The Clinical Implications of the Survival Pathway in Prostate cancer

by

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من طلب العلى سهر الليالي

« Scientia, Iustitia et Libertas »

ABSTRACT

Prostate Cancer (PCa) is the second leading cause of cancer death in North American men. The heterogeneity of the disease, along with the imperfection of the current prognostic determinants is a challenge to physicians who are unable to discriminate indolent cancers from those that will become life- threatening. Previous work has identified 3 molecular subtypes of PCa that correlated with clinical behaviour, and found the PI/AKT pathway genes to be up-regulated in metastatic samples compared to primary ones. Copy number alteration analysis on this group of patients showed frequent 16p13 (*PDPK1*) genomic gain and the 10q23 (*PTEN*) loss. PDK1 activates the PI/AKT survival pathway, while PTEN inhibits it. In this study, we report for the first time the detection of the 16p13.3 (*PDPK1*) genomic gain in lymph node metastasis and their matched primary samples, in castration- resistant prostate cancer, and in unmatched primary prostate cancer samples. This localized gain was enriched in advanced disease compared to primary prostate cancer, and associated with high Gleason grade and high preoperative PSA levels, pointing towards its potential prognostic value. *In vitro*, we characterized a role for PDK1 in PCa cells motility, a crucial process in metastasis. This finding supports the idea of a role for the 16p13.3 (*PDPK1*) gain in PCa progression towards a more aggressive disease, and makes PDK1 a potential therapeutic target in patients with aggressive PCa.

RÉSUMÉ

Le cancer de la prostate (CaP) est la deuxième cause de décès par cancer chez les hommes nord-américains. L'hétérogénéité de la maladie, ainsi que l'imperfection des déterminants pronostiques actuels, constitue un défi pour les médecins qui sont incapables de distinguer les cancers indolents de ceux qui progresseront pour devenir mortels. Une étude antérieure a identifié 3 sous-types moléculaires de cancer de la prostate qui corrèlent avec le comportement clinique, et a rapporté une augmentation dans l'expression des gènes de la voie de signalisation PI/AKT dans les échantillons métastatiques, comparé aux cancers primaires. L'analyse des altérations génomiques dans ce groupe de patients a montré que le gain génomique 16p13 (*PDPK1*) et la perte 10q23 *(PTEN*) sont fréquentes. PDK1 active la voie de survie PI /AKT, tandis que PTEN l'inhibe. Dans cette étude, nous rapportons pour la première fois la détection du gain génomique 16p13.3 (*PDPK1*) dans les métastases et de leurs échantillons appariés primaires, dans des spécimens de cancer de la prostate résistants à la castration, et dans des tumeurs primaire non-métastatiques. Le niveau de gain augmente dans les échantillons de stade avancé, soulignant ainsi une possible valeur pronostique. *In vitro*, nous avons caractérisé le rôle de PDK1 dans la motilité des cellules du cancer de la prostate, un processus essentiel à la métastase. Cette constatation appuie l'idée d'un rôle joué par le gain génomique 16p13 (*PDPK1*) dans la progression du cancer de la prostate vers une maladie létale, et rend PDK1 une cible thérapeutique potentielle chez les patients avec un cancer agressif.

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ABBREVIATIONS

a-CGH: array-CGH

AMF: Autocrine Motility Factor

AR: Androgen Receptor

ATP: Adenosine Tri-Phosphate

BMP: Bone Morphogenic Protein

BPH: Benign Prostatic Hyperplasia

CD: Catalytic Domain

CDK: Cyclin Dependent Kinase

CGH: Comparative Genomic Hybridization

CNA: Copy Number Alteration

CRPC: Castration-Resistant Prostate Cancer

DHT: Dihydrotestosterone

DMT: DNA MethylTransferase

HIF-1α: Hypoxia Inducible Factor 1 α

DDM: DNA DeMethylase

DRE: Digital Rectal Examination

ECM: Extra-Cellular Matrix

EGFR: Epithelial Growth Factor Receptor

EMT: Epithelial-to-Mesenchymal Transition

ETS: E26 Transforming Sequence

F-actin: Filamentous Actin

FAK: Focal Adhesion Kinase

FGF: Fibroblast Growth Factor

FISH: Fluorescent In Situ Hybridization

G-actin: Globular Actin

GLUT: Glucose Transporter

GSEA: Gene Set Enrichment Analysis

GST: Glutathione S-Transferase

GSTP-1: pi-class Glutathione S-Transferase 1

GTP: Guanine Tri-Phosphate

IGF: Insulin Growth Factor

IGFBP: IGF Binding Protein

IHC: Immunohistochemistry

IL-8: Interleukine 8

LH: Leuthanizing Hormone

LHRH: Leuthanizing Hormone Releasing Hormone

LN mets: Lymph Node Metastasis

LOH: Loss of Heterozygosity

LUTS: Lower Urinary Tract Symptoms

MAT: Mesenchymal-to-Amoeboid Transition

MGMT: MethylGuanine DNA MethylTransferase

MLC2: Myosin Light Chain 2

MMP: Matric Metallo-Proteinase

mTOR: mammalian Target of Rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NES: Nuclear Export Sequence

PA: Plasminogen Activator

PCa: Prostate Cancer

PCPT: Prostate Cancer Prevention Trial

PCR: Polymerase Chain Reaction

PDGF: Platelet Derived Growth Factor

PDK1: Phosphoinositide-Dependent Kinase-1

PEPCK: Phosphoenolpyruvate carboxukinase

PFK: 6-Phosphofructo-2-Kinase

PH: Pleckstrin Homology

PI: PhosphatidylInositol

PI3-K: PI-3 Kinase

PIN: Prostate Inter-epithelial Neoplasia

PIP3: PhosphotidylInositol 3,4,5-triPhosphate

PTEN: Phosphatase and TeNsin homologue deleted on chromosome 10

PKB: Protein Kinase B

PKC: Protein Kinase C

PP: Protein Phosphatase

PSA: Prostate Specific Antigen

S6K: p70 ribosomal S6 kinase

SDF-1: Stromal-Cell Derived Factor 1

SGK: Serum and Glucocorticoid-induced protein Kinase

SREBP-1: Sterol Regulatory Element Binding Protein 1

RaIGDS: RaI Guanine nucleotide Dissociation Stimulator

RB: Retinoblastoma

ROCK: Rho-associated Kinase

ROS: Reactive Oxygen Species

RTK: Receptor Tyrosine Kinase

Ser: Serine

siRNA: silencer RNA

Thr: Threonine

TMA: Tissue Micro-Arrays

TMPRSS2: TransMembrane Protease Serine 2

TRAF: TNF receptor-associated factor

TRAMP: Transgenic Adenocarcinoma Mouse Prostate model

TRUS: Transrectal Ultrasound

uPA: Urokine Plasminogen Activator

WHA: Wound Healing Assay

1. INTRODUCTION

1.1 The prostate

The prostate is a small gland that is about the size of a chestnut and is one of the male sex accessory tissues (urogenital system) which include the seminal vesicles, ampullary glands and bulbourethral glands (1, 2). The prostate is located below the bladder, surrounding the urethra and its presence is universal in all male mammals.

1.1.1. Prostate development

Contrary to the other sex accessories that develop from the Wolffian ducts by the 10^{th} week of foetal development upon testosterone stimulation, the prostate starts developing from the urogenital sinus during the 3^{rd} month of foetal growth, and its initiation is directed by dihydroteststerone (DHT) and not foetal testosterone (1). DHT is the product of the metabolic conversion of foetal testosterone by the 5α-reductase enzyme in the urogenital sinus.

The prostate derives from 5 epithelial buds that invade the mesenchyme to form the different zones of the gland (3), as well as the different cell types: the stromal compartment serves as a structural support and consists of connective tissues, smooth muscle cells and fibroblasts, while the epithelial compartment consists of stem cells, basal cells, neuroendocrine cells and differentiated luminal secretory epithelial cells (4).

Post-natal development is thought to be under the control of residual maternal steroids such as estrogens, for the first 5 months, a period during which the prostate undergoes involution (1). During childhood, the gland enters a quiescent state that

persists until puberty when testosterone levels increase, causing the epithelium to proliferate and the prostate to double in size (2). Androgens are required for maintaining normal prostate epithelial cells growth, and their effect is mediated via the androgen receptors.

1.1.2 The mature prostate

1.1.2.1 Function

The prostate contributes to the seminal plasma portion of the ejaculate (around 0.5 ml of the 3 ml seminal fluid is generated by the prostate) (1). The seminal fluid contains non-peptidic secretions (such as citric acid, fructose and polyamines; reviewed in (5)), as well as other secretory proteins (such as PSA= prostate specific antigen, and other Kallikrein family members (6)). The prostate is a major contributor of citric acid, which acts as a very potent metal ion binder (7), as well as PSA, thus keeping the sperm healthy for fertilization.

PSA is the major protein secreted by the luminal cells of the prostate and is encoded by the KLK3 gene, an androgen dependent gene (8). It is a 33-36 KDa glycoprotein that acts as a serine protease (9) and is involved in lysing the clots in the ejaculate, thus better liquefying it (10). PSA is found at 0.70 mg/nL in the seminal plasma (11). Normally, PSA is secreted into the glands lumen, but in the case of proliferative diseases (benign prostatic hyperplasia= BPH, and prostate cancer= PCa), excess PSA is detected in the blood and levels of free (unbound) and complexed (bound) circulating blood PSA can be monitored to screen for prostate diseases (reviewed in (1)).

1.1.2.2 Anatomy

The gland can be divided into four zones: i) the central zone which surrounds the ducts and makes up 25% of the prostate, ii) the transition zone that makes up 5% of the prostate and is the site of BPH, iii) the anterior fibro-muscular zone, composed of fibrous and connective tissues, and finally iv) the peripheral zone, which is the largest zone and the site for around 75% of prostatic carcinomas (reviewed in (12)).

In terms of cellular anatomy, the prostate gland is rich in acini, whose lumen is lined with tall, terminally differentiated secretory cells (13). These cells are inter- connected by cell adhesion molecules and to the plasma membrane by integrin receptors (13). Below the secretory cells, the basal cells are small, undifferentiated and keratin rich, and make up less than 10% of the epithelial cells (1). Basal cells rest on the basement membrane and it has been suggested they act as stem cells (14). The neuroendocrine cells are spread between the secretory cells and are known to be terminally differentiated and negative for androgen receptors and PSA (13, 15).

1.1.2.3 Diseases of the prostate

The prostate gland can be a site for inflammation, known as prostatitis (1, 16) which often associates with pain, difficult urination due to swelling of the gland, and sometimes flu-like symptoms (17). However, prostatitis is not a proliferative disease unlike benign prostatic hyperplasia (BPH) which refers to the enlargement of the prostate causing lower urinary tract symptoms (LUTS) (1). BPH first develops in the transition zone of the prostate unlike cancer that arises in the peripheral zone (18) and several studies have shown that BPH and PCa can occur distinctly and bear distinct gene expression

signatures (1, 19). The prostate is the site of cancer and will be reviewed in more details in the next section.

1.2 Prostate cancer (PCa)

PCa strongly affects the male population with a likelihood of diagnosis around 18% and a lifetime death probability of 3% in North America (1, 20, 21). Indeed the Canadian Cancer Society has classified PCa as the most common cancer in men and the third leading cause of cancer death for Canadian men (20).

1.2.1 Associated risk factors

The established risk fators for PCa are: age, heredity and family history, and ethnicity (reviewed in (22)). **Age** is an important risk factor for PCa with the incidence of the disease increasing after the age of 50 (23, 24). According to the Canadian Cancer Society around 2 of 3 PCa patients are older than 65 years (20). **Heredity and family history** are the second most important risk factors for PCa and studies have shown that the risk of PCa is doubled if one first-line relative has the disease, and increased around 5-11 folds if 2 or more relatives are affected (25-27). The frequency of clinical PCa varies on an **ethnic** basis as well, being the highest in Caucasian populations and lowest in men of Asian origin. Furthermore, black men in the United States were reported to have a 50-60% higher incidence rate of PCa as opposed to Caucasian men ((25, 27) and reviewed in (22)).

PCa incidence also varies on geographical basis with the highest incidence rates in North America and lowest in South-East Asia. However, the frequency of PCa detected at autopsy did not vary between the different parts of the world (28) pointing to the role of

environmental factors in increasing the risk of progression from a latent cancer to a clinical cancer. Although these factors are still not well identified, some studies have shown a role for high animal fat diet along with low intake of vitamin E, selenium, lignans and isoflovanoid, in increasing PCa risk (reviewed in (29) and (22)).The importance of lifestyle in PCa risk was also underlined in migration studies that showed that Asian immigrants to North America adopt higher PCa risks (28, 30, 31).

1.2.2 Natural history of PCa

PCa arises in the peripheral zone, away from the urethra (12). Because of this location, PCa is often asymptomatic and thus clinically undetectable in its early stages, when it is still confined to the prostatic capsule (32, 33). PCa is often a slow growing disease and detection of asymptomatic PCa is incidental and often occurs at autopsy or upon pathologic examination of prostatic tissue removed for reasons other than PCa (33). PCa is often a latent disease and most of the tumours do not become clinically manifest as autopsy studies estimate that around 50% of men older than 50 years of age have PCa although PCa was not the cause of death in those men (34, 35). The incidence of clinically inapparent PCa increases progressively beyond the age of 50 (36), although its prevalence exceeds by far mortality rates. The increasing prevalence of the disease since the 1990s is mostly attributed to the wide-spread use of serum PSA screening rather than to an increase in PCa cases, that otherwise would have gone undetected (1). Still, a fraction of prostate tumours will eventually progress causing a lethal disease. PCa becomes symptomatic when the tumour increases in volume and presses against the urethra causing LUTS, although still confined to the capsule (1, 33).

Eventually, PCa exhibits local extension beyond the prostatic capsule and is manifested by

obstructive symptoms (33). The locally extensive disease infiltrates adjacent structures with invasion of the seminal vesicles and bladder neck, without recognized distant metastasis. If untreated, the average life-expectancy is around 2 to 3 years with a high probability of death (1, 37).

PCa's first site of metastasis, are the lymph nodes although the most common site of clinically recognized dissemination is to bone (33). PCa spreads to well vascularized areas of the skeleton such as the ribs, vertebral column, skull and the proximal ends of the long bones (37). Other sites are less frequent and include lung and liver as sites of metastasis. Bone metastases are symptomatic and may cause bone pain, bone marrow suppression, leukopenia, hypercalcemia and pathologic fractures (reviewed in (37)). Survival of such untreated patients is of the order of 1 year and most of the patients, whether treated or not will die of cancer (1, 33).

1.2.3 PCa diagnostic tools

Before the availability of PSA testing, **digital rectal examination (DRE)** was the only way of detecting PCa. Although DRE is a test with high specificity for PCa, it was shown to be of low sensitivity, missing a considerable proportion of cancer and detecting those at a more advanced stage, when treatment is less effective. An abnormal DRE will often prompt prostate biopsy (24). Combined with PSA, DRE's positive predictive value was improved (38).

Serum PSA is currently used as the first-line screening tool. As stated earlier, excess PSA is released into the blood and could easily be detected as a screening tool for PCa. A currently accepted threshold for serum PSA screening is 4 ng/ml (24) and any increase beyond this threshold will prompt further investigation. Thus, any increase in PSA

levels, which reflects an increase prostatic volume, or an abnormal digital rectal examination, will require a biopsy (39).

Transrectal Ultrasound (TRUS) is used as a diagnostic tool by urologists for volume measurement of the prostate (40). Although it seems to be very useful in directing the biopsy needle to different prostate sites, its diagnostic value is limited by low accuracy (24).

Since it is impossible to localize the tumour within the prostate, the **biopsy** of the gland is systematically done throughout the different prostatic lobes, and the biopsied cores are histologically assessed. Initially, a sextant biopsy was performed (6 cores) (41); however, this approach revealed a significant rate of false negatives and has been replaced by extended biopsy schemes with 10-13 cores (42). Needle biopsy, followed by the histological assessment is used to make the final diagnosis and is often used to confirm or reject suspicions of PCa.

1.2.3.1 Limitation of the diagnostic tools

As stated earlier, DRE is a test with low sensitivity and can only detect tumours at a more advanced stage. As far as PSA levels are concerned, setting a clear cut-off remains debatable as the Prostate Cancer Prevention Trial (PCPT) has shown (43). In fact, it was shown that no serum PSA level could be used as a definitive cut-off to define patients that are at risk of PCa, given that around 7% of men with PSA levels lower than 0.5ng/ml still develop the disease (24, 43). The dilemma of whether to screen with PSA and with which threshold was also addressed in the European Randomized Study of Screening which showed some benefits of mass PSA screening, including a 20% reduction in PCa

mortality in the screening arm (44).

However, this benefit of reduced mortality comes at costs of over-treatment, with 48 patients to be treated in order to avoid one death (44). Thus the use of PSA increases detection rate of PCa leading to detection of those that are more likely to be confined. Another caveat to consider is the daily variation of PSA levels (45) that can result from inflammation/infection, pre-test ejaculation (46), digital rectal examination (47) and prostatic massage (48). Furthermore, PSA does not have the ability to discriminate between PCa, BPH and prostatitis given that they all result in increased PSA levels (1). Thus, the interpretation of PSA values should always take into account the presence of other prostate disease.

The effectiveness of biopsy as a diagnostic tool remains limited by the probability of picking the most informative adenocarcinoma zone, a probability that decreases with increased gland volume (24, 41).

1.2.4 PCa prognostic tools

Clinical staging is the only source for PCa prognostic determinants prior to surgery (preoperative PSA, Gleason Grade and TNM staging system). Pathologic staging on the other hand is determined post-surgery, after removal of the prostate and is more accurate in estimating the burden of the disease (49).

Preoperative PSA: In addition to its use as a diagnostic tool, preoperative serum PSA levels are used in clinical staging as a predictive tool for the course of the disease. Indeed studies have shown that 80% of men with PSA less than 4.0 ng/ml have organ-confined disease, and more than 50% of men with PSA greater than 10.0 ng/ml have extra-prostatic disease (50, 51). Furthermore, 20% of men with PSA greater than 20 ng/ml and 75% of

those with PSA greater than 50 ng/ml are found to have pelvic node involvement (52).

Histological grading on biopsy specimen: The most common histological system to grade PCa is the Gleason grade (39, 49), which describes a score of 5 grades, each with a distinguishable pattern and the final score, which is the sum of the 2 most occurring patterns, varies between 2 (least aggressive/most differentiated) and 10 (most aggressive/least differentiated) (53). This grading system is considered as the most reliable and powerful index for prediction of aggressive disease, and a recent study has shown that the percentage of Gleason grade 4/5 cancer had a strong independent predictive value for regional pelvic lymph node metastases, and interestingly, was a predictor of disease recurrence, regardless of metastasis (39). The only caveat of this prognostic tool is- beside the initial variability due to biopsy- the empirical aspect of the system along with the subjective pathological assessment (24, 39).

TNM clinical staging assessment (described in (54)): the T-staging refers to the local staging of the tumour based on its volume, findings from DRE and the number of positive biopsies. A T0 staging refers to the lack of evidence of a tumour while T1 refers to a tumour that is present but not detectable clinically. T2 stage is when the tumour is palpable on examination but has not spread outside the prostate. T3 and T4 refer to a more advanced stage in the disease where the tumour has extended through the prostatic capsule (T3) and invaded nearby structures (T4). The tumour is resectable via surgery up until stage T3. The N- staging refers to the lymph node status and is obtained by operative lymphadenectomy. A N0 stage refers to no spread to the regional lymph nodes, and an N1 stage informs of lymph node involvement. Finally, the M-staging is the skeletal metastasis and is best assessed by bone scan. A M0 stage denotes no distant metastasis

contrary to M1, where metastasis is detected. These parameters have been organized into nomograms in order to better predict the outcome of the disease (55). Based on the TNM system, along with information about the life expectancy (56), the treatment of PCa can vary from watchful waiting to a combination of hormonal therapy and/or radical prostatectomy and radiation therapy as well as chemotherapy for later stages (23).

The TNM surgical staging uses similar parameter to assess the extent of the tumour. This kind of staging takes place on the resected prostate, post-surgery, and is thus more informative and inclusive compared to clinical staging performed on biopsy specimen (1). However surgical staging is possible only when the patient has already undergone surgery.

1.2.5 Treatment of localized PCa

Treatment options for this category of patients include: radical prostatectomy, radiation therapy, conservative management (active monitoring and watchful waiting), and other treatments such as adjuvant therapies and cryoablation (1).

1.2.5.1 Radical prostatectomy

Radical prostatectomy is a surgical intervention where the prostate gland is removed. It was the first treatment used for PCa and has been performed for more than a 100 years (1). A good candidate for this surgical intervention is a healthy man, free of comorbidities that make the surgery risky, with a life expectancy more than 10 years and with a tumour judged to be biologically significant and completely resectable base on preoperative clinical staging parameters (57). The known disadvantages of radical prostatectomy are first

the hospitalization and recovery period they require, the possibility of incomplete resection (positive margins), as well as the risk for erectile dysfunction and urinary incontinence (1). Despite its potential disadvantages, it remains the gold standard given that other treatments such as hormone therapy radiation therapy and chemotherapy were proven to never be curative. Radical prostatectomy can also be performed as a salvage step in patients in whom other treatments have failed (58, 59).

However, given the imperfection of those parameters discussed above, who should undergo radical prostatectomy, and who should resort to other treatment modalities remains uncertain.

1.2.5.2 Radiation therapy

External beam radiotherapy consists of using beams of gamma radiation directed at the prostate and surrounding tissues through multiple fields (60). This intervention has outcomes roughly comparable to radical prostatectomy and seems to be effective when beams are administered in a dose escalating trend (1). However, radiation therapy does not seem to be able to eradicate all cancer cells consistently and is associated with injury to the microvasculature of the bladder, the rectum, the sphincter muscle and the urethra (1).

