facteur transcriptionnel. Cependant, les observations en microscopie confocale ne montrent pas d'intégration du RA Q640X dans les complexes d'activation transcriptionnelle formés par NFAT.

Le bilan de ce volet d'étude qui fait l'objet d'une publication en cours de rédaction [Marcias et al., 2009c], nous permet d'avancer trois hypothèses suivantes, en effet le RA Q640X pourrait :

- stimuler l'expression d'un ou de plusieurs ligands capables d'activer des récepteurs membranaires ;
- conduire à une augmentation de l'expression d'un effecteur intracellulaire ou à la libération de calcium intracellulaire ;
- augmenter l'expression d'un récepteur membranaire.

Conclusions

Les études menées jusqu'à maintenant au laboratoire sur cette nouvelle classe de RA tronqués montrent que la perte de la région CTE conduit à un facteur de transcription ligand-indépendant dont les activités pourraient être liées à trois étapes fondamentales de la progression du cancer de la prostate, l'invasion tumorale, les métastases osseuses, et l'échappement à la privation androgénique. L'ensemble des données de mes travaux suggère que la voie EGFR/PI3K pourrait représenter une cible thérapeutique conduisant à l'inhibition des activités hormono-independentes des RA tronqués dans les cancers de la prostate.

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Constitutively active androgen receptor in prostate cancer: interconnection with kinases pathways

The emergence of androgen receptor (AR) mutations is a key event in the progression of prostate cancer (PCa) toward androgen-independency.
The AR protein consists in four functional domains: 1) the N-terminal region containing the activation function 1(AF-1) that is responsible for ligand-independent activity of the AR; 2) a central DNA binding domain (DBD) containing two zinc fingers; 3) the hinge region that encompasses a nuclear localization signal (NLS); 4) the C-terminal end (CTE) that includes the ligand binding domain (LBD) and AF-2.

Mutations issued from clonal selection conferring new properties to the AR may be involved in PCa progression, bone metastasis and tumour growth under hormonal-treatment. The analysis of human tumour samples from localized and metastatic PCa allowed the identification of a new class of CTE-truncated mutant ARs. These mutant ARs the result of somatic nonsense mutations, and were twenty –fold more frequent in hormone-refractory PCa that in hormone-naïve PCa. Furthermore, recent data proposed aberrant splicing as an alternative mechanism for CTE-truncated ARs. In the present study, we confirmed the co-existence of these two mechanisms, which offers a new preclinical model of hormone-resistant PCa.

Investigations on the CTE-truncated Q640X AR have revealed in previous studies the constitutive nuclear localisation and the ligand-independent transcriptional activities of this CTE-truncated AR. Since the AR is a phosphoprotein, we explored the role of kinases signalling pathways in the activity of the Q640X AR. Our present results suggest that this AR mutant requires activating phosphorylation by PI3K/Akt and the MEK1-2 proteins, for full transactivation or transcriptional activities. Furthermore, we demonstrated that serine at position 213 in the AR sequence remains fundamental for signal transduction from PI3K/Akt kinases.

In LNCaP cells, we have previously shown that the Q640X AR stimulates transcriptional activities of transcription factors such as NFAT and AP-1. We demonstrate that activation of Phospholipase C is a prerequisite for NFAT activity. In addition, we provide evidence that the crosstalk between truncated AR and NFAT or AP-1 factors depends on upstream signalling pathways.

Altogether, our data indicate that Q640X AR requires activating phosphorylation by growth factors signalling pathways for full transcriptional activities. Moreover, this CTE-truncated AR may enhance some ligand/membrane receptors signalling to activate NFAT and AP-1.

In conclusion, expression of CTE- truncated ARs may provide a mechanism for resistance to hormonal therapy. Understanding their functional properties ARs will allow the identification of new targeted therapies for the treatment of hormonal resistant PCa. Targeting the PI3K/Akt pathway may be a useful strategy for treating patients with hormone-refractory PCa positive for CTE truncated ARs.