Brachytherapy is another form of radiation therapy where the radioactive sources are directly implanted into the prostate gland to deliver a high dose of radiation to the tumour while sparing the bladder and the rectum. Brachytherapy has been shown to have excellent short-term control rates (61) although with more common urinary symptoms and erectile dysfunction compared to external beam radiotherapy. Still, it reduces the occurrence of

rectal and bladder injuries (62, 63).

1.2.5.3 Conservative management

Watchful waiting refers to monitoring the patient until he develops metastatic disease while active monitoring refers to delaying primary treatment if there is no evidence of cancer progression. Conservative management is usually advised for insignificant tumours (volume less than 0.2 ml, Gleason score below 6 and confined disease) or when other types of intervention might put the patient at risk (1, 64, 65). For example, deferred treatment is an option for men with life expectancy of less than 10 years and a low Gleason grade PCa, although recent studies are evaluating the usefulness of active monitoring in younger patients (66, 67). The consequence of active monitoring for men without an obviously aggressive disease is that those with a clearly aggressive one will be treated immediately while those with a less aggressive disease will be monitored, thus reducing over-treatment of PCa. However a randomized control trial reported that patients with clinically localized PCa, managed with watchful waiting had significantly higher rates of local progression, metastasis and PCa-specific death, compared to those initially treated with radical prostatectomy (68). Furthermore, studies have shown that treatment of PCa is more likely to be successful if administered earlier, when the tumour is smaller, while deferred treatment is more appropriate for older patients with a limited life expectancy or comorbidities (67). The usefulness of conservative management remains thus controversial.

1.2.5.4 Other treatments for localized PCa

Other treatment options are also available for localized PCa although less commonly used. Such options include primary hormone therapy, which refers to endocrine treatment (will be discussed later) as a first line of treatment. Neo- adjuvant androgen deprivation (prior to surgery) is also less used as results have shown that this procedure does not improve cancer-specific survival although improving local control, and reducing biochemical recurrence of the disease after the surgery (1, 69). Finally, cryoablation destroys the prostate tissue through freezing. The initial results of this practice were reported to be poor with incomplete eradication of the tumour and high complication rates (urinary retention, incontinence, chronic rectal and perianal pain and loss of erection) (70).

1.2.6 Treatment of advanced / metastatic PCa

At least 10% of men with newly diagnosed PCa have locally advanced disease or metastatic PCa at the time of diagnosis, and around 35% of patients treated for localized disease will recur with a PSA elevation following radical prostatectomy (71). These patients contribute to a majority of PCa mortality (1). The treatment of advanced disease by a single modality was shown to be associated with significant risk of recurrence and current studies focus on combining different therapies such as hormone therapy (androgen deprivation), radical prostatectomy, radiation therapy and chemotherapy. The use of radical prostatectomy for the management of locally advanced disease has decreased given that many men with clinical stage T3 have regional spread and might not

benefit from such a procedure (72). The focus in this class of patients thus is on hormonal therapy and later, chemotherapy.

Hormonal therapy: Similarly to normal prostate epithelial cells, PCa cells have retained the ability to proliferate upon stimulation with androgens, resulting in tumour growth (73). Endocrine therapy was first proposed as a hypothesis in 1947, known as Huggins hypothesis, which stated that prostate cancer epithelium, like benign prostatic epithelium, will undergo atrophy when androgen hormones are reduced (74). The hypothesis was confirmed in the University of Chicago on 21 patients with advanced PCa who were subjected to surgical castration, and the report was the first to describe the benefits of androgen ablation (75). Since, androgen deprivation therapy has been one of the most effective therapies in treating advanced PCa, as most patients will respond to it. The current forms of androgen deprivation therapy function either by lowering the levels of circulating androgens, or by blocking the binding of androgens to their receptors, or both. There are four strategies of hormonal therapy: i) ablation of androgen sources (by resection of the testis, known as Orchiectomy), ii) inhibition of androgen synthesis through drugs that interfere with the steps of steroidogenesis (5α- reductase inhibitors), iii) the use of antiandrogens which block the binding of androgens to the androgen receptors and finally iv) the inhibition of Leuthanizing hormone releasing hormone (LHRH) which uses LHRH agonists to desensitize LHRH receptors in the anterior pituitary, thus shutting down the production of Leuthanizing hormone (LH) and thus testosterone (reviewed in (1)).

Antiandrogens have been shown to cause serious liver toxicity as a possible side effect although Bicalutamide (the most commonly used antiandrogen) was shown to have

efficacy that is equivalent to that of medical or surgical castration for advanced disease (76). Orchiectomy is effective at reducing testosterone by 90% within 24 hours of surgery but is associated with serious psychological consequences of an empty scrotum (1, 77). Finally, all LHRH agonists induce a testosterone increase on initial exposure, which can result in a severe, life- threatening exacerbation of symptoms (78). As a solution, LHRH agonists are co- administered with anti-androgen to block the effect of the initial surge of testosterone.

LHRH agonists and orchiectomy are equivalent in terms of outcome (castration) although one is a medical intervention while the other is surgical. Currently, LHRH antagonists are commonly used in order to reduce levels of testosterone, along with androgen-deprivation aimed at blocking any remaining testosterone (79).

General side effects of androgen deprivation therapy include osteoporosis, hot flashes, sexual dysfunction, cognitive function alteration and anaemia, caused by the decrease in levels of androgens (reviewed in (1)). Most importantly, although patients respond well to androgen deprivation therapy especially if administered early in the disease, almost all of them will experience resistance to treatment and see their disease recur after one to three years, and resume growth despite hormone therapy (79).

Treatment of castration-resistant PCa: Docetaxel is currently the standard treatment for castration-resistant metastatic PCa, with a median survival benefit of 2 to 3 months (80). It was shown to prolong progression-free and overall survival, along with improvement of pain and quality of life when administered with other agents such as mitoxantrone and prednisone (81, 82). However the associated toxicity is not negligible with fatigue, edema, neurotoxicity and changes in liver function (1).

Palliative management: Although the mechanisms of drug resistance in PCa are under investigation with several proposed mechanisms, it is still not well understood how resistance occurs. Thus, patients with failed hormonal therapy and those that develop resistance to Docetaxel are left with no cure. Palliative care is then the only option and aims at reducing: back pain, reduction of sensory levels, leg weaknesses, and changes in bladder of bowel control. Palliative management includes magnetic resonance imaging of the spine along with high- doses intravenous corticosteroids (1).

SUMMARY: It is challenging for physicians to discriminate between an indolent PCa and a lethal one at early stages of the disease, given the imperfection of the available tools. At the same time, there is currently no cure for advanced disease except for palliative care. Thus it is important to develop markers that can predict the course of the disease at an early stage, mainly the ones that are to become aggressive/metastatic in order to treat them early on. Those markers must be specific, objectively measured, and reproducible.

1.3 Metastasis

1.3.1 Overview

Metastasis is the spread of a tumour from an organ to another distant one. It is the most deadly aspect of cancer responsible for over 90% of cancer-related deaths; once tumour cells spread, it becomes impossible to eradicate the tumour by surgery or localized radiation (83, 84).

On the anatomical level, metastasis is a multi-step process: it first consists of tumour cells invading local tissue, a step that requires the disruption of adhesive mechanisms that hold

epithelial cells together (85). At this stage, cancer is still considered to be localized until intravasation happens; intravasation is the penetration of cells into blood vessels, a tough process that only one in a thousand cells will be able to perform (83). After entering, cells travel through capillaries and must face several sources of stress such as lack of oxygen and nutrients, as well as low pH, reactive oxygen species (ROS) and inflammatory reactions (83). Finally, colonization takes place at a distant site after the cells undergo extravasation (exit of tumour cells from capillaries). Colonization is seen as a selection process where most malignant cells are selected for their ability to survive and proliferate in a new environment (reviewed in (83, 84, 86)).

1.3.2 Epethelial to mesenchymal transition (EMT)

EMT is a crucial step in metastasis that allows cells to detach from each other and thus from the primary tumour in order to invade the surrounding tissue (87). The main steps of EMT consist of, the loss of cell-cell contacts, the remodelling of the cell-matrix adhesion sites and progression through the extracellular matrix (84, 85, 87). During EMT, epithelial cells de-differentiate, disassemble, and migrate away from their parent epithelium as separate individual, non-polarized and invasive mesenchymal cells (83, 88). On a molecular level, the hallmarks of EMT are the inhibition of E-cadherin, the up-regulation of Twist and Snail transcription factors and the overexpression of N-cadherin and vimentin (85, 88-90). The tumour micro-environment plays a very important role through stromal, extracellular matrix (ECM) and endothelial secretions.

1.3.3 Angiogenesis

Angiogenesis refers to the formation of new blood vessels and is an essential requirement for metastasis (83). As tumour cells proliferate, those located in the center of the growing

tumour will suffer from hypoxia, and will secrete the Hypoxia Inducible Factor 1 α (HIF-1α) to activate pro-angiogenic factors and thus promote the formation of new blood vessels (91). The recruitment of adequate blood supply ensures enough oxygen and nutrient supply to the growing tumour, and increases the chance of metastasis.

1.3.4 The tumour micro-environment

Another important factor is the tumour micro-environment. Put simply, cells surrounding the tumour are in constant cross talk with cancer cells which can mobilize their surroundings to their advantage (83, 84, 87). Important contributions to this cross-talk are from: the ECM, the stromal cells, and the endothelial cells.

a- The ECM: the extra-cellular matrix is made of fibronectin, collagen and laminin. The ECM interacts with cells through integrins which are linked to cytoplasmic complexes (such as FAK and SRC), and this interaction mediates signals to the actin cytoskeleton (84, 85, 87, 88).

b- Stromal interactions: the stroma refers to the connective tissue that supports the epithelial cells. Cancer cells can signal to macrophages and induce them to produce Fibroblast Growth Factors (FGF), Epithelial Growth Factor Receptor (EGFR) ligands and Platelets Derived Growth Factors (PDGF) along with proteases needed to degrade the ECM. Furthermore, signaling to myo-fibroblasts in the stroma induces secretions of stromal cell-derived factor-1 (SDF-1) which enables the recruitment of endothelial cells. There is thus a constant epithelial-stromal interaction that provides selective pro-metastatic advantages (84, 87).

c- Endothlial cells: endothelial cells are a significant source of chemokines such as Groβ

and interleukine 8 (IL8) which increase the contractile forces and cytoskeletal remodelling needed for cell movement during metastasis. Chemokine secretions are increased through cross-talk between endothelial cells and cancer cells (87, 88).

1.3.5 Motility

Many of the cancer metastasis steps discussed above require cell motility (92). In fact, failure to regulate cell motility has been shown to be a critical determinant of many cancer metastases (93, 94). Thus, it is important to understand the structures, the mechanisms and the molecular pathways associated with cell motility, specifically in cancer.

1.3.5.1 The cytoskeleton

The cell cytoskeleton is implicated in several functions besides motility. In fact, the cytoskeleton is essential for proper functioning of cells, and is needed for cell's organization in space, its structure, the mechanical interactions with its environment, motility, cellular division (mitosis, cytokinesis), and cellular transport (83, 88, 92, 93, 95, 96). The cytoskeleton is a complex system of filaments and more than a hundred accessory binding proteins (83, 95). The three major protein families responsible for the cells' spatial organization and mechanical properties are the **intermediate filaments**, **the microtubules** and the **actin filaments.**

a- Actin filaments: actin is found in the cell under two forms: The globular actin (G-actin), which is the monomer form of actin, and the filamentous actin (F-actin), a polar polypeptide that consists of two parallel proto- filaments (95). They are present in high concentrations at the cortex of the cell, underneath the plasma membrane (83) and thus

determine the shape of the cell surface. Actin is also heavily required for cell locomotion, such as in lamelipodia and filopodia.

b- Microtubules: microtubules are long hollow cylinders made of 13 parallel protofilaments of the protein tubulin. Tubulin is a polar hetero-dimer of α and β subunits. Microtubules are involved in positioning of organelles, directing intracellular transport, the mitotic spindle, and locomotion in cilia and flagella (reviewed in (83, 95)).

c- Intermediate filaments: unlike actin and microtubules, intermediate filaments are not polar (83); they are made of fibrous subunits and occur in different types: Nuclear, Vimentin-like, Epithelial and Axonal.

1.3.5.2 Mechano-biology and cell movement

A key factor responsible for the diverse functions of the cytoskeleton is the dynamic and adaptable property of its filaments, which allows for an enormous range of structures. For that, the main filaments are assisted by a wide range of accessory proteins, including the motor proteins. For actin, several distinct proteins have been identified (83) and can be classified into seven groups in addition to the actin motor proteins (myosin family) (83, 97, 98): the actin nucleation proteins (eg. formin, Arp2/3), the G-actin binding proteins (e.g. thymosin, profiling), the actin-depolymerization proteins (e.g. cofilin), the actin severing protein (e.g. gelsolin), the stabilizing proteins (e.g. capping protein, tropomyosin) and the filament bundling/cross-linking proteins (e.g. fimbrin, filamin, α-actinin). The microtubule cytoskeleton is also supported by major accessory proteins (83, 95) such as the nucleation proteins (e.g. γ-TuRC), the αβ tubulin dimers binding proteins (e.g. stathmin

and +TIPs), the microtubules disassembly proteins (e.g. kinesin 13), the microtubules severing proteins (e.g. katanin), the microtubules stabilizing proteins (e.g. MAPs and XMAP215) and filament bundling/cross- linking proteins (e.g. tau, MAP-2).

Mechano-biology: β-tubulin and G-actin are bound to GTP and ATP respectively (83, 95, 98). As filament elongation occurs, hydrolysis takes place, and results in GDP and ADP. The T-form (ATP or GTP) has a higher affinity to other monomers and tends to be found at the nascent end of the proto-filament while the D-form (ADP or GDP) has a lower affinity and tends to dissociate much easier. Thus the balance between addition and dissociation of filaments depends on the rate of ATP/GTP hydrolysis (83). Given the physiological differences (such as Mg^{2+} concentration (98)) between the positive and the negative ends of the filament, depolymerisation tends to occur faster at the negative end, making the positive end the site of elongation, in a phenomenon known as tread-milling (83, 92, 97, 98).

Motor proteins: Motor proteins are distinct from the other accessory proteins mentioned above. There are several types of motor proteins, differing in the type of filament they bind to, the direction of movement along the filament, the cargo carried (if any) and the function in cell's motility (flagella beating, cell division or filament sliding) (83). However, they all have a similar structure in common with a head region that contains the motor domain and bind/hydrolyzes ATP along with determining the identity of the track and the direction followed. The tail domain determines the identity of the cargo for microtubules, and is used for bundling in the case of actin (83, 97, 98).

Myosin II: is an actin-based motor protein consisting of two heavy chains that end with a globular head domain at the N-terminus and a C-terminal tail that binds other

myosin proteins to form thick filaments. Myosin uses ATP hydrolysis to direct itself towards the positive end of the actin (83, 97).

Kinesin and dynein are both microtubule motor proteins. While kinesin is similar in structure to myosin II, dynein is made of 2-3 heavy chains and a variable number of intermediate chains. Kinesin walks towards the positive end of microtubules and carries organelles and/or binds other microtubules. Dynein on the other hand, is a negative-end directed protein specialized in vesicle trafficking (83).

Cell movement: In animals, most of the cells move by crawling rather than using cilia or flagella to swim (83). Cell crawling depends on the actin-rich cortex located beneath the plasma membrane and occurs in cycles of 3 distinct steps: protrusion, attachment and traction

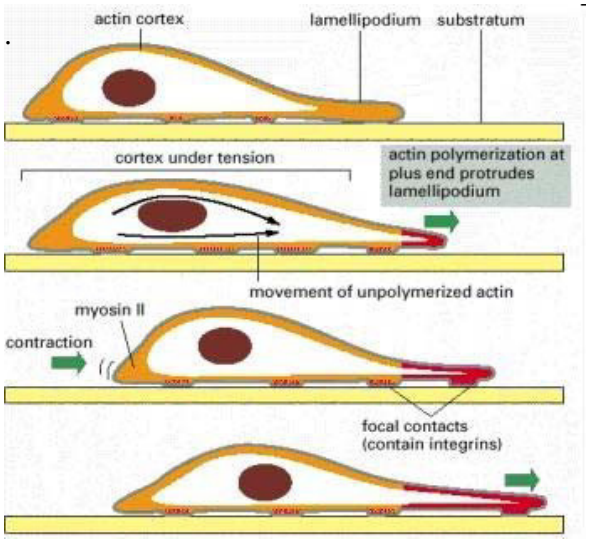

Figure 1. Cell crawling depends on the actin-rich cortex (red) andoccurs in cycles (from (83)).

The first step, protrusion occurs at the leading edge and relies on extensive actin polymerization to push the plasma membrane outward. Different cell types generate different protrusion structures such as filopodia in migrating cones, lamellepodia in epithelial cells and fibroblasts, and pseudopodia in amoeba and neutrophils (83).

During the crawling process, actin filaments remain stationary and motility is achieved through directional tread-milling, a result of collaboration between actin on one hand, and several actin-binding proteins on the other hand (83, 95, 97, 98). First, extensive actin nucleation is ensured through the Arp2/3 protein at the leading edge, while cofilin -just behind the leading edge- severs D-actin. In parallel actin capping proteins ensure uni-directionality of movement (83, 95). Following protrusion, cells adhere to new sites of attachment at cell front, and these attachments persist until the rear of the cell is pulled forward, in a process known as traction. Although still poorly understood, it is clear that the driving force of traction is the actin-myosin II interaction where myosin motor proteins contract and use the stationary actin cytoskeleton to exert traction (92, 98).

1.3.5.3 Cancer cells motility

Most of what is currently known about cell motility was generated in 2- dimentional environments. Recent advances have shown that 3-dimentional motility takes place using similar mechanisms to those described above. However, given the complexity of a 3-D environment, cells employ additional mechanisms, mainly the secretion of proteolytic enzymes to degrade the ECM (83, 87, 95), along with a higher degree of flexibility that

permits repeated adaptation of cell shape to environment changes (94).

Another concept observed in 3-D motility but not in 2-D, is the one of blebbing (83, 99, 100) which results from a hydrostatic gradient, caused by different levels of acto-myosin contraction between the front and the rear of the cell.

Cancer cells can transit between different migration strategies depending on the tumour microenvironment; the observed changes in the environment are underlined by molecular changes such as a decrease in E-cadherin (that usually acts at a cell-cell junction) during the epithelial-to-mesenchymal transition (EMT) or a decrease in Rac and an increase in Rho signalling during the mesenchymal-to-amoeboid transition (MAT) (85). The two mechanisms are not mutually exclusive and a rapid switch can occur between the two states depending on the ECM environment (85, 94). The table (Table 1) below shows the main differences between the two migration strategies, also represented in Figure 2 (83, 85, 94).

	Mesenchymal	Amoeboid
Cell shape	Elongated, spindle-like shape	Round shape
Protrusion	One or more leading Pseudopodia	Bleb-like protusion
Molecular control	Controlled by Rac and Cdc-42	Controlled by Rho-Rock pathway along with p-MLC
Cycles	Cycles of protrusion-adhesion-stress fiber formation-contraction/detachment and ECM-degrading enzymes secretion (MMP, cathepsins).	Cycles: expansion and contraction mediated by extensive myosin-actin contractions
Strategy	Path-creating: need proteases to degrade ECM	Path-finding: protease independent and receptor-mediated adhesion independent; cells squeeze through gaps in ECM

Table 1. Migration strategies in cancer cells.

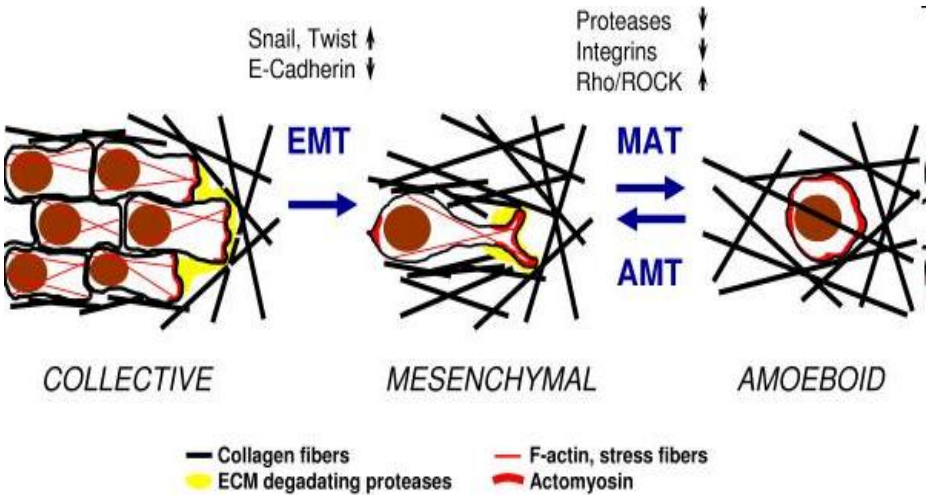

Figure 2. Cells can transition between 3 motility strategies depending on the surrounding environment. The transition between the strategies shows underlying molecular changes (adapted from (85)).

The Rac/Rho pathway: The Rac/Rho pathway lies at the heart of cell motility and most cell-surface receptors pathways converge towards it. Rac acts on several targets such as POR1 and WAVE, which are involved in actin re-organization, and LIM kinase, which promotes actin turn-over in order to promote outgrowth at the leading edge.

Rho on the other hand focuses on its associated kinase (ROCK) and promotes actin filament stability, phosphorylation of myosin, assembly of acto-myosin filaments and finally the formation of focal adhesion (88, 92, 96, 101).

Beside motility, Rho/Rac family members are involved in regulating cell proliferation/apoptosis, survival and cell polarity, and are over-expressed in several cancers (96, 102).

The AKT/PKB (protein kinase B) in motility: AKT is a crucial player in the PI3K

pathway which is linked to survival, growth, migration and invasion (103-106).

Its importance in promoting cancer was confirmed by demonstrating its hyper-activation in many aggressive pathways, its ability to increase tumorigenesis in mice and the fact that PTEN (negative regulator of AKT) is hypo-activated in several cancers (103, 107). However, little is known about AKT's role in cancer cell motility as different studies show conflicting outcomes (104, 106, 108-110). It has been proposed that distinct AKT isoforms may function in opposing manners depending on the isoform, the cell and the tissue type (103, 108) which might explain the different conclusions regarding the role of AKT in motility.

Recently, PDK1, an upstream, activator of AKT has been shown to be involved in motility (100).

1.3.5.4 Prostate cancer cell motility

The prostate cancer cells tumour environment is very complex, with secretions generated from the ECM, endothelial cells, smooth muscle cells, stromal cells and specialized epithelial cells (basal, exocrine and neuroendocrine cells) (93). This complex environment makes prostate cancer cells exposed to a variety of signals, some of which have been shown to play a role in motility.

Such signals include (reviewed in (93)): EGF (increases motility in DU145 prostate cells), the autocrine motility factor (AMF; associated with increased metastatic potential of PC-3M prostate cells), IGF (very enriched in bone and act as a chemoattractant), neuroendocrine secretions (bombesin increases migration of PC-3 cells (111, 112), Calcitonin increases LNCaP cells motility and PTH increases

motility of DU145 and PC-3 cells), stromal cells secretions, and proteases (PA: plasminogen activator associates with metastatic potential; uPA: urokine plasminogen activator and MMP9: matrix metalloproteinase 9).

Despite extensive literature elucidating the role of these signals in motility, the molecular mechanisms underlying prostate cancer in general, and motility in specific, are still poorly understood. Recent evidence points towards a role for EGFR upregulation in promoting androgen independence through the activation of MAPK, ERK and PI3/AKT pathways (105, 113). It has also been shown that DU145 prostate cancer cells proceed to motility through the EMT mechanism with a decrease in E-cadherin and an increase in Vimentin expression (113). Furthermore, the study showed that the EGFR-induced migration of DU145 was driven through AKT activation as inhibition of AKT using LY294002 suppressed the EFG-mediated cell migration as well as EMT. However one should note that the LY294002 is a PI3-K inhibitor and is not specific to AKT. The effect of this inhibition is thus upstream of AKT.

1.3.6 Molecular basis of metastasis and clinical implications

The advances in molecular genetics have allowed scientists to explore the mutations linked to cancer and metastasis, and thus the model of tumour progression is constantly being remodeled. Traditionally, metastasis has been explained by the idea that somatic mutations accumulate sequentially and result in rare cells capable of invading and colonizing other sites (reviewed in (84)). However more recent models see metastasis in terms of a dynamic heterogeneity and clonal selection principle. In other words, some unstable metastatic variants are already present in the population of cells, and will

eventually prevail under proper conditions to cause metastasis (114, 115). The use of gene expression microarrays and the discovery of gene signatures in primary tumours have allowed scientists to investigate novel ways to target genes that might be related to metastasis at an early stage (84, 115).

A clear understanding of the molecular basis of metastasis has important clinical implications such as the identification of molecular prognostic factors that allow the prediction of the course and the outcome of the disease; targeting those specific genes can also be used as a new treatment modality. Currently, animal models are used along with functional validation approaches. For example, c-Met, the metastatic initiation gene is being targeted using ARQ 197, a small inhibitor molecule, in phase II clinical trials. Other examples include the RANK ligand inhibitor (Denosumab; clinical trial phase III) and the TGFβ inhibitor (monoclonal antibody; clinical trial phase I), which are two metastatic virulence genes (reviewed in (84)).

SUMMARY: metastasis is the deadliest aspect of human cancer, and motility is a critical component of it. Several pathways are involved in cancer cells motility, including the AKT pathway. PCa shows complex interactions between the tumour and its environment that act as a source of several signals shown to be involved in motility. Understanding the molecular basis of prostate cancer cells motility is important to identify molecular prognostic factors on one hand, and novel targets for therapy on the other hand.

1.4 Molecular biology of PCa

1.4.1 Genomic, epigenetic changes and oncogenes

1.4.1.1 Oncogenes

An oncogene is a gene whose expression can confer malignancy on a cell in a dominant fashion, and arises from a genetic damage to a normal proto-oncogene, thus resulting in a gain of function (116). These damages could result from chromosomal translocations or rearrangements (117, 118), DNA amplification (116), deletion of a regulatory sequence or mutations. Thus, an oncogene in the classical term is associated with the initiation of the disease.

KRAS gene, which encodes the Ras protein (119) is a proto-oncogene that is mutated in a wide spectrum of cancers (120), but rarely in primary PCa (116, 121). It has been suggested that, given the infrequency of Ras oncogene activation, it could be a late event in PCa progression (116). Indeed, a recent study (122) unveiled a novel mechanism of Ras activation detected in a rare subset of metastatic PCa patients.

MYC is another oncogene that was speculated to play a role in PCa progression given that its expression was highest in PCa samples compared to benign prostatic hyperplasia or normal prostate samples (123). Another study on PCa patients showed that those with elevated Myc protein expression were derived from tumours with Gleason scores of 5 or higher (124), while another one found that Myc levels were elevated in all PCa specimens regardless of the Gleason grade (125). Myc up-regulation could be the result of defective gene regulation (116, 123, 124) as well as gene amplification (126) of chromosome 8q24. This amplification and the resulting overexpression were shown to predominate in lymph

node metastasis samples compared to unmatched primary PCa samples (126).

FAK is another candidate oncogene, also located at the amplified 8q24 region (126). Focal-Adhesion Kinase (FAK) plays an important role in mediating signalling from growth factor receptors to downstream targets (127). Interestingly, a study has pointed to a role for FAK phosphorylation at tyrosine (Y) 801 in transforming Ras in fibroblasts (127) which might explain the advantage of the amplification at 8q24 in lymph node metastasis samples described above.

Overall, and unlike other cancers, it seems that the classical oncogenes might be a common feature of a late phase of progression rather than an initiation phase (116). This is also confirmed by the fact that PCa cell lines- PC3, LNCaP and DU145 –which are all derived from metastases- have their oncogenes activated (128, 129).

1.4.1.2 Epigenetic changes

Epigenetic changes are heritable changes in gene function that are not due to changes in DNA sequence (130). In human PCa epigenetic changes arise at the earliest steps of transformation and persist through invasion and metastasis (131). Epigenetic changes of PCa progression can be linked to two widely accepted mechanisms (130, 132): DNA methylation and histone tail acetylation.

a- Changing patterns of DNA methylation

Patterns of DNA methylation depend on the relative activities of DNA methyltransferase (DMT) and DNA demethylase (DDM) (130). DMT recognizes short dinucleotide sequences known as CpG islands, located at the promoter region (133).

Methylation interferes with DNA repair, recombination, replication and most importantly transcription (reviewed in (130)). Hypomethylation (decreased methylation) leads to the activation of previously silenced genes and is seen more in advanced cancers (134). Hypermethylation (increased methylation) on the other hand is linked to transcriptional silencing and occurs at specific regulatory sites such as promoters of tumour suppressor genes (130).

In PCa, DNA hypermethylation plays an important role in DNA damage repair (130): hypermethylation of the pi-class glutathione S-transferase gene (GSTP1) is the most common alterations in human PCa (135) and occurs at early stages of the disease. Hypermethylation of E-Cadhetin promoter results in decreased E- cadherin expression, linked to poorly differentiated cancers (130). This alteration is also involved in the control of hormonal response genes such as the androgen receptor (AR) promoter methylation, which is reported to be more prevalent in castration-resistant prostate cancer (CRPC) compared to primary cancer (136-139). Cell cycle genes are also regulated by methylation, and hypermethylation of CDKN2, an inhibitor of CDK, inactivates it (130). Furthermore, hypermethylation of methylguanine DNA methylatransferase (MGMT) was shown to induce silencing of the repair mechanism. Hypermethylation of known tumour suppressor genes such as RB1, MLH1 and von-Hippel-Lindau gene is rare in PCa (132).

b- Histone tail acetylation/deacetylation

Acetylation/deacetylation of histones acts as a molecular communication link between chromatin and signal transduction pathways (140); it is a reversible, yet heritable process that preserves states of activity and inactivity of some genes (130). Acetylation

facilitates transcription by allowing access of transcription factors to DNA by displacing histones (141) resulting in a relaxed chromatin structure.

In PCa, IGF binding proteins (IGFBPs) regulate the levels of IGF, and their level of expression was linked to PCa progression, with increased IGFBP 2, 3 and 5 linked to increased Gleason score and aggressiveness (130, 142). It was shown that increased levels of IGFBP2 expression occurs through histone acetylation (143). Activation of CDK inhibitors p21 is linked to the promotion of cell growth in CRPC (144), and was shown to occur via hyper-acetylation of histone H4 (145). Finally, AR interacts with co-activators to stimulate gene expression, and some co-activators such as SRC1 have an intrinsic histone acetylatransferase activity that acts on H3 and H4 to facilitate AR transcriptional activity (reviewed in (130)). Co-repressors such as Rb recruit a de-acetylase thus causing the opposite effect (130).

c- Clinical significance

Changes in methylation are a good source of cancer biomarkers (132): they can be detected using PCR, and the acquired DNA methylation is very well reported in every human cancer. Furthermore, DNA methylation appears likely to discriminate aggressive vs. non-aggressive disease (reviewed in (130)). Both DNA methylation and histone acetylation appear consistently in all cancer cases early, and can be potentially reversible. Thus, from a clinical perspective, modulation of DMT/DDM and histone acetylation state seem a promising target, and drugs have been developed for that purpose (Vidaza, Zebularine as DMT inhibitors, and Zolinza, Valproic acid as histone deacetylase inhibitor) (132).

1.4.1.3 Gene expression in PCa

In order to determine the molecular pathways underlying PCa progression, microarrays have been used to examine levels of gene expression across primary and metastatic PCa samples. Several studies (146-151) have examined the differential gene expression between primary PCa samples on one hand and metastatic samples on the other hand as a strategy to determine the genes that might play a role in PCa progression as well as expression patterns that might be associated with certain clinical behaviours. In 2002, a microarray study showed that some genes were differentially expressed in metastatic samples compared to primary ones and those were involved in cell cycle regulation, DNA replication and DNA repair mechanisms, as well as other functions such as transcriptional regulation, signaling, cell structure and motility (148). These functions are well known to characterize metastasis and a better functional understanding of their role might reveal some therapeutic targets. More recently in 2011, a study compared primary PCa samples on the basis of the Gleason grade and showed that high grade tumours were more enriched in androgen receptor pathway genes, as well as growth factor and cytokines (151) while another study looked specifically at bone metastasis specimens from autopsies and showed that 664 genes were unique to bone metastasis samples and included genes involved in bone morphogenesis (BMPs) (149). In 2004, Lapointe and colleagues (147) performed a genome-wide gene expression microarray on a set of 64 primary PCa specimens and 9 unmatched lymph node metastasis samples. The study identified 3 subgroups of prostate tumours that exhibited distinct patterns of gene expression, and interestingly, these subtypes correlated with clinical behaviour: tumours from subtype 1 were the least clinically aggressive ones while those in subtype

2 and 3 were clinically aggressive. The metastasis samples shared similar patterns of gene expression with the clinically aggressive tumours of subtypes 2 and 3. Further analysis of these data in our laboratory (unpublished) using Gene Set Enrichment Analysis (GSEA) found enrichment in genes related to the PhosphatidylInositol (PI) cell survival and growth pathway in metastasis compared to primary tumours. GSEA is a computational technique that compares a certain set of genes between two different biological states (152). These findings point to a potential contribution of the PI pathway genes to the metastatic process.

One should also note that PCa gene expression profiling has allowed the discovery of the TMPRSS2 (transmembrane protease serine 2) fusion with members of the ETS family (E26 transforming sequence), the first gene fusion discovered in PCa (153). This fusion appears to be a genetic trigger for the PIN-to- adenocarcinoma transition while not being detected in benign prostate samples (118, 154, 155). The fusion gene is also detected in more aggressive CRPC metastatic PCa via qPCR and FISH validation. *In vitro*, introducing ERG (member of the ETS family) fusion into immortalized benign prostate epithelial cells induced invasion without increasing proliferation (117). However the prognostic and functional role of the fusion in metastatic PCa is still under investigation.

1.4.1.4 DNA copy number alterations (CNA) in PCa

Copy number alterations in PCa consist of chromosomal gains via genomic amplification of oncogenes and chromosomal loss via deletion of tumour suppressor genes.

Early studies using conventional Comparative Genomic Hybridization (CGH) have reported common alterations in prostate cancer specimen, and these include losses at 8p, 13q,1p, 22, 19, 10q and 16q and gains at 8q, 7q, Xq and 18q ((156) and reviewed in (157)). Furthermore, genomic losses were reported to be more common than chromosomal gains in primary PCa (reviewed in (158)).

More recently, array Comparative Genomic Hybridization (a-CGH) has allowed the detection and the mapping of alterations in higher resolution. The study of Lapointe and colleagues used (126) high resolution gene microarray-based Comparative Genomic Hybridization (aCGH), and described specific losses linked to patients subtypes, such as 5q21, 6q15 and 8p21, and other losses common to all the patients, such as 13q14. The study also revealed that primary PCa had more frequent losses than gains, similar to previous findings.

The loss of *NKX3.1* gene at 8p21 chromosome is the most common event in early carcinogenesis (157, 158). *NKX3.1* is involved in regulating cell differentiation and proliferation (157), and its loss was detected by Fluorescent *in situ* hybridization (FISH), aCGH (126) and allelic imbalance analysis (reviewed in (158)). *In vivo*, targeting *NKX3.1* in mice leads to defects in prostate ductal morphogenesis, and *NKX3.1* mutant mice developed PIN lesions early on, without progressing to cancer (159). The loss of *PTEN* gene at 10q23 chromosome is a frequent event as well, but occurs more in carcinoma than in PIN lesions (158). *PTEN* is inactivated by mutation in PC-3 and LNCaP cell lines (160). On a functional level, PTEN is a negative regulator of AKT and its loss results in AKT activation, thus reduction of cell death and aberrant proliferation. Loss of 10q was reported to be frequent in

carcinoma and was detected by several independent strategies such as LOH (161, 162), FISH (160, 163) and CGH (126, 162), while low PTEN levels were detected by IHC (161, 164-170).

Clinically, loss of heterozygosity at 10q23 was found to associate with cancer progression with increasing frequency correlating with stage and tumour grade (157). In an aCGH study performed in 2007, Lapointe et al showed that loss at 10q23 was a very frequent event in patients with lymph nodes metastasis (126), again pointing to the importance of PTEN as a tumour suppressor gene in prostate cancer.

The loss of 13q4 (*RB1*) occurs in around 50% of prostate tumours, and re- introduction Rb into Rb (-) prostate cell lines inhibits tumourigenesis. Unlike *NKX3.1*, the losses of *PTEN* and *RB1* are more common events in clinically localized PCa, and more advanced PCa (157, 169, 171). In the study by Lapointe et al 2007, the loss of 13q4 was detected in primary PCa samples that exhibited different clinical behaviour as well as in the lymph node metastasis samples (126).

1.4.1.5 CNA in advanced/metastatic PCa

Compared to primary PCa, where chromosomal losses seem to be a more common genetic change (157, 158), samples of local recurrence show significantly more gains than in primary samples, with gains mostly at 8q, X and 7 (172, 173).
This difference in the patterns of CNA between primary and more advanced/metastatic PCa suggests that the progression of PCa, on one hand, and the development of aggressive/metastatic state, on the other hand, may have different genetic basis. The study by Lapointe et al, 2007, identified CNAs in the same set of patients used for the gene expression

study described above (126). The study revealed that, compared to the primary tumours, the LN mets samples had a higher frequency of alterations and were associated with more frequent gains at 8q24 and 16p13 as well as losses at 10q23 (*PTEN*), 13q24 (*RB1*), 17p13 (*TP53*) and 16q23. These alterations were present in the unmatched primary PCa samples with the exception of the 16p13 genomic amplification. Five of the 9 patients with LN mets had concurrent gains at 8q24 and 16p13. Table 2 summarizes the common alterations that were detected in the set of 9 lymph node metastasis samples, along with the AR mRNA levels of expression.

The 8q24 region contains the genes *MYC* and *PTK2* (encodes FAK) and was shown to be associated with PCa progression (172). The 10q23 (*PTEN*) loss was reported as a predictor of PCa recurrence and metastases (169) and the 16q23 contains two candidate tumour suppressor genes *ADAMTS18* (174) and *WWOX* (175). The 16p chromosomal amplification has been reported in breast, prostate and lung cancer (176-179), but the 16p13 amplicon has not yet been characterized. Five of the 9 patients with LN mets all shared a common minimal region of gain (2.9 MB) which contains *PDPK1* (along with 48 other genes), meaning that the gene was amplified across the 5 samples. *PDPK1* encodes the phosphoinositide-dependent kinase-1 (PDK1), an upstream regulator of the PI/AKT survival pathway which, as stated earlier was found to be enriched in the metastatic samples compared to the primary ones. Compared to other genes, *PDPK1* genomic gain correlated the best with mRNA levels.

Gene loci	LN mets								
	PL116	**PL194**	**PL129**	**PL27**	**PL133**	**PL118**	**PL114**	**PL115**	**PL122**
16p13.3 (*PDPK1*)	amp	amp	amp	amp	-	-	-	-	amp
10q23 (*PTEN*)	del	-	-	del	del	del	-	del	del
8q24 (FAK, MYC)	amp	amp	amp	amp	-	-	-	-	amp
16q23	del	del	del	del	-	-	-	-	del
AR mRNA status	++	++	+	-	-	-	++	ND	-

Table 2. Genomic alterations of PCa lymph nodes metastases (Unpublished data from (126) and (147)). Amplifications (amp) and deletions (del) of loci of interest in LN Mets from different patients. Minus (-) symbol represents the absence of genomic alterations. Lower portion shows AR mRNA expression level in each LN Mets: high (++), medium (+) or non-detectable (ND).

SUMMARY: detection of genomic alterations specific to metastatic PCa can serve as a marker for this aggressive disease. Gene expression data has showed the genes of PI/AKT pathway to be up-regulated in metastatic PCa compared to primary samples. Array-CGH data has reported the loss at 10q23 that maps to the *PTEN* gene and the gain at 16p13 that maps to *PDPK1.* PTEN and PDK1 have antagonist roles in the regulation of the PI/AKT pathway and their respective loss and gain, along with findings from gene expression data points to an important role for this pathway in PCa metastasis.

1.4.2 The PI/AKT pathway

1.4.2.1 Overview

PI3Ks (phosphoinositide 3-kinases) phosphorylate membrane inositol phospholipids substrate and are implicated in cell proliferation, growth, survival, metabolism, migration and membrane trafficking (180).

PI3Ks are activated by receptor tyrosine kinases (RTK) upon binding of growth factors such as PDGF, insulin and EGT to their receptors, causing them to dimerize and cross-phosphorylate (181, 182). Activated RTK recruits PI3K heterodimers to the plasma membrane, where they phosphorylate PIP2 into PIP3 (183). PIP3 recruits and activates several PH domain-containing proteins and this includes i) GEFs (Guanine exchange factors) for activation of Rac/Rho involved in actin cytoskeleton and migration (184), ii) PDK1 and AKT (also known as protein kinase B; PKB) which results in activation of AKT involved in proliferation and survival (185) and finally iii) the TSC2 (tuberin) and mTOR (mammalian target of rapamycin) resulting in activation of mTOR that promotes translation and thus cell growth (186).

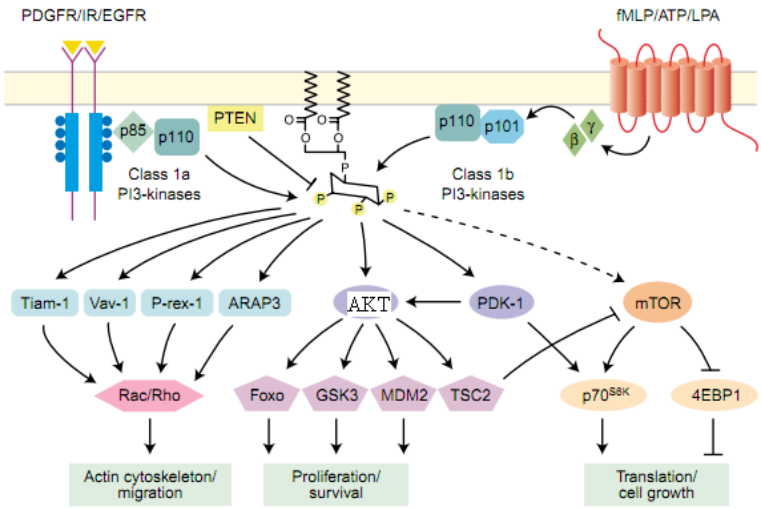

Figure 3. The PI3K signalling pathway: upon its activation following activation of receptor tyrosine kinases (RTK), the PI3K phosphorylates and activates several targets involved in cytoskeleton regulation and motility, proliferation and survival, as well as translation and cell growth (Adapted from (181)).

1.4.2.2 AKT pathway and cellular functions

Activation of AKT induces cellular proliferation by inhibiting GSK3β, thus preventing degradation of cyclin D1(187, 188). Furthermore, AKT inhibits cell cycle inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ (189).

AKT also promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of pro-apoptotic BAD (190), and inhibits the caspase cascade by phosphorylating and stabilizing PED/PEA 15, an inhibitor of caspase-3 (191). Additionally, AKT phosphorylates FOXO3.1 and promotes its cytoplasmic retention, thus preventing its transcriptional activity (192).

In terms of metabolism, AKT phosphorylates and mediates membrane translocation of

Glucose transporter GLUT1 and GLUT4 (193) and stimulates glycolysis via phospho-Fructokinase 2 (PFK2) (189). Cell growth is controlled by AKT via regulation of mTOR that restricts cell cycle progression when growth conditions are less than ideal (194); it also phosphorylates TSC2 (tuberin) and disrupts its interaction with TSC1 (hamartin) (187), leading to increased mTOR activation. Finally, AKT was shown to have oncogenic functions, with sustained angiogenesis, unlimited replicative potential and tissue invasion and metastasis (reviewed in (195)).

1.4.2.3 AKT alteration in human cancers

Amplification (at chromosome 19q23) and overexpression of AKT 2 (or AKT-β) was first reported in ovarian cancer, then in breast and pancreatic cancer (196). AKT-1 gene amplification has also been observed in PCa (197, 198), and AKT1 (or AKT α) protein levels have been reported to be elevated in breast, ovarian and prostate cancer where AKT 1 rather than AKT 2 seems to play an important role (187, 199, 200).

AKT is activated in a broad range of human malignancies including carcinomas (cancer of epithelial origin), glioblastomas multiform and haematological malignancies (187). Increased AKT 1 kinase activity was reported in around 40% of breast and ovarian cancer, and more than 50% in prostate cancer. Activation of AKT2 was observed in 30-40% of ovarian and pancreatic cancers (187) while AKT3 activity was detected in estrogen receptor-negative breast cancer and CRPC cell lines, pointing to its potential role in increasing aggressiveness (198).

However, other components of the pathway present with alterations in human cancers (gastric, ovarian, colorectal, glioblastomas, breast and lungs) such as

amplification of PIK3CA (163, 201), PIK3CA somatic mutations (202) or such as loss of PTEN, a negative regulator of AKT (203, 204).

1.4.2.4 AKT in PCa

In PCa, AKT is activated via PDK1-mediated phosphorylation at threonine 308 residue, and inhibited by PTEN (187). Although AKT1 seems to be highly expressed in PCa, it is still unclear what the role of other AKT isoforms (AKT 2 and 3) is, and their contribution to carcinogenesis (187). Recently, AKT was shown to be activated independently of PI3K/PDK1 only when caveolin (cav-1) was overexpressed (205). Cav-1 seemed to mediate AKT activation via inhibition of serine/threonine protein phosphatses PP1 and PP2 which regulate AKT (205, 206).

Elevated AKT 1 expression and kinase activities in PCa were shown to associat with hormone-resistant phenotypes, and poor prognosis (197-199). Amplification of PIK3CA was also reported in PCa (197) and associated with functional loss of PTEN in advanced prostate cancer (203, 207-209). AKT in metastatic PCa was shown to be activated as well (210, 211).

1.5 PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome10) is a tumour suppressor gene found to be mutated in brain, breast and prostate tumours (160). The gene maps to the 10q23 loci while the protein is a lipid phosphatase that removes the phosphate from the D3 position of phosphotidylinositol 3,4,5-triphosphate (PIP3) (212), thus antagonizing the effect of the AKT pathway.

PTEN deletion was shown to activate the AKT survival pathway and increase tumourigenesis in prostatic epithelium, as well as play a role in mammalian cell migration where selective PTEN inactivation in the brain caused defects in neuronal migration (213).

1.5.1 PTEN deletion in prostate cancer

In 2006, a study performed on 40 localized prostate cancer revealed that 21% of the samples showed a homozygous PTEN deletion while 19% of samples showed a heterozygous deletion, using fluorescent in situ hybridization (FISH), and results were concordant with protein levels using immunohistochemistry (IHC) (162). However, previous results had also pointed to the existence of PTEN deletion in PIN samples, seen as a pre-neoplastic cell transformation (157, 158). The same year, another study using the same tool assessed the PTEN status in benign prostatic epithelium (n=6), low grade PIN (n=12), high grade PIN (n=13) and primary prostate cancer (n=35). The study reported no deletion in benign and low grade PIN, 23% deletion in HGPIN, and 68% deletion in PCa samples; results were in concordance with low levels of PTEN measured by IHC as well (170). Interestingly, the study found no correlation between PTEN deletion and tumour stage, Gleason Grade and pre-operative PSA levels. The reason of discrepancy could have been the sample size, the method of detection used and whether the tumour was naïve or not to treatment by hormones. Thus, the same group conducted another study on a group of 107 samples obtained by radical prostatectomy, with 10 benign prostate for control. Results showed a PTEN deletion in 44% of cases, with most of the cases (39%) harbouring heterozygous deletion (169). Although deletion associated with an earlier recurrence, no correlation with other clinical-

pathological features (PSA, tumour volume, Grade and stage) was detected (169). A recent study by McCall et.al, 2008 (165) investigated PTEN protein expression and clinical outcome, and reported low levels of cytoplasmic PTEN to independently associate with shorter time to relapse in a set of 68 hormone-sensitive patients.

1.5.2 PTEN expression and Gleason grade:

As described above, the findings of Yoshimoto et.al (169, 170), did not point towards an association between PTEN deletion (detected by FISH) and Gleason grade, in contrast to other studies (165, 166).While the sample size does not seem to be a variable (since a sample of 35 primary tumours and 107 tumours, using FISH gave similar results), other studies using a different method of detection did find a correlation between PTEN deletion and Gleason grade.

A study done on 109 primary prostate cancer specimens embedded in paraffin assessed the protein levels of PTEN expression using IHC, and used DU145 and PC-3 cell lines as positive and negative controls respectively. The study reports 20% of the cases to be completely negative and 64.2% to be mixed between positive and negative staining (166). Clinical follow-up was available for 69 patients and analysis showed that complete loss of PTEN correlated with high stage and increasing grade. The main difference between the study and previous ones (Yoshimoto et.al), pertains to the technique: in fact, while IHC as a technique has the ability to reflect non-genomic mechanisms associated with PTEN inactivation (such as hypermethylation), it fails to clearly point to a genomic deletion at 10q23 on one hand, and cannot distinguish homozygous deletions from heterozygous ones on the

other hand. Thus, the samples with completely negative staining could reflect a homozygous deletion, an epigenetic silencing or a combination of deletion and silencing. In fact, the detection rate of completely negative PTEN staining by IHC (20%) is much higher than what was reported for homozygous deletion by FISH (6% in Yoshimoto et.al) pointing to the fact that the cases with complete negative staining could represent extreme cases where PTEN deletion and silencing are combined; and this would explain the correlation between such cases and Gleason score.

Another study assessed PTEN level of expression in 130 untreated patients who had undergone radical prostatectomy to remove local tumours. IHC was performed on 118 of the sample, while a subset of 86 patients had fresh tissue available for immunoblotting as well. Results showed that 39/118 (33.1%) of patients had absent to weak PTEN immuno-histochemical staining, while 76/86 (88%) of patients showed a lower PTEN expression on western blot compared to tumour-free tissue (214). In both cases of protein detection, low PTEN expression showed a significant association with high Gleason grade.

1.5.3 PTEN and androgen receptors (AR)

Previous studies have hinted to a role for PTEN in modulating AR transcriptional activity, and thus may play a role in progression towards androgen-independent PCa. In fact, inhibiting the PI3K signalling by using LY294002 or by inducing PTEN in LNCaP cell line (PTEN negative) prevented known effects of AR (measured in terms of proliferation and PSA levels) compared to controls transfected with an empty

vector and stimulated by androgens (215). Another study used the Shionogi mouse model that is initially androgen-dependent and mimics the pattern of regression of androgen-independent PCa following hormone therapy. The study reports that inhibition of PTEN (using anti-sense oligonucleotides) in Shionogi mammary mice reduced the androgen-withdrawal induced regression and accelerated the androgen independence progression when compared to control mice (164). In the same study, analysis of PTEN levels of expression in human benign prostate samples and hormone-naïve and androgen- independent prostate cancer samples showed that patients who developed androgen-independence had a significantly reduced PTEN expression levels compared to the benign samples on one hand, and the hormone-naïve patients on the other hand (164).

This was followed by a study that hypothesized that the restoration of PTEN expression in a PTEN -/- androgen independent PCa cell line alters its growth sensitivity to androgens. Thus the C4-2 cell line- a PTEN null cell line derived from LNCaP for its ability to form tumours in castrated male mice- was used and PTEN was re-expressed under inducible control of doxycycline treatment. That resulted in nearly 80% inhibition of cell growth in vitro (216). Furthermore, when PTEN was expressed with Casodex treatment (an anti-androgen) growth was inhibited, and cells became more susceptible to the effects of the anti-androgen. This further pointed to an additive effect of PTEN on anti-androgen treatment. More interestingly, PTEN and Casodex were shown to decrease the phosphorylation of p-AKT, the activator of the AKT survival pathway, but not Casodex alone. This has pointed to the fact that PTEN and anti-androgens act on two different pathways. The importance of the study comes from the fact that two explanations were possible regarding the PTEN-AR relationship: first, that the AKT and the AR pathways

are parallel, and are both needed for full activation of C4-2 cell growth, and that AKT can be modulating AR signalling directly (216). More recently, a study examined a sample of patients who had been treated with hormones and had their tissue samples collected pre and post- relapse. Both FISH and IHC were used in the study and showed that while 23% of hormone-sensitive tumours had PTEN deleted, 52% of their matched refractory tumours harboured the deletion (165). Furthermore, low cytoplasmic PTEN in hormone-sensitive tumours associated with earlier relapse when compared to high cytoplasmic PTEN tumours. Although the study did not directly examine the relation between AR and PTEN, it showed that PTEN is indeed involved in the transition to androgen-independent PCa. In 2008, a study directly addressed the co-expression of PTEN and AR measured by IHC on a sample of 10 benign tissue, 20 androgen dependent PCa and 17 androgen independent PCa. However, the major difference with the previous study is that assessment was done on samples collected before treatment, as the study was retrospective: the androgen dependent/independent status was determined later by looking at clinical follow-up. Results of the study showed that low nuclear AR and low cytoplasmic PTEN levels are co-expressed in androgen independent PCa (167). Furthermore, androgen-dependent PCa patients who showed the co-expression had a shorter relapse-free survival while androgen- independent PCa patients had a reduced survival. While the study underlines the involvement of low PTEN levels in androgen independent cancer, the low levels of AR still remain to be clarified. In fact, if normal levels of PTEN were to prevent androgen-independent PCa by modulating sensitivity to androgens, cells with deleted PTEN should be more sensitive to androgens as the literature suggests, and thus have higher levels of AR. However, other studies have

outlined the existence of alternative pathways that do not require AR for activation of downstream AR targets (217). The implications of such studies are that presence/absence of an AR nuclear staining might not be reflective of whether or not a tumour has developed resistance to hormone therapy.

Finally, a recent study was done on a sample of 59 hormone-refractory non- metastatic PCa patients and assessed AR and PTEN expression. Results showed that 78% of the samples showed high AR levels which could mean that tumours have found other ways to activate AR without using androgens (168).

Furthermore, PTEN loss correlated with high levels of AR, which is consistent with literature that proposes PTEN as a negative regulator of AR expression (164, 215-217) but not with the study of 2008 (167). In our laboratory, analysis of data from (126) and (147) revealed that out of 9 patients with LN mets, 6 had the PTEN deletion and 5 of these 6 patients had non-detectable to low levels of AR mRNA, thus pointing towards a possible association between PTEN deletion and low levels of AR expression in advanced disease, similar to (167) (Table 2).

1.5.4 PTEN mutations

Earlier, before the investigation of 10q23 loss, PTEN mutations were known to be frequently detected in patients with autosomal dominant disorders: Cowden's disease, Lhermitte-Duclos disease and Bannanyan-Zonana syndrome (160, 213).

A review by Chow et. al, 2006 suggests that like other tumor suppressor genes, PTEN germ line mutation of one of the allele might result in hereditary cancer

predisposition (213) and might thus be subject to the Knudson's two-hit hypothesis (218). Mutations of PTEN are not localized to particular region of the gene and include missense mutations, nonsense mutation, insertions/deletions and splice-site mutation (reviewed in (213)).

In cancer, PTEN is frequently inactivated by somatic mutation in a wide array of human tumours, where it appears to be a late event in glioblastoma, melanoma and prostate cancer and rare in breast and renal cancer (reviewed in (219-221)). Given the role of PTEN as a regulator of the AKT survival pathway, mutations in PTEN clearly underlie the importance of this gene in cancer. This being said, it is thus important to keep in mind the potential existence of such mutations in patients when conducting genomic investigations or analyzing results such as those presented earlier, either by FISH or IHC.

1.5.5 PTEN mouse-model

The study of PTEN in human PCa has also made use of the mouse model. In fact the murine PTEN prostate cancer model described in Wang et.al, 2003 showed that the invasive PTEN null PCa cells in the mouse respond to androgen ablation initially but are later capable of proliferating even in the absence of androgen (211). Furthermore, PTEN null tumours witnessed an increase in p-AKT levels and a decrease in latency of PIN formation: enlarged prostate glands were observed by 6 weeks compared to 9 months in heterozygous PTEN +/-, and invasive carcinoma was observed by 9 weeks (211).

Another study addressed the effect of PTEN doses on cancer progression and using mice with decreasing PTEN activity, found that as PTEN levels decreased there was a progressive increase in cell proliferation and activation of the AKT pathway (222). Finally, a study also approached the role of PDK1- an up- regulator of the AKT survival pathway- and built a model with a hypomorphic PDK1 in PTEN+/- mice. Results showed that compared to PTEN +/- mice alone (larger externally visible tumours after 9 months with 72% having tumours: lymphoma, endometrial carcinoma, prostate and testicular carcinoma), the $PDK1^{hyp}$; PTEN +/- mice had no externally visible tumours even after 14 months and specifically no endometrial or testicular cancers (223).

SUMMARY: PTEN is a negative activator of the AKT survival pathway, and has been shown to have tumour suppressor activity both *in vitro* and *in vivo*. It has also been suggested that PTEN plays a role in the regulation of androgen receptors transcriptional activity and might thus mediate transition to androgen-independent PCa. Deletions at 10q23 (*PTEN*) were reported to be frequent in PCa, and were detected in lymph node metastasis samples as well. These findings further support the need to investigate the role of the AKT survival pathway in mediating PCa progression towards an aggressive state.

1.6 PDK1

1.6.1 Discovery

The discovery of PDK1 was first published in 1997 by Alessi et al (224), making it a relatively recent protein under investigation.

PDK1 discovery was part of effort aimed at elucidating the insulin transduction pathway (225) and mainly the role of AKT in this pathway. AKT was found to be activated upon its phosphorylation at two residues: the threonine 308 and the serine 473 residues (226). This phosphorylation and the resulting activation seem to depend on the PI3-K, as inhibition of this kinase by wortamannin abolished it. However, the kinase or potential kinases, were yet unknown. Using Glutathione S-transferase-AKT-α (GST-AKT-α) from unstimulated embryonic kidney 293 cells, and a QAE-sephadex column, PDK1 was first isolated from rabbit skeletal muscle extracts as a 67 KDa protein that became phosphorylated upon addition of MG-ATP (224, 225). This 67 KDa protein was able to phosphorylate AKT-α in presence of phosphatidylinositol-3,4,5-triphosphate (PIP3) only (224, 225). In fact, removing the effect of PIP3 (by omitting it, by triton-X100 or by incubating it at 55° C) abolished the activation and phosphorylation of GST-AKT-α (224). The protein was thus termed phosphoinositide-dependent kinase-1 (PDK-1). Subsequent studies further elucidated the mechanism of function of PDK1. An AKT-α mutant that was made unable to interact with PIP3 could not be phosphorylated by PDK1, and co-localization studies revealed that the interaction with PDK1 and AKT with PIP3 plays a crucial role in AKT recruitment to the plasma membrane, and its

phosphorylation by PDK1 (227).

1.6.2 PDK1 phosphorylation of AKT

AKT phosphorylation at both Thr308 and Ser473 is essential for maximal activity (226). However, the use of radiolabelled phosphate (^{32}P) showed that a ^{32}P- labelled GST-AKT-α (phosphorylated upon PDK1 addition) had one ^{32}P at the Thr308 residue, and none at the Ser473 residue (224). This meant that *in vitro*, PDK1 phosphorylated AKT at Thr308 only.

While the kinase that phosphorylates AKT at Ser473 remains elusive, work in mouse embryonic stem cells (ES) that were made PDK1$^{-/-}$ showed that while AKT was not activated, the Ser473 residue was still phosphorylated, but not the Thr308. Furthermore, PI3-K inhibition (by LY294002 and wortamannin) inhibited this phosphorylation at Ser473 (228). This means that a kinase(s) other than PDK1, but still PI3-K-dependent, is still to be characterized. Recently, it was suggested that Ser473 residue could be phosphorylated by PDK2, a possible modified version of PDK1(229) . A homologue of PDK1, structurally and functionnaly was characterized in *Drosophila melanogaster* flies (230): DSTPK61 kinase was found to share high sequence homology with PDK1 and is involved in regulation of sex differentiation, oogenesis and spermatogenesis in flies. Interestingly, and similar to PDK1, DSTPK61 also phosphorylates a DPKB (drosophila PKB), the homologue of the mammalian AKT. DPKB plays a role in regulating cell growth, with loss of function resulting in smaller flies.

1.6.3 Structure and cellular signalling

1.6.3.1 Structure

PDK1 protein is encoded by the *PDPK1* gene, located at 16p13.3 chromosome. Upon initial isolation, it was determined to be a 556 amino acids protein with a kinase domain at the N-terminus, and a Pleckstrin Homology (PH) domain at the C-terminus (224) needed for its interaction with PIP3. The nuclear export sequence (NES) was later discovered (231) and is needed to export PDK1 from nucleus (Fig4).

In vitro experiments showed that PDK1 has the intrinsic ability to phosphorylate its own T-loop at the Serine241 residue, thus remaining fully constitutively active (232). In fact, PDK1 kept a high catalytic activity when immuno-precipitated from either growth factor stimulated or un-stimulated cells (230, 232). Furthermore, PDK1 expression in bacteria showed it remained stochiometrically phosphorylated at Ser241 (232).

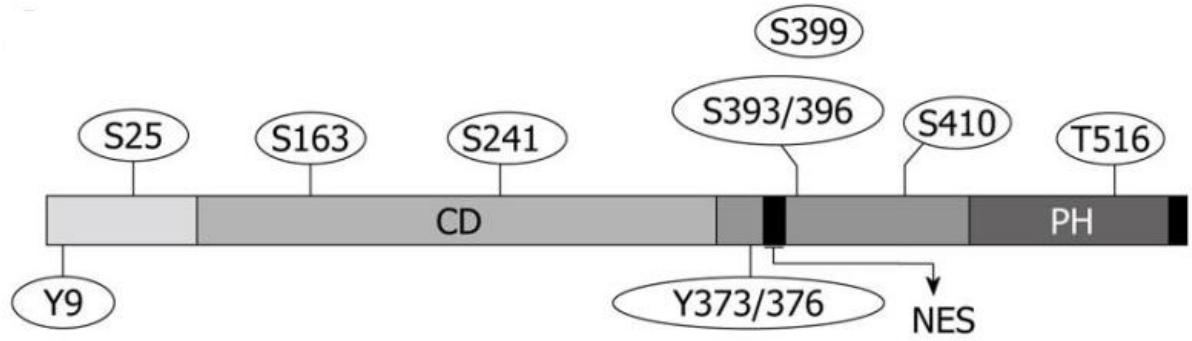

Figure 4. Structure of PDK1 revealing the C-terminus Pleckstrin Homology (PH) domain, the N-terminus catalytic domain (CD) as well as the nuclear export sequence (NES) located in the tyrosine rich region (Figure adapted from (231)).

Recently, PDK1 crystal structure (233) increased our understanding of the protein (Figure 4): unlike other AGC kinases family members, PDK1 does not possess a C-terminal hydrophobic motif usually phosphorylated for activation (232). Instead, it has a 5 Å hydrophobic pocket, known as the PIF-pocket, which acts as a docking site for other AGC kinases hydrophobic motifs (225, 231, 234).

In fact, L155E, a PIF-pocket PDK1 mutant was unable to bind or phosphorylate S6K or SGK. The crystal structure also revealed the classic bi-lobal kinase fold with the α-C-helix that plays a key role in the kinase core (225, 230).

1.6.3.2 Signalling

Besides activating AKT, PDK1 also phosphorylates p70 ribosomal S6 kinase (S6K) (235, 236), which controls protein synthesis and is required for cell growth and amino acids storage (225).

Serum-and glucocorticoid-induced protein kinase (SGK), involved in transport regulation (237), and the less characterized atypical PKC isoforms are also targets of PDK1. The PKC family includes 12 isoforms that have been classified as the conventional (PKCα, PKCβ and PKCγ), the novel (PKCδ, PKCε, PKCε and PKCζ), the atypical (PKCδ, and PKCλ) and finally the PKC-related category (PKN1, PKN2 and PKN3). The PKC family members mediate the effects of growth factors and hormones and were shown to be involved in cancer, cardiac disease, and spinocerebellar ataxia (reviewed in (238)).

Evidence for these kinases being substrates for PDK1 was mainly generated from ES cells where $PDK1^{-/-}$ cells, stimulated with insulin growth factor 1 (IGF-1) did not activate

the AKT-α, S6K and SGK, while wild type PDK1 did (225, 228, 239). The above mentioned PDK1 targets are members of the AGC kinases family, and are activated by inducing phosphorylation at two highly conserved residues: a threonine located in the T-loop of the enzyme (activation loop), and a serine residue located at the C-terminal hydrophobic motif (238).

a- Regulation of AKT phosphorylation

Both AKT and PDK1 share a PH domain that allows their respective binding to PIP3 (224, 232). Through this interaction, PDK1 and AKT co-localize to the membrane enabling PDK1 to phosphorylate AKT (224, 230-232). In vitro experiments showed that PDK1 can only phosphorylate AKT in the presence of PIP3 vesicles (224, 240), and that this activation induces recruitment of AKT to the plasma membrane (241). Furthermore an AKT mutant with a deleted PH domain was not able to interact with PIP3, and unable to translocate to the plasma membrane to be phosphorylated (185). However, one should note that the binding of AKT to PIP3 does not activate it, but induces a change of conformation in AKT that enhances its phosphorylation by PDK1 (232). Evidence of this was generated through life time imaging microscopy (242).

b- Regulation of S6K and SGK

Neither S6K nor SGK have a PH domain (224, 232, 235). They are both phosphorylated at the same rate with or without the presence of PIP3 (232). In fact, the S6K/SGK-PDK1 interaction occurs through the PIF-pocket of PDK1 that interacts tightly with the C-terminal hydrophobic motif of S6K and SGK, allowing

PDK1 to phosphorylate their T-loop. Thus, the mechanisms by which PDK1 recognizes AKT, on one hand, and S6K/SGK, on the other hand, are different (Figure 5).

c- Cellular localization of PDK1

The PI3K signalling pathway is well documented in the cytosol (243), and PDK1 was initially purified from cytosolic extracts (224). The fact that PDK1 co-localizes with and activates AKT at the plasma membrane through their PH domains, and that, on the other hand, it can phosphorylate S6K and SGK independently of PIP3, raised the question about PDK1 sub-cellular localization within the cytoplasm (244). It is not clear whether PDK1 is constitutively associated with the plasma membrane or whether it can be found in an unbound, –free– form within the cytoplasm. Different studies have come to conflicting conclusions with some pointing towards a translocation to the plasma membrane upon PI3K activation, while another found that PDK1 formed a pool by the membrane (reviewed in (232)).

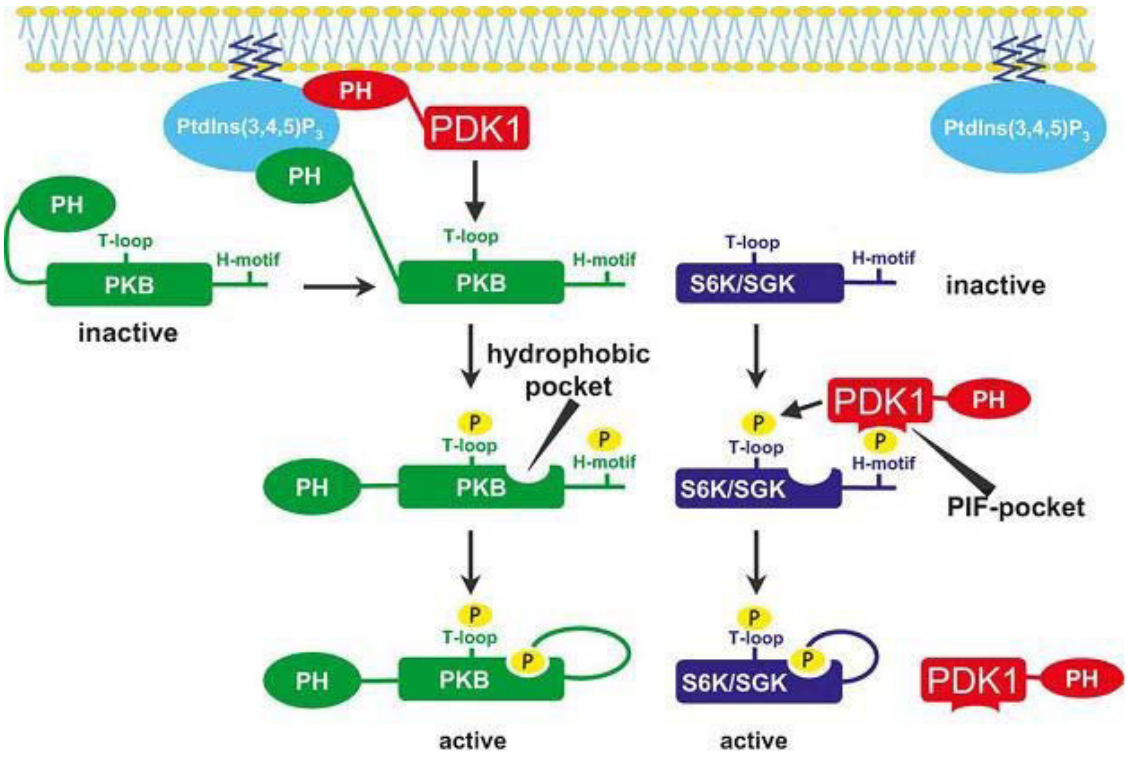

Figure 5. PDK1 activates AGC kinases family members via distinct pathways (Adapted from (232)).

Less is known about PDK1's nuclear function, where an autonomous PI3K pathway is thought to operate (243). In fact, a study in 2003 showed that PDK1 is a nuclear-cytoplasmic shuttling protein (231), and that nuclear localization is increased by insulin and in PTEN deficient cells. The study also noted the presence of a NES at amino acid 382-391 along with the ability of nuclear PDK1 to induce a reduction in cell's anchorage-independent growth, and a decrease in the preventive effect against UV, compared to wild type PDK1 (231).

Later, an independent group confirmed the results showing that PDK1 can shuttle between the cytoplasm and the nucleus, as the inhibition of the nuclear export

receptor CRM1 provoked nuclear accumulation of PDK1 (243). Furthermore, the model suggested that this shuttling is dependent on a serine rich motif between Ser 389 and Ser 396 of PDK1, in direct proximity to the NES. The residues and mainly S396 (most proximal to the NES) were shown to undergo rapid and transient phosphorylation following growth factors activation, causing nuclear translocation. The study took a step further and looked at the effects of this translocation on signaling using an NES-mutated PDK1 (accumulates in the nucleus). They showed that PDK1 nuclear localization induced AKT co-localization to the nucleus, and FOXO3a cytoplasmic retention, and thus a better suppression of FOXO3a transcriptional activity.

The role of nuclear PDK1 is still poorly understood. This shuttling might act as a PDK1 regulator mechanism that sequestrates PDK1 and reduces it kinase activity in the cytoplasm; or it could be that PDK1 might have nuclear targets yet to be explored (231, 243, 244).

d- PDK1 kinase-independent function

PDK1 has been reported to have non-catalytic activities as well, mainly as a docking site for other proteins (reviewed in (243)). Tian et al, 2002 ((245) showed that PDK1 interacts with the N-terminal region of the Ras effector protein RaI guanine nucleotide dissociation stimulator (RaIGDS). Another kinase independent role was revealed in T-cells development where PDK1 recruits the CARD11 scaffold protein (246). More recently, a kinase-independent role for PDK1 in motility was also suggested where PDK1 competes with RhoE for ROCK binding, thus preventing ROCK inhibition by RhoE (100).

1.6.4 Physiological functions of PDK1

PDK1 was first shown to play a role in regulating development and size in mice (247). Hypomorphic mouse ES cells ($PDK1^{fl/fl}$) were injected into murine blastocysts. Although mice were viable and fertile, they were born at a lower than expected Mendelian frequency with a 5 fold lower PDK1 kinase activity and an overall size 30% smaller than wild type littermates, a difference maintained through adult life (247). Comparison of organs volumes showed a 50% reduction in volumes of kidney, pancreas, spleen and adrenal glands. The reduction on volume was shown to result from a similar number of cells that are 45% smaller then wild type mice, rather than from fewer cells. $PDK1^{-/-}$ mice were not recovered post-natively as the double deletion caused embryonic lethality at embryonic day E9.5. Also, by E7, embryos of $PDK1^{-/-}$ mice were smaller in size and by day E8 showed developmental abnormalities with embryos shorter in length, with no somites or posterior mesoderm and a smaller allantois (247).

Another study used the Cre/loxP technology to generate $mPDK1^{-/-}$ mice that lack PDK1 in cardiac muscle (248). Those mice were apparently healthy until 5 weeks of age and died suddenly afterwards while wild type PDK1 littermates were viable. By 6 weeks, $mPDK1^{-/-}$ mice showed thinner ventricular walls, enlarged atria and right ventricle, along with a reduction in cardio-myocyte volume rather than number, consistent with previous findings (247). The death of these mice of heart failure was confirmed by echocardiography analysis.

This study revealed a role for PDK1 in regulating cardiac viability and preventing heart failure. On a molecular level, PDK1 deficiency resulted in no activation of AKT, S6K and 6-phosphofructo-2-kinase (PFK). Using the same Cre/loxP system, another study

investigated the role of PDK1 in the liver (249) through L-PDK11$^{-/-}$ mice that lack PDK1 in hepatocytes. This resulted in the failure to activate AKT. L-PDK1$^{-/-}$ mice were glucose intolerant when injected with glucose, and exhibited 10 fold lower levels of hepatic glycogen. They were also unable to normalize blood glucose within 2 hours after insulin injection (249), and died between 4-16 weeks due to liver failure. On a molecular level, PDK1 deficiency translated to a deregulation of genes required for controlling gluconeogenesis (such as the phosphorenolpyruvate carboxykinase-PEPCK-, the glucose 6 phosphate –G6Pase-, the sterol regulatory element binding protein 1-SREBP1-, as well as the insulin like growth factor binding protein 1-IGFBP1- and the insulin receptor substrate 2-IRS2-, underlying an important role for PDK1 in regulating glucose homeostasis and controlling the expression of insulin-regulated genes (249).

1.6.5 PDK1 in cancer

Although PDK1 is the key kinase upstream of AKT, not much importance was given to PDK1's role in promoting cancer (250).

In a human glioblastoma cell line (U87-MG), which is PTEN inactive (by truncation), PDK1 was targeted with siRNA to knockdown its expression levels (251). This knock-down resulted in an inhibition of AKT activation measured by a decrease in levels of phosphorylation at both Thr308 and Ser473. The knockdown also reduced the levels of S6K phosphorylation and caused a dramatic inhibition of proliferation, as a result of a decrease in cell division and an increase in apoptotic activity as expected. This study suggested that PDK1 could be a potential target in human neoplasm. In breast cancer cells, a descriptive study using Western blots and immunohistochemistry on tissue micro-

arrays (TMA) of breast cancer specimen compared the levels of PDK1 and AKT phosphorylation between cancer and normal samples (251). The reported results showed elevated levels of phosphorylation of both PDK1 and AKT in breast cancer specimen compared to normal ones, while the levels of total PDK1 and AKT remains invariable. However, the results remain controversial for PDK1 phosphorylation as the authors used an antibody that detects the phosphor-Ser 241 residue, which is known in literature to be constitutively phosphorylated.

A transgenic adenocarcinoma of the mouse prostate model (TRAMP mouse) was used in another study to assess the effect of PDK1 inhibition by a PDK1 inhibitor (OSU03012) (252). The results showed that PDK1 inhibition had a chemopreventive effect, with a reported decrease in the weight of the four prostate lobes of the treated mice, a lower incidence of carcinoma and metastasis, and a lower Gleason grade, again underlying the importance of PDK1 in cancer, and prostate cancer specifically.

In 2006, a study gave a closer look at the role of PDK1 within the process of carcinogenesis with a focus on its role in primary tumour progression towards metastasis (253). This study was the first reported evidence for PDK1 involvement in invasion. The authors showed by using the PDK1-expressing mouse mammary epithelial cells (Comma/PDK1) that higher levels of PDK1 increased invasion dramatically as assessed by the Boyden Chamber assay. In parallel, an increase in matrix metalloproteinase 2 (MMP2) secretions were also detected by zymography. Interestingly, the group also looked at the effect of PDK1 expression on gene expression microarrays analysis, and reported the upregulation of a set of 21 genes, some of them involved in ECM regulation and invasion. The most robust changes were observed with an 18 fold increase in

Decorin, an 11 fold increase in type I pro-collagen and a 10 fold increase in collagen VI, all reported to play a role in mammary tumourigenesis. Down-regulated genes included the 26-fold down-regulated WDNM1 gene, a breast cancer tumour suppressor and TIMPP3m an inhibitor of MMP-2. Results were further confirmed by real time PCR (q-PCR).

In 2009, and during the course of this work, a study took a step forward and looked at PDK1 status at the genomic level (using Fluorescent *in situ* hybridization) along with its levels of expression in breast cancer (176). The study reported that the total PDK1 protein (detected using the PKB kinase antibody from Santa-Cruz®) and mRNA levels were over-expressed in the majority of breast cancer samples compared to normal adjacent tissue, and that 21% of the examined tumours had an amplification of *PDPK1* located on chromosome 16p13.The study also notes that more samples had a protein over-expression than those who had the genomic amplification suggesting that other mechanisms might be regulating the expression of PDK1. The *PDPK1* amplification associated with a poor patient survival and the protein over-expression was associated with upstream lesions of the PI3K pathway such as PTEN loss and ERBB2 amplification (176). Finally, in cell lines, PDK1 increased expression resulted in increased AKT phosphorylation at Thr308, increased growth and migration and a higher resistance to PI3K inhibition. This study was the first to look at the 16p13 amplification as a possible mechanism behind PDK1 high expression in cancer.

Relative to the importance of PDK1 in activating the AKT pathway which has been shown to be over-expressed in several cancers, a better characterization and understanding of its role might be crucial in cancer genes targeted therapy.

1.6.6 PDK1 and motility

Previous studies have demonstrated PDK1's involvement in inducing chemotaxis in *Dictyostelium* (254), as well as its role in regulating chemokines in the migration of circulating leukocytes (255) and neutrophils (256). PDK1 deletion has also been linked to developmental abnormalities in the brain linked to the inability of cells to migrate properly (257). The role of PDK1 in cancer motility has not yet been studied or reported extensively. In 2007 a study by Primo et.al (258) shed some light on PDK1's role in regulating endothelial cells migration. The study showed that the effect of PDK1 on motility required the PH (Pleckstrin Homology) domain of PDK1 as well as its catalytic activity -using PDK1 mutants with a deleted PH domain and a kinase-dead (K111A) status respectively- and that it occurred through AKT-1. In 2009, a study by Pinner and Sahai (100) demonstrated a non-catalytic role for PDK1 in promoting cell motility in a melanoma cell line model. Based on evidence presented in the study, they proposed a mechanism by which PDK1 physically displaces RhoE, which normally binds and inhibits ROCK1, thus allowing the formation of blebs. That same year a study on PDK1's role in breast cancer showed that increased PDK1 expression stimulated growth and motility of breast cancer cell lines (176). Besides these few studies, no other findings were reported regarding the involvement of PDK1 in cancer motility, and namely prostate cancer motility.

1.6.7 PDK1 inhibitors in cancer

Fourteen years have passed since the discovery of PDK1 with no clinically approved PDK1 inhibitor. As suggested in a recent review (259), the reason behind the absence of effective, clinically-approved inhibitors might be the issue of selectivity as PDK1 is the first node in the PI3K signalling pathway, and its de- regulation affects several kinases that play different roles in the cell regulation (259). UCN-01 (7hydroxystaurosporine), a PDK1 inhibitor, was being evaluated in a clinical trial, and was shown to induce dephosphorylation of AKT, turning off the survival signals and causing apoptosis (260). It was shown to inhibit PDK1 at an IC_{50} of 33nM. Another PDK1 inhibitor, OSU-03012, mentioned earlier, was shown to effectively inhibit PDK1 in rhabdomyosarcoma cells (261). However, neither of these two inhibitors was approved, mainly due to selectivity issues, and the resulting toxicities they generate (259). More recently, two inhibitors have been in clinical trial: BX-795 which inhibits PDK1 in vitro with a low IC_{50} of 6nM and GSK2334470 which has an IC_{50} of 15nM (259). While BX-795 has already encountered issue of selectivity (off-targets such as ERK8), GSK2334470 reflected an impressive selectivity against 110 protein kinases (259). However, this inhibitor also showed differential sensitivity: while highly inhibiting S6K and SGK, it showed a lower effect on IGF-1 stimulated AKT and a slower effect on RSK with a latency between 8 to 24 hours (259).

SUMMARY: Previous data has reported gain at the 16p13 region that maps to *PDPK1* in metastatic PCa, but not in unmatched primary tumours. PDK1, the protein product of *PDPK1* is a relatively recently studied protein and its

functions have not been extensively characterized. Very recent work in cancer showed that PDK1 plays a role in breast cancer and melanoma, but no previous study has addressed its function in PCa progression. Given the lack of data regarding the 16p13 amplification further investigation is needed to better characterize its role in PCa progression to metastasis. Furthermore, understanding the role of PDK1 in PCa cells might make it a target for therapy.

1.7. Rationale, hypothesis and aims

1.7.1 Rationale

Prostate cancer (PCa) is a heterogeneous disease and its clinical manifestations can range from an indolent to an aggressive/metastatic lethal disease. It is thus challenging but important for physicians to be able to stratify patients at early stages of the disease, when PCa is still asymptomatic in order to treat those with a potentially aggressive disease, and spare indolent ones harmful treatment that can be avoided. The current prognostic determinants (pre-operative PSA, Gleason grade and the TNM staging system) do not seem to be able to properly discriminate between the patients. Better prognostic makers, that are specific, reliable and objectively measured, are thus needed.

Previous gene expression profiling analysis and aCGH studies on PCa samples have generated molecular signatures and specific genomic alterations associated with metastasis. Of interest was the genomic gain at 16p13 where the *PDPK1* gene resides, a genomic region that has not been characterized in PCa and which presence correlated with an increase in PDK1 mRNA levels. Interestingly, gene set enrichment analysis revealed the AKT survival pathway to be enriched in LN mets compared to primary PCa,

indicating that the pathway was highly activated in those patients.

1.7.2 Hypothesis

Based on the rationale presented above, we hypothesized that the 16p13 gain, which contains *PDPK1*, can be detected in primary PCa samples, and can be used as a prognostic marker for metastatic PCa. We also hypothesized that PDK1, the protein product of *PDPK1* gene, plays an important role in mediating PCa progression towards a more aggressive disease.

1.7.3 Aims

In order to test our hypothesis, we adopted the following strategy:

AIM 1: we surveyed a selected cohort of patients with metastatic PCa for the 16p13 genomic gain and tested if it could be retrieved in their matched primary tumours using Fluorescent *In Situ* Hybridization (FISH).

AIM 2: We surveyed a selected cohort of patients with primary PCa for the 16p13 gain with clinical and pathological features.

AIM 3: We investigated *in vitro* the functional role of PDK1 in PCa progression by specifically looking at cell proliferation and motility using MTT proliferation assay and wound healing assay, respectively.

The tissue samples consisted of formalin fixed paraffin embedded (FFPE) tissue represented on tissue micro-arrays (TMA). **FISH** is considered the gold standard method to detect gene amplification or deletion in tissue (262). Briefly, a 16qh centromeric control probe and a 16p13.3 locus-specific probe are labeled with

differenct fluorochromes, and then are co-hybridized to a tissue section. Probe signal is then visualized and counted under a fluorescent microscope.

FISH is currently used in pathology laboratories to detect the Her-2 genomic amplification in breast cancer, and was reported to give more reproducible results than immunohistochemistry (263).

In vitro functional assays were carried on the following 3 prostate cancer cell lines: The PTEN-negative AR-negative PC-3 cells, the PTEN-negative AR- positive LNCaP cells and finally the PTEN-positive AR-negative DU145 cells. The different cell lines with their PDK1, PTEN and AR protein levels of expression are represented in the Figure 6 as determined by Western blots.

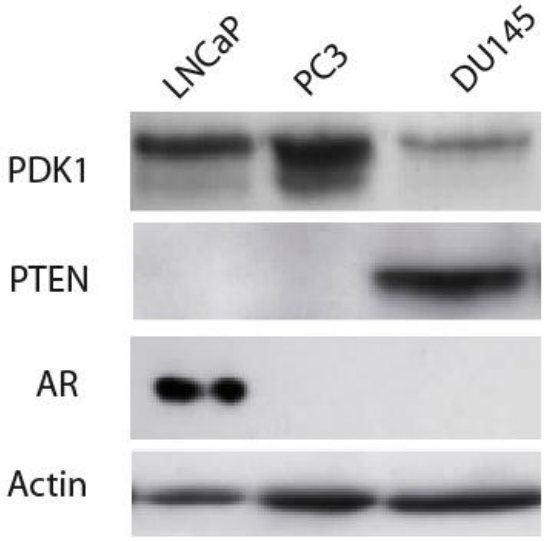

Figure 6. Protein levels of PDK1, PTEN and AR in the LNCaP, PC-3 and DU145 prostate cancer cell lines.

Growth and survival were assessed using the **MTT proliferation assay**, a colorimetric assay that is commonly used to monitor cell proliferation *in vitro*. It

measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by succinate dehydrogenase of the mitochondria. MTT enters the cells and passes to the mitochondria where it gets reduced to give a dark purple coloured product. Cells are then solubilized and the released purple product is measured spectrophometrically.

Cells motility was assessed *in vitro* **by Wound healing assay** (WHA). Briefly, the cells are plated to form a confluent monolayer before being scratched thus creating a wound. Closure of the wound by migrating cells is then monitored over time and motility is assessed in terms of percent recovery of initial wound surface area.

2. MANUSCRIPT

The 16p13.3 (*PDPK1*) genomic gain in prostate cancer: a potential early biomarker of metastasis

Contributions:

- All the FISH analysis, except for 7 cases, *in vitro* work in DU145 and LNCaP cells, and the writing of the manuscript was performed by Khalil Choucair.
- *In vitro* work on PC-3 cells was performed by Karl-Philippe Guérard.
- FISH on the 7 cases was performed by Maisa Yoshimoto in Dr. Jeremy Squire's lab.
- Joshua Ejdelman contributed to the assembly of the primary prostate cancer tissue microarray.
- Landan Fazli from Dr. Martin Gleave's lab provided the CRPC and the 3 metastases samples on TMA.
- Eleanora Scarletta from Dr. Simone Chevalier's lab provided tissue samples.
- Drs. Kanishka Sircar and Fadi Brimo provided their pathological expertise.
- Dr. Cunha provided the metastatic PCa tissue samples.
- Dr. Jacques Lapointe: supervisor.

The 16p13.3 (*PDPK1*) genomic gain in prostate cancer: a potential early biomarker of metastasis

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ABSTRACT

Prostate cancer (PCa) is a leading cause of cancer death and distinguishing life threatening tumors from indolent ones is a major challenge. The identification and characterization of genomic alterations associated with advanced disease may lead to the development of new markers of progression and more efficient therapeutic approaches. In this study, we performed Fluorescence *In Situ* Hybridization (FISH) to detect the copy number gain of chromosome 16p13.3 in 10 lymph node (LN) metastasis samples and their matched primary tumors, 9 transurethral resections of prostate (TURP) tissue samples of castration-resistant prostate cancer (CRPC), and 46 additional primary PCa specimens with clinicopathological parameters. We detected the gain in 5/10 LN metastases and 3/5 matched primary tumors, 3/9 CRPC samples, and 9/46 (20 %) primary tumors. In the latter set of samples, the 16p13.3 alteration was associated with high Gleason score and elevated preoperative prostate specific antigen (PSA) levels. The levels of 16p13.3 gain were higher in LN metastasis and CRPC specimens compared to primary PCa. Chromosome mapping revealed a focal gain that spans *PDPK1* encoding the 3-Phosphoinositide-dependent protein kinase-1 (PDK1). RNA interference-mediated knock down of PDK1 in three different PCa cell lines reduced cell motility without affecting growth. Our findings support a prognostic value of the 16p13.3 gain and a role of PDK1 in promoting PCa progression, through migration, which is key for metastatic lethal disease.

INTRODUCTION

Prostate cancer (PCa) is the most common cancer diagnosed in North America and respectively the third and second leading cause of cancer death among men in Canada and in USA (1, 2). PCa is heterogeneous in its clinical outcome ranging from relatively indolent to aggressive metastatic disease. Key issues in the management of PCa are to distinguish latent from clinically significant tumors to primarily treat patients with life threatening disease and to identify molecules contributing to risks of metastatic progression and resistance to treatment. The current prognostic tools such as preoperative PSA levels, clinical TNM staging and Gleason grading of biopsy specimens cannot accurately predict individual clinical outcome. Most advanced cancers will respond to androgen deprivation therapy, but will invariably relapse and become castrate resistant PCa (CRPC) (3). There is currently no cure for metastatic PCa.

The identification and characterization of genomic alterations associated with advanced PCa may lead to developing new progression markers and more efficient therapeutic approaches. DNA Copy Number Alterations (CNAs), gains and losses, have been described in PCa and include known or candidate tumor suppressors such as *NKX3-1* (8p21), *PTEN* (10q23), *RB1* (13q14), *TP53* (17p13), oncogenes such as *MYC* (8q24), and the *AR* (Xq12) in CRPC (4). Studies using a genome-wide approach such as array-CGH to detect and map CNAs in PCa have confirmed earlier findings implicating losses at 8p, 10q and gains at 8q in disease progression (5-8). The 10q23 deletion has been shown to predict PCa recurrence (9) and earlier prostate cancer specific death (10). PTEN is a negative regulator of the PI3K /AKT survival pathway known to be up-regulated in several types of cancer (11). 3-Phosphoinositide-dependent protein kinase-1 (PDK1)

phosphorylates and activates the AGC kinase members regulated by PI3-kinase, including AKT which is activated by phosphorylation at Thr308 (12).

Lapointe et al. found that the 16p13.3 gain was among the most frequent genomic alterations in LN PCa metastases (13), a gain not yet characterized. In this study, we have mapped the focal 16p13.3 genomic gain and identified *PDPK1* encoding PDK1 as the driver of the gain with consequences on PCa cell migration.

MATERIALS AND METHODS

Tissue samples. Formalin-fixed paraffin-embedded prostate tissue samples were collected at McGill University Health Centre, Hospital do Câncer, São Paulo and University of British Columbia with their respective research ethics board's approval. Specimens included 10 LN metastases and their matched primary tumors, 9 transurethral resections of prostate (TURP) tissue samples of CRPC (defined by a rising serum PSA during androgen ablation therapy despite testosterone at castrate levels), and 46 primary tumors and adjacent benign tissues from radical prostatectomy. Gleason score, surgical stage and preoperative PSA were available for the latter 46 specimens. Duplicate tissue cores (1mm diameter) were assembled into tissue microarrays (TMAs). Haematoxylin/Eosin (H&E)-stained TMA sections were reviewed to map representative tumor and benign areas for scoring.

FISH. Dual-color FISH was carried out on TMA sections using as probes, the BAC clone RP11-20I23 (BACPAC Resources Center) mapping to *PDPK1* locus on chromosome 16p13.3 region and the recombinant DNA clone PHUR-195 (ATCC), which maps to the 16qh centromeric region. RP11-20I23 and PHUR-195 DNA were respectively labeled

with SpectrumOrange-dUTP and SpectrumGreen-dUTP (Enzo) using the Nick Translation Reagent Kit (Abbott Molecular). Mapping the regions flanking 16p13.3 was done using 6 additional BAC probes (Supplementary Table 1, tissue section processing and hybridization detailed in Supplementary Note 1).

FISH data analysis. In order to evaluate the 16p13.3 copy number, we counted fluorescent signals in 100 non-overlapping interphase nuclei for each sample (as identified on corresponding H&E) counterstained with 4',6-Diamidino-2-phenylindole, *(DAPI III,* Abbott Molecular*)* to delineate nuclei. Based on hybridization in 30 benign prostate cores, the 16p13.3 gain was defined as present at a threshold of ≥15% (mean + 3 standard deviation in controls) of tumor nuclei containing 3 or more 16p13.3 locus signals and by the presence of two PHUR-195 signals. Images were acquired with an Olympus IX-81 inverted microscope at 96X magnification using ImageProPlus 7.0 software (MediaCybernetics).

Cell lines. The metastatic human PCa cell lines PC-3, DU145 and LNCaP (ATCC) were cultured in RPMI 1640 medium with 10% fetal bovine serum (HyClone), 1% L-Glutamine and 1% penicillin-streptomycin (Invitrogen) at 37°C and 5% CO_2.

siRNA transfection. Non-targeting and specific siRNAs (Ctrl, siPDK1-1 and siPDK1-2) were used in silencing experiments (sequences in Supplementary Note 2). 24-hours post-plating, 10 nM siRNA was transfected using HiPerfect reagent diluted 1:200 according to manufacturer instructions (Qiagen). Experiments were started 72h post-transfection.

Plasmid construction and transfection. PDK1 cDNA (GenBank: AF017995.1) was PCR-amplified from reverse-transcribed RNA extracted from RWPE-1 prostate cells,

sequence-verified and inserted into pcDNA6/V5-HisA (Invitrogen) in-frame with sequences encoding the V5 epitope (primers listed in Supplementary Note 3). After rendering the pcDNA6-PDK1-V5 construct resistant to siPDK1-1 by synonymous nucleotide changes using site-directed mutagenesis (Stratagene, Supplementary Note 4), siRNA-treated cells were co-transfected with pcDNA6-PDK1-V5 or empty vector (2 or 1.25 μg per well in 6-well and 24-well plates, respectively) using HiPerfect as described above.

Protein extraction and Western blotting. Whole-PCa cell protein extracts were prepared using RIPA buffer. Twenty-five μg of protein were loaded on SDS-PAGE, transferred to nitrocellulose membrane, and probed with primary antibodies against: PDK1 (ECM Biosciences), actin (CHEMICON), Akt, phospho(p)-Akt (Thr308) (Cell Signaling), V5 (Invitrogen), AR (NeoMarker) and PSA (DakoCytomation). Signals were revealed using HRP-conjugated antibody (Jackson ImmunoResearch) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo) (Supplementary Note 4 for details).

Growth assay. Survival/proliferation was measured using MTT assay in 24-well plates as described in Supplementary Note 5.

Migration assay (wound healing assay). Cells were plated in 6-well plates at $1.2x10^5$ cells per well for PC-3 and DU145 and at $3x10^5$ for LNCaP cells. 72h after transfection, a linear scratch was performed using a pipette tip. Motility was assessed in duplicates at 3h, 6h and 12h by computing the percentage of wound closure relative to initial surface area (0h). At each time point, images were captured with ImageProPlus 7.0 software on an

Olympus IX-81 inverted microscope at 10X magnification. For each experiment, MTT assay was done in parallel as described above.

Statistical analysis. Growth and migration assays were done in triplicate and duplicate respectively and each experiment was replicated at least twice. Significance of PDK1 knock-down response on growth and motility was determined using two-samples *t*-test and $P<0.05$ was considered to be significant. Clinicopathological tumor characteristics and their association with the 16p13.3 gain were assessed using Fisher's Exact test. Levels of gain were compared between specimen categories with the U-Test (Mann-Whitney).

RESULTS AND DISCUSSION

We used FISH to assess CNAs at the 16p13.3 region on 10 PCa samples of LN mets and their matched primary tumors, 9 CRPC samples from TURP, and 46 primary tumors from radical prostatectomy with corresponding clinicopathological parameters. We found that 5 of 10 LN mets samples harbored the 16p13.3 copy number gain, a frequency in line with the previous array-CGH study (13), and 3 of these 5 LN cases showed the gain in their matched primary samples (Figure 1A, B). One of the 5 LN metastasis with no 16p13.3 gain showed the gain in its respective primary specimen (case F), while 4 cases did not harbor the gain in their primary or their metastatic samples. Although the number of metastases available was limited, the detection of this gain in most matched primary tumors (60 %) is in agreement with earlier data (14) and more recent studies (5, 15) showing that metastatic genomic profiling reflects the original primary tumor. In most LN metastases with 16p13.3 gain analyzed here, we observed a

deletion of 10q23 (*PTEN*) ((9) and unpublished observations) as found in the previous array-CGH study (13), which may indicate cooperation between PDK1 and PTEN in the metastatic process. For cases with no alterations at 10q23 and 16p13.3, alternative genetic pathways may have lead to their metastatic behavior.

The gain was also detected in 3/9 CRPC samples and in 9/46 additional primary tumors (Figure 1C), the latter supporting the idea of an early marker for aggressive PCa. In the 46 radical prostatectomy specimens, the gain was significantly associated with Gleason score ≥8 (P=0.002, Table 1) and preoperative PSA levels (P=0.047), but not with surgical stage (P=0.258). Recently, a report has shown that the 16p13.3 gain was associated with poor survival of breast cancer patients (16). A cytogenetic study on lung tumors has found the 16p13.3 gain to be associated with poor differentiation and late stage disease (17). Despite the importance of testing its prognostic value on a larger population of PCa patients with survival data, these findings suggest a strong clinical relevance for 16p13.3 gain in several cancers. Whether this genomic alteration is involved in the development of CRPC is unknown. The AKT pathway was shown to be activated in CRPC along with *PTEN* deletion (18). Whether the AKT pathway is further activated in tumors with 16p13.3 gain needs to be assessed, as the observations in LN metastasis suggest that harboring the *PTEN* deletion along with the 16p13.3 gain may lead to a worse clinical outcome.

We compared the level of gain in primary samples with CRPC and metastatic samples and more specifically evaluated the average percentage of nuclei with >3 copies of 16p13.3 across the specimens with gain. CRPC and LN metastasis samples harbored

higher percentages of nuclei with >3 copies than the primary (P<0.05, Table 1), which supports a link between 16p13.3 and disease progression.

To define the extent of 16p13.3 gain, we mapped the flanking genomic region with FISH using 6 different BAC probes (Supplementary Table 1) on the 9/46 primary PCa samples with gain for which additional TMA sections were available for multiple probes hybridization. The size of the gain varied from 0.57 to 9 MB (Figure 1D). The minimal region of gain common to the 9 samples was 0.57 MB in size and included 20 genes according to the UCSC Genome Browser, Feb 2009 assembly. To prioritize candidates at this locus, we looked at the previous PCa array-CGH study by Lapointe et al. which reported the 16p13.3 gain in LN mets (13) and the corresponding gene expression data (19) since we did not have gene expression data for the 9 samples mapped here. Both CNAs and gene expression data were retrieved for 11 of 20 genes. *PDPK1* gene had the highest DNA/RNA correlation coefficient and the only one above 0.5 (Supplementary Table 2). Although we cannot exclude a role for the other genes in the 16p13.3 region, mapping results and PDK1 mRNA levels correlating with CNAs in the microarray datasets suggested *PDPK1* as a candidate driver of the gain. Our results concord with a recent report on PDK1 overexpression in breast cancers with increased *PDPK1* CNA (16).

PDK1 is expressed in PCa cell lines with different PTEN and AR status: LNCaP, PC-3, and DU145 (Supplementary Figure S1). The AR negative (AR-)/PTEN- PC-3 and the AR+/PTEN- LNCaP express high levels of PDK1 while the AR-/PTEN+ DU145 cells express the lowest. To explore the role of PDK1 in PCa progression, we down-regulated its expression in these 3 cell lines with siRNAs and assessed the resulting

consequences on growth and migration. siPDK1-1 and siPDK1-2 were used in PC-3 and siPDK1-1 was used in DU145 and LNCaP cells. Cell migration was significantly reduced when PDK1 was siRNA down-regulated across the 3 cell lines and the difference with siCtrl was noticeable at 3 hours into the assay ($P<0.01$, Figure 2 A, B). Western blots were performed in parallel to assess the effect of siRNAs on PDK1 protein expression and downstream cell signalling. In all 3 cell lines, siRNAs effectively down-regulated PDK1 expression (assessed by band quantification relative to actin levels (Figure 2 C). In DU145 cells, down-regulation of PDK1 was the most effective (84% knock-down) and resulted in a down-regulation of AKT phosphorylation at Thr308 residue while the knock-down in LNCaP and PC-3 cells did not substantially affect this phosphorylation site. In all 3 cell lines, knocking down PDK1 did not reduce cell growth as measured by MTT assay (Figure 2 D), a finding consistent with *in vitro* results in mouse embryonic fibroblast derived from PDK1 knockout mice (20).

To further validate our findings, we re-expressed PDK1 using the human V5-tagged cDNA resistant to siPDK1-1. Transfection with a control vector (Mock) did not affect the ability of siPDK1-1 to reduce PC-3 cell motility, while re-expressing PDK1 was effective in rescuing motility and restoring levels to the control value (Figure 3). Over-expressing PDK1 in siCtrl transfected cells did not affect motility. The effects of PDK1 down-regulation and PDK1 re-expression on motility were noticeable at 3 hours ($P<0.006$) and were even more significant at 6 and 12 hours ($P<0.001$). Figure 3 shows the effective downregulation of PDK1 expression by siRNA and its subsequent re-expression. Levels of phospho-AKT did not significantly vary across experimental conditions.

Recent reports suggested that PDK1 regulates endothelial (21) as well as breast cancer cell migration (16, 22) through phosphorylation of AKT at Thr308. The kinase and PH domains of PDK1 were necessary for this effect in endothelial cells (21) and PKCζ was involved in one of the breast cancer studies (22). In our study, phosphorylation of AKT at Thr308 was affected by the reduction of PDK1 levels in DU145 but not in PC-3 and LNCaP cell lines while motility was consistently diminished in all cell lines. Although reduced by siRNA, it is possible that the levels of PDK1 remained sufficient to maintain the AKT phosphorylation in PC-3 and LNCaP cells, considering that their basal level of PDK1 is higher than that of DU145 cells (Supplementary Figure S1). PC-3 and LNCaP do not express a functional PTEN which may further contribute to the deregulation of AKT phosphorylation. Similar observations were previously reported in animals showing that AKT phosphorylation remained normal in PDK1 hypomorphic mice expressing reduced levels of PDK1 (20).

Absence of expected effects on the AKT pathway suggests that PDK1 may modulate PCa cell motility by another mechanism. PDK1 has been reported to bind ROCK1, a mediator of cell motility, at the plasma membrane without use of its kinase domain (23). Loss of PDK1 diminishes ROCK1 activity and consequently reduces motility. Further experiments are needed to determine whether such a mechanism underlies the effect of PDK1 on PCa cell motility.

Given the involvement of PDK1 in motility, it is possible that the 16p13.3 gain detected in primary and LN metastases contributes to cancer cell migration outside the prostate and one can expect to find it in circulating tumor cells (24). In recent CNAs surveys of PCa, this gain was also detected in distant metastasis sites such as the bone,

liver, and adrenal (5, 15), and associated with PCa liver metastases (5). The latter observation suggests a role for the 16p13.3 gain in PCa cell migration to distant organs which ultimately leads to lethal PCa.

Taken together, our results support that the 16p13.3 gain is relevant to PCa progression and may represent an early marker of metastasis, since retrieved in primary PCa which is sampled by biopsies at time of diagnosis. PDK1, encoded by *PDPK1* at 16p13.3, is implicated in PCa cell motility, a critical step for progression to metastasis.

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FIGURES AND TABLES

Figure 1. Dual color FISH analysis of 16p13.3 gain in PCa samples. (A) Results of interphase FISH for chromosome 16p13.3 on 10 LN mets (A-J) and their matched primary tumors. **(B)** On the left panel, representative FISH images of LN mets without gain and their matched primaries show two red signals (16p13.3 locus) and two green signals (centromere 16) in most of the nuclei. On the right panel, FISH images show ≥3 red signals (16p13.3 locus) and two green signals per nucleus indicating a 16p13.3 gain. **(C)** Representative images of the 16p13.3 gain in a CRPC sample (upper panel) and in a primary sample (lower panel). **(D)** Mapping of flanking regions of the 16p13.3 gain in 9 primary PCa samples using BAC probes. Each probe was co-hybridized with the centromere 16 probe. Gains are indicated by red boxes for each sample. BACs and their chromosomal locations are indicated in Supplementary Table 1. The horizontal dotted lines delineate the minimal region of gain with, on the far right, the list of genes mapping to this region (UCSC Genome Browser, Feb 2009 assembly).

Figure 2. PDK1 knockdown reduces PCa cell motility. Panel **(A)** illustrates wound healing assays for PC-3, DU145 and LNCaP cell lines at 0 and 12 hours with control siRNA (siCtrl) compared to siPDK1 (siPDK1-1 and -2 for PC-3, siPDK1-1 for DU145 and LNCaP). Dotted lines show areas used for quantification. Migration results are

represented in panel **(B)** (from left to right: PC-3, DU145 and LNCaP) in terms of % recovery of initial surface area after wounding (h), with accompanying Western blots in panel **(C)**. Residual expression (% relative to siCtrl and normalized to actin) is indicated for PDK1 and p-AKT (relative to siCtrl and normalized to AKT). Full blots are available in Supplementary Figure S3. Panel **(D)** shows the effect of PDK1 knockdown on growth in PCa cell lines. MTT assays were performed on PC-3, DU145 and LNCaP cells, on the day of transfection (0h) and 72h post-transfection at time intervals indicated. Cells were transfected with control (siCtrl), siPDK1-1 or siPDK1-2. Differences between control and PDK1-targeting siRNAs were not significant with $P \geq 0.83$.

Figure 3. Ectopic expression of siRNA-resistant PDK1 rescues motility in siPDK1-treated PCa cells. Left panel shows the quantified results of migration comparing PC-3 cells transfected with siCtrl or siPDK1-1 in combination with an empty vector (Mock) or a siRNA-resistant PDK1-V5 expressing vector at 3, 6 and 12 hours after wounding. Accompanying Western blots are shown on the right**,** representing expression levels of PDK1, AKT and p-AKT. Full blots are available in Supplementary Figure S4.

Table 1. 16p13.3 gain, clinicopathological parameters and levels of gain

SUPPLEMENTARY FIGURES AND TABLES

Supplementary Figure S1. PDK1 expression in LNCaP, PC-3, and DU145 PCa cell lines. Cells were grown in RPMI1640 medium supplemented with 10% FBS and assessed for basal levels of expression of PDK1, PTEN, AR and the androgen regulated PSA by Western blotting. Actin was used as control. Full blots are available in Supplementary Figure S2.

Supplementary Figure S2. Full Western blots used for supplementary Figure S1, where black inserts show parts of the blots used for Figure.

Supplementary Figure S3. Full Western blots used for Figure 2C, where black inserts show parts of the blots used for Figure.

Supplementary Figure S4. Full Western blots used for Figure 3, where black inserts show parts of the blots used for Figure.

Supplementary Table 1. List of the BAC probes used in FISH experiments with their corresponding mapped genomic location.

Supplementary Table 2. DNA copy numbers/RNA expression correlation coefficient of 11 genes mapping to 16p13.3 minimal region of gain.

Supplementary Note 1

The 5 µm TMAs sections were de-paraffinized in 6 changes of xylene before immersion in 95% ethanol. The slides were then placed in 0.2 N HCl solution at RT° for 20 min followed by a 2-hour incubation at 80°C in 10 mM citric acid buffer (pH 6) for pre-treatment. Specimens were digested in 0.1 mg/ml protease I (Abbott Molecular), and then fixed for 10 min in formalin before dehydration in ethanol series. The two probes and target DNA were co-denatured at 73°C for 6 min and left to hybridize O/N at 37°C using the ThermoBrite system (Abbott Molecular). Post-hybridization washes were performed in 2xSSC and 3% NP40/0.2xSSC at 73°C for 2 min and 1 min respectively, followed by a 30s incubation at RT° in 2xSSC.

Supplementary Note 2

Two specific siRNAs were used to silence PDK1 expression. Targeted sequences were: 5'-UAAUACGUCCUGUUAGGCGUG-3' (siPDK1-1) obtained from Qiagen and 5'-AAAUUCUUGGCCUCUGGUC-3' (siPDK1-2) as reported in (23) and non-targeting (Ctrl) from Qiagen: 5'-ACGUGACACGUUCGGAGAAUU-3'.

Supplementary Note 3

PDK1 RT-PCR primers: PDK1-Left with the *Eco*RI restriction site: 5'-GATGTGAATTCCCCATGGCCAGGACCACCAGCCAG-3' and PDK1-Right with the *Not*I restriction site: 5'-CAGTATGCGGCCGCTGCACAGCGGCGTCCGG-3'. The PCR amplified cDNA was inserted into the *Eco*RI and *Not*I sites of pcDNA6/V5-HisA (Invitrogen) The pcDNA6-PDK1-V5 construct was rendered resistant to siRNA by synonymous nucleotide changes (AACAGGACGTAT to AATCGTACATAC) into the targeted sequence of siRNA PDK1-1 by site-directed mutagenesis (QuikChange Lightning kit, Stratagene). After deletion of the DNA region to be mutated, the changed sequences were re-inserted in two steps using the following primers: 5'-CTTTGTCCACACGCCTAATATCTGATGGACCCCAG -3' and

5'-CTGGGGTCCATCAGATATTAGGCGTGTGGACAAAG-3' (for deletion), 5'-AAACTTTCTTTGTCCACACGCCTAATCGTATATCTGATGGACCCCAGCGGGAAC -3' and

5'-

GTTCCCGCTGGGGTCCATCAGATATACGATTAGGCGTGTGGACAAAGAAAGT

TT-3' (for re-insertion of first part), and 5'-

AAACTTTCTTTGTCCACACGCCTAACATACTATCTGATGGACCCCAGCGGGA

AC -3' and

5'-

GTTCCCGCTGGGGTCCATCAGATAGTATGTTAGGCGTGTGGACAAAGAAAGT

TT -3' (for re-insertion of second part).

Supplementary Note 4

RadioImmunoPrecipitation Assay (RIPA) buffer: 50 mM HEPES pH 7.2, 150 mM NaCl, 2mM EDTA pH 8.0, 1% NP-40, 0.5 % Deoxycholate, 1mM Na_3VO_4, 10mM $Na_4P_2O_7$, 1 mM NaF and Complete Protease Inhibitor Cocktail by ROCHE. Briefly, cell culture medium was removed and cells were washed twice with ice-cold Phosphate-Buffered Saline (PBS) before adding RIPA buffer and scraping the cells off. Cell lysate was then collected and left to shake on ice for an hour. After overnight freezing, lysates were centrifugated at 17000 Xg for 10 min and supernatants were transferred to new tubes. Protein concentration was determined using Bio-Rad RC DC Protein Assay. Primary antibodies used were mouse anti-PDK1 at 1:5000 (ECM Biosciences Cat no. PM1461), mouse anti-actin at 1:100,000 (CHEMICON Cat No. MAB1501R0), rabbit anti-Akt at 1:10,000 (Cell Signaling cat No. 9272), rabbit anti–phospho(p)-Akt (Thr308) at 1:1000

(Cell Signaling Cat No. 4056), anti-V5 at 1:5,000 (Invitrogen Cat No. R960-25), mouse anti-AR at 1:1000 (NeoMarker cat No. AR441), and rabbit anti-PSA at 1:2000 (DakoCytomation cat No. A0562). Antibodies were diluted in 5% nonfat dry milk or 5% bovine serum albumin (BSA for anti-p-AKT) in a solution of Tris-Buffered Saline (TBS)/ 0.1% Tween-20. Secondary anti-mouse or -rabbit HRP conjugated antibody (Jackson ImmunoResearch; Cat No 715-035-150 and 711-035-152 respectively) were used at 1:20,000 and results were revealed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo).

Supplementary Note 5

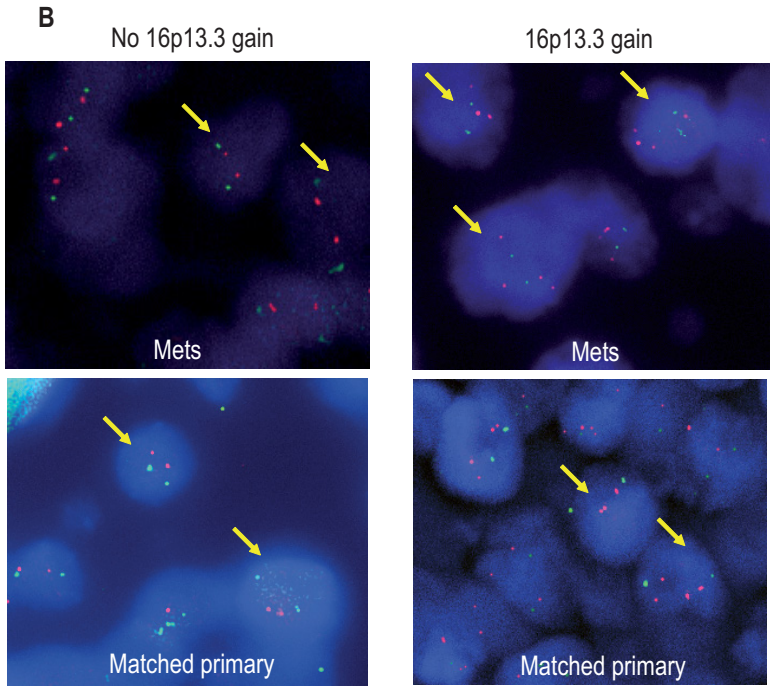
Proliferation was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 24-well plates in triplicates. Cells were plated at $2.2x10^4$ cells per well for PC-3 and DU145 and at $5.5x10^4$ for LNCaP cells. 5 mg/ml MTT in PBS was added to each well at a final concentration of 0.5mg/ml, and plates were incubated at 37°C for 4 hours in a 5% CO_2 environment. The medium was then carefully removed, dimethyl sulfoxide (DMSO) was added to cells and left to incubate for 10 min at 37°C and 5% CO_2. Readings were done at wavelengths of 595nm for MTT reagent and 690 nm for the background (Victor^{3}V 1420 multilabel Counter plate reader, PerkinElmer).

Figure 1

A

Case	Mets	Primary
A	gain	gain
B	gain	gain
C	gain	gain
D	gain	-
E	gain	-
F	-	gain
G	-	-
H	-	-
I	-	-
J	-	-

16p13.3 gain: gain
No 16p13.3 gain: -



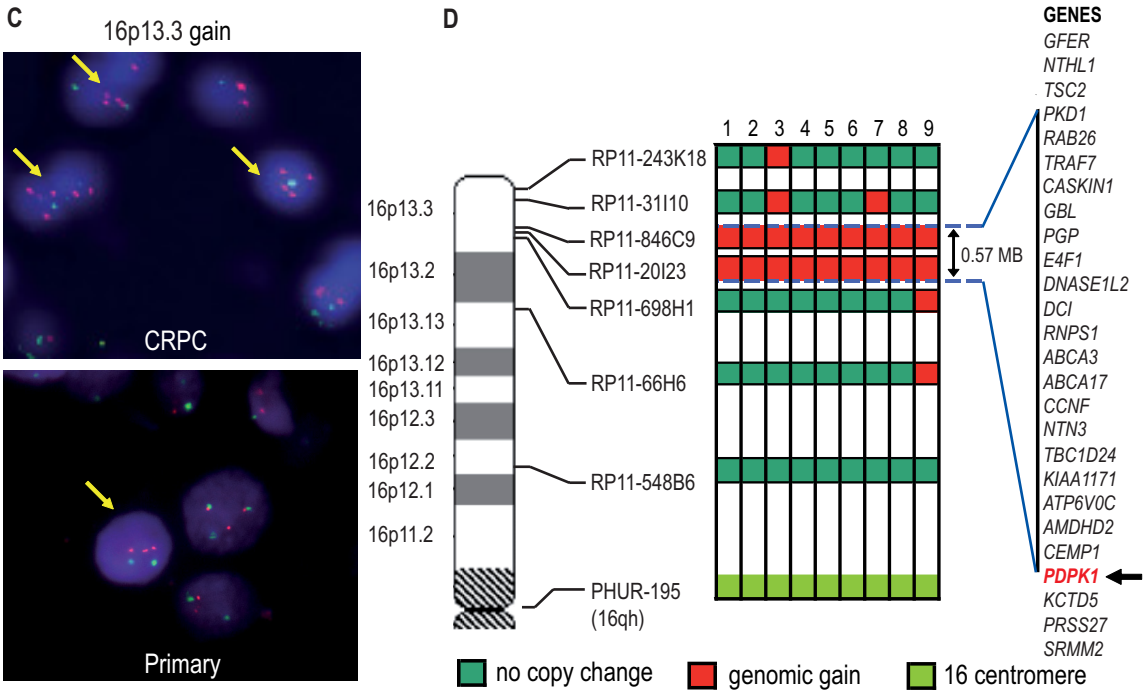


Figure 2

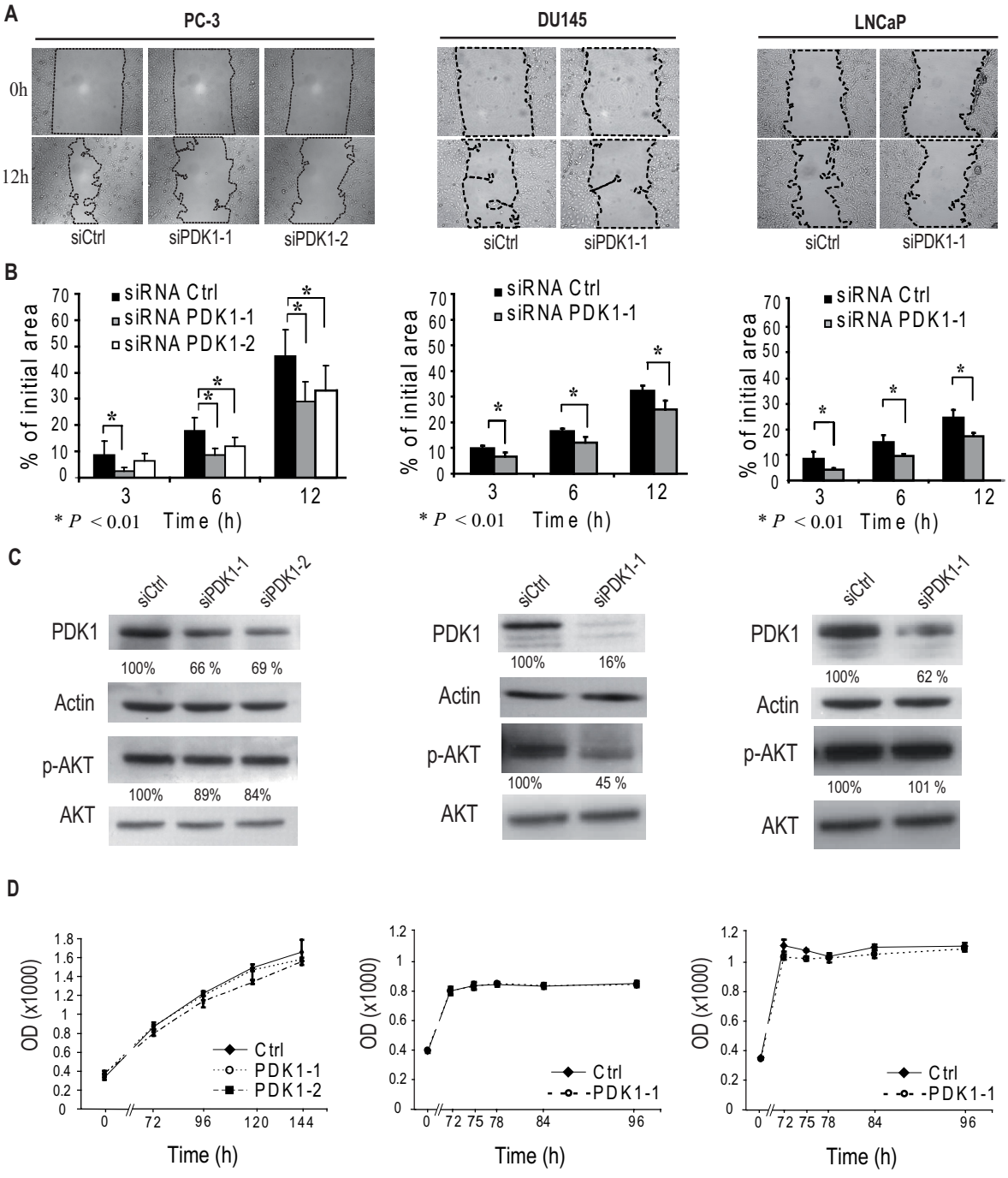

A
PC-3
DU145
LNCaP
0h
12h
siCtrl
siPDK1-1
siPDK1-2
B
siRNA Ctrl
siRNA PDK1-1
siRNA PDK1-2
% of initial area
Time (h)
* P < 0.01
C
PDK1
Actin
p-AKT
AKT
100%
66 %
69 %
16%
62 %
89%
84%
45 %
101 %
D
OD (x1000)
Ctrl
PDK1-1
PDK1-2
Time (h)

Figure 3

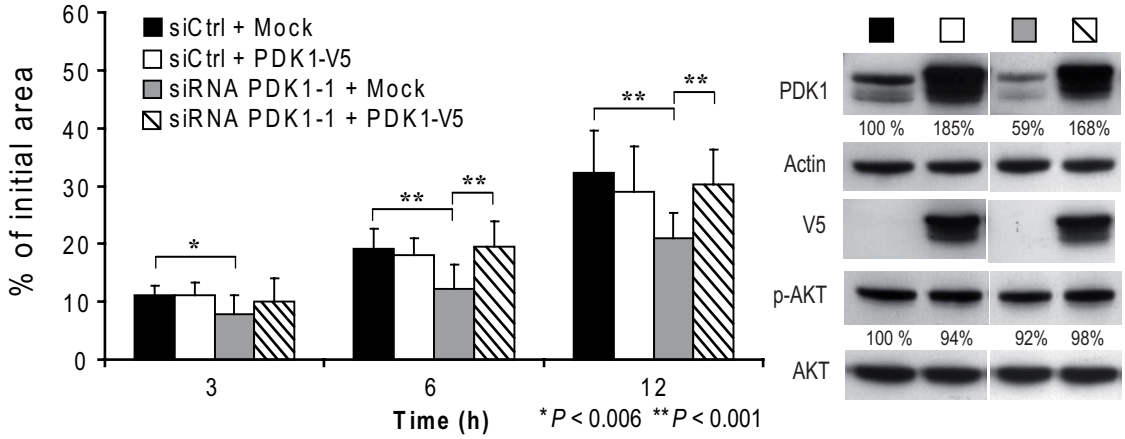
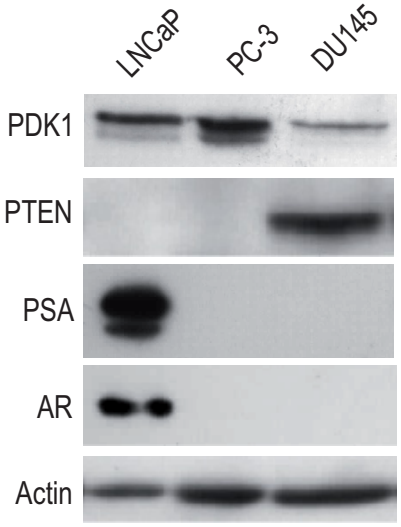

siCtrl + Mock
siCtrl + PDK1-V5
siRNA PDK1-1 + Mock
siRNA PDK1-1 + PDK1-V5
% of initial area
60
50
40
30
20
10
0
3
6
12
Time (h)
*P < 0.006 **P < 0.001
PDK1
100 %
185%
59%
168%
Actin
V5
p-AKT
100 %
94%
92%
98%
AKT

Table 1. 16p13.3 gain, clinicopathological parameters and levels of gain

Clinicopathological parameters (n=46)	Number of cases	16p13.3 status			
		Not gained	Gained		
				Mean % of nuclei with 3 copies	Mean % of nuclei with >3 copies
Gleason score					
< 8	39 (85%)	35 (90%)	4 (10%)		
≥ 8	7 (15%)	2 (29%)	5 (71%)		
			*P=0.02		
Surgical stage					
≤ T2	29 (63%)	25 (86%)	4 (14%)		
≥ T3	17 (37%)	12 (71%)	5 (29%)		
			*P=0.238		
Preoperative PSA ($ng.ml^{-1}$)					
< 10	30 (64%)	27 (90%)	3 (10%)		
≥ 10	16 (36%)	10 (62.5%)	6 (37.5%)		
			*P=0.047		
Tumour type					
Primary PCa	46	37 (80%)	9 (20%)	20.0	3.4
	10 (matched)	6 (60%)	4 (40%)	19.0	13.75
LN mets	10	5 (50%)	5 (50%)	21.2	19.4;**P<0.05‡
CRPC	9	6 (67%)	3 (33%)	20.7	15.0;**P<0.05‡‡

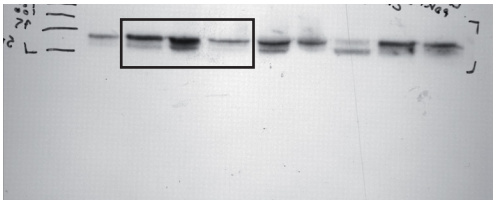
* Fisher's Exact Test
** Mann–Whitney *U* test
‡ LN mets VS. Primary PCa
‡‡ CRPC VS. Primary PCa

Supplementary Figure S1- PDK1 expression in LNCaP, PC-3 and DU145 PCa cell lines.

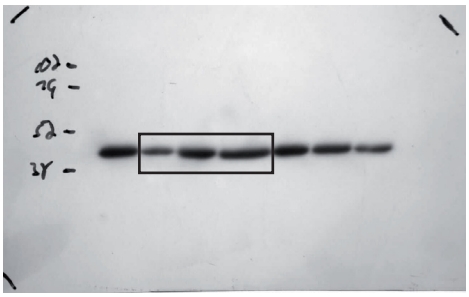


Supplementary Figure S2- Full images of western blots. Inserts show parts used in Supplementary Figure S1.

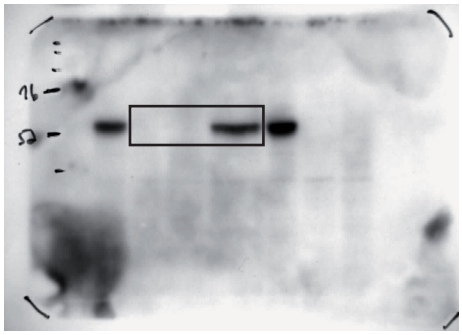
PDK1



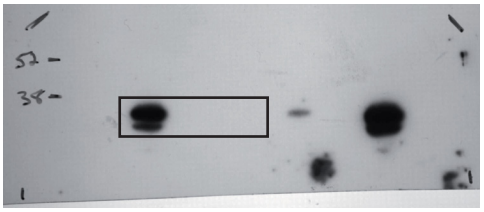
Actin



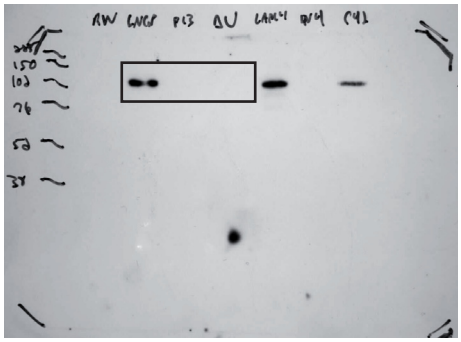
PTEN



PSA



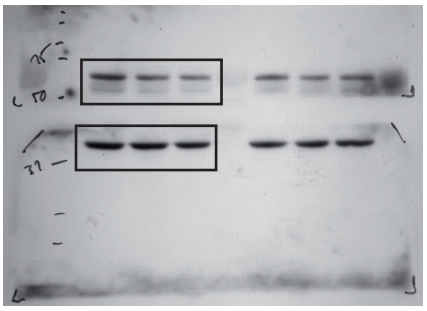
AR



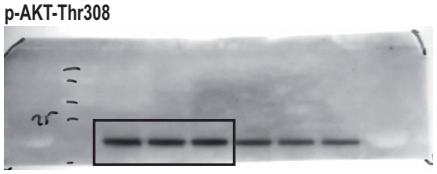
Supplementary data Figure S3 - Full images of western blots. Inserts show parts used in Figure 2C.

PC-3

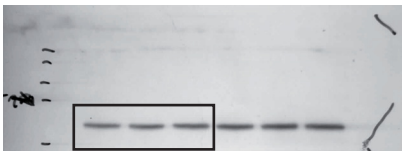
PDK1 and Actin



p-AKT-Thr308

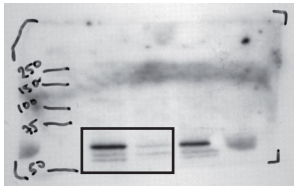


AKT

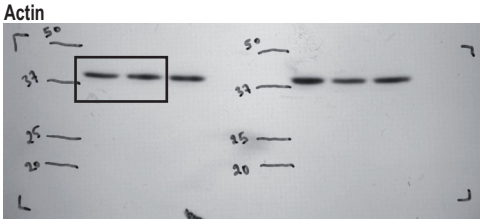


DU145

PDK1



Actin



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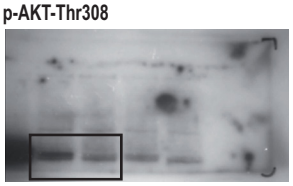
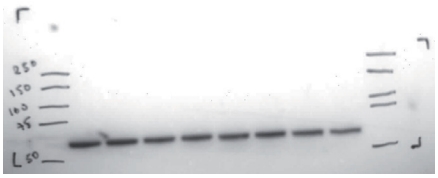
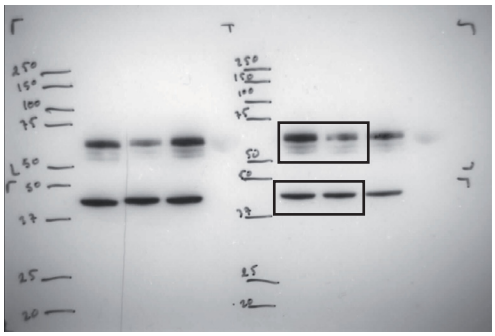


AKT



LNCaP

PDK1 and Actin



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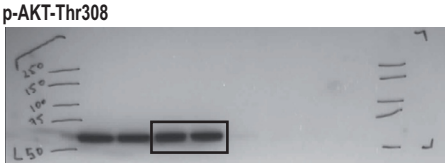
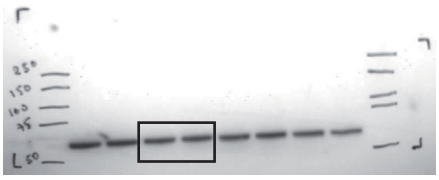


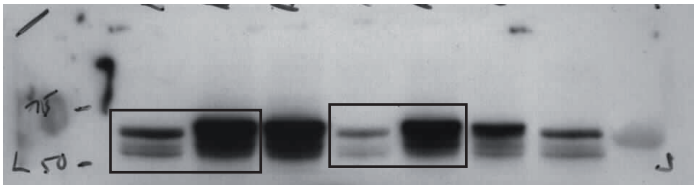
AKT



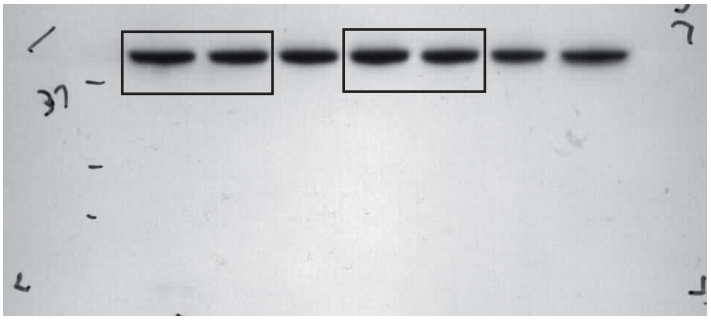
Supplementary data Figure S4 - Full images of western blots. Inserts show parts used in Figure 3.

PC-3

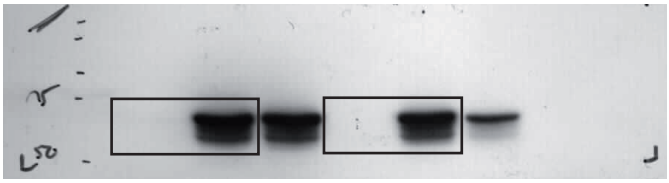
PDK1



Actin



V5



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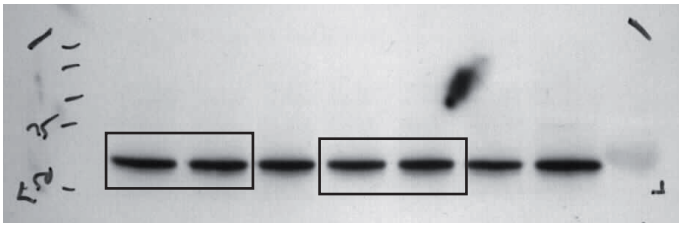
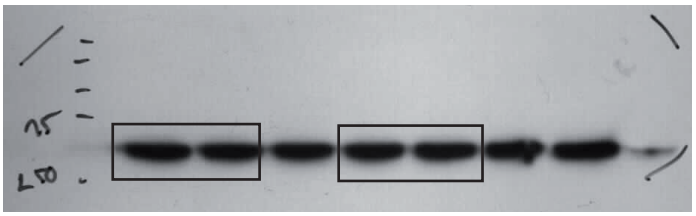


AKT



Supplementary Table 1. List of the BAC probes used in FISH experiments with their corresponding mapped genomic location.

BAC	Mapped Genomic location
RP11-243K18	326,993-491,802
RP11-31I10	1,812,991-1,978,785
RP11-846C9	2,144,245-2,364,301
RP11-20I23	2,485,370-2,643,240
RP11-698H1	2,715,720-2,886,066
RP11-66H6	11,036,514-11,203,598
RP11-548B6	23,671,608-11,203,598

Supplementary Table 2. DNA copy numbers/RNA expression correlation coefficient of 11 genes mapping to 16p13.3 minimal region of gain

Gene	Correlation DNA/RNA
PKD1	n/a
RAB26	0.29
TRAF7	0.06
CASKIN1	n/a
GBL	0.10
PGP	0.19
E4F1	-0.04
DNASE1L2	n/a
DCI	0.19
RNPS1	0.13
ABCA3	0.28
ABCA17	n/a
CCNF	0.22
NTN3	n/a
TBC1D24	n/a
KIAA1171	n/a
ATP6VOC	n/a
AMDHD2	-0.09
CEMP1	n/a
PDPK1	**0.57**

Data from refs 13 and 19 of the paper. n/a: data missing from either DNA or RNA analysis.

3. DISCUSSION AND CONCLUSION

In this thesis, our goal was to assess the 16p13.3 genomic gain as a potential prognostic marker of PCa progression towards metastasis. For that purpose, we surveyed 10 samples of LN mets and matched primary PCa samples for the 16p13.3 genomic gain, as well as 9 CRPC and 46 primary PCa with clinicopathological parameters. We report the detection of the gain in the three types of PCa tissues. *In vitro*, we investigated the role of PDK1 in PCa cell growth and motility and showed it affects PCa motility without affecting growth.

We have confirmed previous a-CGH data of Lapointe and colleagues regarding the detection of the 16p13.3 genomic gain using FISH in LN mets samples (126). The 16p13.3 region of genomic gain was narrowed down to a 0.57 MB region that contains *PDPK1* by mapping of 9 primary PCa samples (Figure 1 D, manuscript). Analysis of the data from Lapointe and colleagues revealed a 2.9 MB genomic region of gain at 16p13, common to 5 patients with LN mets (126). In terms of patterns of gain, the size of the region varied: in one patient, a 0.83 MB region of gain extended upstream the *PDPK1* locus while another had a 9 MB region that begins upstream of *PDPK1* and extends downstream towards the centromeric side. In the a-CGH study, the patterns of gain showed variation as well, and these ranged from 2.9 MB common to all the patients, to 33MB and span the whole 16p chromosome.

Compared to a 2.9 MB region detected by a-CGH, we were successful at more closely defining the region of gain and the genes involved beside *PDPK1*, by FISH. This discrepancy with the a-CGH can be attributed to the fact that our characterization was done on primary PCa tumours while their findings were on metastatic tumours, known to

harbour a higher degree of alterations (126, 173), and to the larger number of samples that we mapped (9 compared to 5).

Our region of gain includes 19 other genes along with *PDPK1*, for 11 of which we have the RNA/DNA correlation that points towards *PDPK1* as a driver of the gain (Supplementary Table 2, manuscript). Among those genes, some have been shown to be involved in cancer and might play a role in PCa progression. For example, RAB26, a Ras oncogene family member is up-regulated in non-small lung carcinoma (264) and uveal melanoma (265). High expression of CCNF (G2-mitosis specific cyclin F) was reported in oesophageal and breast cancers (266, 267), and has been shown to be involved in mediating resistance to chemotherapy in colon and ovarian cancers (268, 269). Thus, one could think that these genes might play a role along with *PDPK1* in PCa progression.

In our work, a clear functional link still needs to be made with the PDK1 protein in PCa specimens. Our investigations were limited by the lack of appropriate antibody against PDK1 in IHC as the available one showed inconsistent and non-reproducible results in our hands. Of note, *PDPK1* genomic status along with PDK1 protein levels were assessed in breast cancer and the study reported that high protein levels were not always indicative of *PDPK1* amplification (176). Still, the amplification was of prognostic value. Given the absence of data on PDK1 protein levels in the surveyed samples, one cannot rule out that *PDPK1* might be a passenger of the gain, although the RNA/DNA correlation indicates that *PDPK1* is likely the driver.

We have assessed 10 pairs of LN mets samples and their matched primary PCa and found the 16p13.3 gain in 5 LN mets and 3 matched primary samples (Figure 1A, manuscript).

Our observations support the idea of 16p13.3 gain as a potential signature for metastatic PCa, detectable in primary samples. Our results are unique in comparison to previous ones as we report for the first time the 16p13.3 gain by FISH in primary samples, matched to LN mets. The detection of the gain in matched primary PCa samples raises the question of clonal origin whereby a certain clone already present in the primary tumour accumulates certain alterations that provide it with a selective advantage to grow and metastasize (270). In our work one could think that acquiring the 16p13.3 genomic gain might be conferring cells with a selective advantage to metastasize to LN. However, in order to establish the genomic gain as a change present in the clone of origin, a larger sample size of LN mets samples and their matched primary PCa samples would be needed. Indeed, we estimate that a sample of 30 LN mets and their matched primary samples are required to achieve statistical significance for $\alpha=0.05$. Furthermore, PCa preferentially metastasize to bone (1, 71), which represents the most advanced stage of the disease. Thus, in order to better understand the role of 16p13.3 gain in promoting aggressive/metastatic disease, it might be interesting to investigate its presence in bone metastasis samples.

Five LN mets samples did not harbour the 16p13.3 genomic gain (Figure 1A, manuscript). In those patients, the occurrence of the 10q23 deletion, which maps to the *PTEN* gene, might offer an explanation for the patients' progression towards metastasis. In fact, results from the a-CGH study (126) showed that 3 of the 4 patients with no 16p13.3 gain had a deletion at the 10q23 (Table 2). In our study, most of the LN mets samples showed the deletion ((169) and unpublished results). PTEN is a negative regulator of the AKT survival pathway (207-209), while PDK1 activates AKT (230) and

plays a role in PCa cell motility (Figure 2, manuscript). Thus one can imagine that acquiring both the deletion and the 16p13.3 gain might have an additive effect that further promotes metastasis, where both alterations upregulate the AKT pathway and PDK1 increases motility.

For patients with the 10q23 deletion and no 16p13.3 gain, one could suspect the existence of other alterations along with *PTEN* deletion. More specifically, the amplification at 8q24 and the deletion at 16q23 (Table 2) were common alterations in LN mets samples. The 8q24 region contains loci encoding FAK and MYC, both shown to be involved in PCa progression (101, 111, 112, 123, 126, 129). The 16q23 region contains two tumour suppressor genes, *ADAMTS18* (174) and *WWOX* (175), which role remains unknown in PCa. It would thus be useful to investigate these alterations in our samples. Finally, other alternative pathways might explain the progression towards metastasis in patients with none of the alterations mentioned above, such as patient PL114 (Table 2).

The 16p13.3 gain was also detected in 3/9 CRPC samples, another evidence for the prevalence of the gain in aggressive disease. Whether the gain actually plays a role in development of CRPC is unknown. It was shown that AKT can activate AR via phosphorylation, independently of androgen availability (271). Furthermore, the 10q23 (*PTEN*) deletion was shown to associate with high AKT phosphorylation and AR expression in CRPC samples (168). Any role for PDK1 in regulating AR signaling is unknown. One could hypothesize that the 16p13.3 gain might increase the levels of PDK1, and consequently the phosphorylation of AKT, leading to the activation of AR. Thus, the finding that 16p13.3 gain is detected in CRPC samples, along with the literature about AKT and androgen receptors constitute an interesting starting point to further

investigate a potential role for PDK1 in mediating androgen-independence. Determining the status of *PTEN* and androgen receptors in our set of CRPC patients should consequently be done.

In our work, the 16p13.3 gain was detected in 20% of primary PCa samples with clinicopathological parameters. In the a-CGH study by Lapointe and colleagues (126), the gain was only detected in lymph node metastasis patients and not in the unmatched primary samples. One possible explanation for this discrepancy, besides the difference in the patient populations, is the use of different detection tools: in our study, we used FISH with a BAC probe specific to the 16p13.3 region. FISH detects specific alteration in a discrete and absolute manner that can be visualized in each nucleus in the sample while a-CGH is only able to detect copy number imbalances relative to other DNA regions in a tissue homogenate (272, 273). Thus the results obtained by a-CGH are affected by the noise in the background and might not be able to detect amplifications in small or few glands because of a dilution effect that results from other cells not showing the alteration.

The gain in primary PCa samples associated with high Gleason score and high preoperative PSA levels (Table 1, manuscript). Furthermore, we noted that the level of gain was significantly higher in CRPC and LN mets samples compared to primary ones (Table 1, manuscript). Most 16p13.3 gains consisted of one extra copy in primary samples, compared to higher levels of gains reported (5 copies) in primary breast cancer (176), which might suggest different biological mechanisms underlying the different natural histories of the two diseases. One could argue that in PCa, one extra copy might be enough to cause an effect on PCa progression.

Genomic alterations have been used to generate prognostic markers. In PCa, the loss of *PTEN* has been shown to correlate with disease recurrence and PCa specific death (169, 274). It would thus be interesting to investigate the co-occurrence of the 16p13.3 gain and the 10q23 deletion in our primary PCa samples and its ability to predict PCa recurrence compared to each alteration separately. We have established that the 16p13.3 genomic gain in primary PCa samples associates with high Gleason score and preoperative PSA levels, both markers of poor outcome. This observation supports the idea of an early signature for advanced PCa. The 16p13.3 genomic gain could be used as a marker for metastatic PCa progression, better coupled with other markers such as the 10q23 and 16q23 deletions and 8q24 amplification. Developing such a panel of markers, including the 16p13.3 gain, could be useful in the context of the molecular heterogeneity of PCa and might prove beneficial to detect patients who might be at risk of progressing towards metastasis. Ultimately, the value of the 16p13.3 gain as a link it to disease recurrence (biochemical recurrence) should be made and compared to established markers. We estimate that 288 samples would be required to achieve statistical significance.

In PCa, no previous study has addressed the role of PDK1 in cell growth and survival. Our results suggest that PDK1 does not affect growth in 3 PCa cell lines, with different genetic background (Figure 2, manuscript). Several studies have shown results that are concordant with our findings where the knock-out of PDK1 did not affect cell proliferation (247, 248, 275). In the study of Lawlor et al (247), results on mice showed that knock-out of PDK1 was lethal at early embryonic development while hypomorphic mice (*PDPK1*$^{+/-}$), were fertile and smaller in size. However, the reduction in size was independent of cell proliferation. These results, similar to our findings, support the idea

that PDK1 does not affect cell proliferation. Other studies have however shown the opposite where knock-out of *PDPK1* gene in immortalized mouse embryonic fibroblasts (MEFs) resulted in the arrest of cell proliferation (276). A study used stable RNAi to target PDK1 and observed a reduction in cell proliferation (176). Similar to our method of silencing, transient siRNA-mediated PDK1 knock-down inhibited proliferation of U-87MG human glioblastoma cells (251).

One might hypothesize that the observed discrepancy might be attributed to the difference between our cell lines of prostatic origins, and cells of other origins. In fact, a study by Ross and colleagues assessed gene expression in a set of 60 cell lines of different origins using gene-expression microarrays (277). They report different patterns of gene expression across the different cell lines depending on the tissue origin, and attribute these differences to physiological variations of the same gene across different tissues. More importantly, they showed that glioblastoma cell lines clustered in a separate distinct branch with a unique gene expression pattern, while DU145 and PC-3 cells that we used clustered with ovarian cancer cell lines. One might thus suspect that the contribution of PDK1 towards the regulation of cells proliferation cells might be more important in other cell lines than in prostate cancer cells.

We have shown that PDK1 down-regulation reduced PCa cell migration in 3 different cell lines (Figure 2, manuscript). In addition to breast cancer, melanoma and endothelial cells discussed in the manuscript, PDK1 has been shown to play a role in motility in a wide spectrum of processes: in inducing chemotaxis *Dictyostelium* (254), in regulating chemokines, in the migration of circulating leukocytes and neutrophils (255, 256), and in neuronal migration (257).

PDK1 knock-down had the most dramatic effect on motility in PC-3 cells. We thus chose this cell line to test the specificity of the observed effect via ectopic re-expression of PDK1 in siPDK1 treated cells. In these cells, ectopic re-expression of PDK1 rescued motility (Figure 3, manuscript). Motility was not enhanced in cells treated with PDK1 re-expression vector without prior treatment with siPDK1. A possible explanation is that PC-3 is a metastatic PCa cell line that already expresses high levels of PDK1 protein and the excess of PDK1 might have no effect since the cells had already reached their metastatic potential.

Knock-down of PDK1 in LNCaP and PC-3 did not affect the levels of p-AKT, while levels of p-AKT were reduced in DU145 cells following PDK1 knock-down. The work of Lawlor et al (247) further supports our observations in PC-3 and LNCaP, as it showed that decrease in levels of PDK1 did not affect the phosphorylation of AKT in response to insulin stimulation. Despite the variation in the AKT phosphorylation status upon PDK1 down-regulation, motility was consistently reduced across the cells lines while growth was not affected. Our observations support the idea that the PDK1-driven motility is independent of the AKT pathway, in concordance with other studies (100, 109, 110, 113). Several studies have however shown the involvement of AKT in cancer cells motility (103, 104, 258). In PCa, a recent study in 2010 showed that inhibition of AKT reduced motility in DU145 but not in PC-3 cells (113), thus suggesting that the effect observed when PDK1 is down-regulated is independent of AKT. It seems that the AKT pathway might not be the main mechanism by which PCa cell motility is controlled.

In this thesis, we did not define the mechanism by which PDK1 could be affecting motility. PDK1 could be mediating motility via its kinase domain (258). To that purpose,

we have developed a K111A kinase-dead PDK1 mutant that will be tested for its ability to rescue motility in siPDK1 treated cells. Based on previous studies, the PH domain seems to be essential for the effect of PDK1 on motility (100, 258) and we have therefore developed a PDK1 mutant that lacks the PH domain. These experiments should help clarifying the mechanisms of PDK1-driven PCa cells motility.

Previous work on PCa cells motility has shown that cells migrate via EMT (113, 278). Whether PDK1-driven PCa cell motility also occurs via the EMT mechanism is unknown. In breast cancer, it was shown that PDK1 played a role in promoting invasion and activation of matrix metalloproteinase, hallmarks of EMT (253). In order to examine the mechanism in question, it would be interesting to assess the levels of E-cadherin, Snail, matrix metalloproteinase production namely MMP-9 and invasion. Another important factor in understanding the mechanism in question is to examine potential partners for PDK1 in motility. The first candidates are the members of the Rac/Rho pathway that lies at the heart of motility and promotes actin-reorganization (279). Rho-associated kinase (ROCK1) has already been shown to interact with PDK1 in a melanoma cell model whereby PDK1 had a non-catalytic activity (100): PDK1 binds with ROCK1 preventing this way the binding of RhoE, a negative inhibitor of ROCK1. ROCK1 then phosphorylates myosin-light chain 2 (MLC-2) in order to activate acto-myosin contraction, needed for cell movement. It would thus be interesting to assess the interaction between PDK1 and ROCK1, as well as the levels of phosphorylation of MLC-2.

Another class is the family of AGC kinases (to which ROCK1 belongs (238)) that share the characteristic of being regulated by PDK1 itself termed the master of AGC kinases

(232). Of this family, RSK (90 kDa ribosomal S6 kinase) has been shown to be involved in cell motility via phosphorylation of FLNA and L1CAM at serine 2152 and 1152 respectively, both involved in regulation of actin cytoskeleton (280). Another set of members are the PKC isoforms, which are under the control of PDK1, have been shown to regulate motility (281-285). One would thus have to test these PKC isoforms and their potential role in PDK1-driven motility. Unpublished work from the lab shows that phosphorylation of S6, the direct target of the 70 kDa ribosomal S6 kinase, was modulated by PDK1. S6K is also a member of the AGC family of kinases and has been shown to be involved in motility and invasion (286-288). One of the studies has shown a direct interaction between S6K and Rac whereby inhibition of Rac prevented the activation of S6K similar to the effect of PI3-K inhibition on S6K activation. Furthermore, the study showed that S6K localized to the actin-rich cortex of migrating cells and that inhibition of S6K using rapamycin reduced cells motility while activation of S6K using nitric oxide donors enhanced it (286). It was further demonstrated that inhibition of S6K reduced hepatocyte growth factor-mediated ovarian cancer cells motility and that a constitutively active S6K on the other hand was sufficient to induce invasion in ovarian cancer cells accompanied by increased proteolytic activity of matrix metalloproteinase 9 (MMP-9) (288). Finally a more recent study (287) clearly showed that activation of S6K promotes epithelial-to-mesenchymal transition assessed by loss of E-Cadherin, and up-regulation of Snail transcription factor as well as an increase in N-Cadherin and Vimentin along with a cellular morphological change. Inhibition of S6K using siRNA was sufficient to reverse this transition. These findings along with ours show that S6K may play a role in PCa motility, especially that levels of p-S6 seem to be constantly modulated by the changes in PDK1 levels of expression.

In order to investigate the mechanism behind the PDK1-driven PCa cells motility, we could assess the levels of EMT markers on our proteins extracts from the WHA experiments. If EMT is the mechanism in question, we expect a decrease in E-Cadherin and an up-regulation of Snail, N-Cadherin, and Vimentin (85). Immuno-precipitation can also be performed to assess PDK1 potential partners such as ROCK1. Furthermore, we extracted enough RNA from the WHA experiments to perform gene expression microarrays to unveil potential new pathways involved in PDK1-driven motility. Results from such experiments might prove to be instrumental in understanding the partners of PDK1 and the pathways that are involved in PDK1-driven motility.

In conclusion, we have reported the detection of the 16p13.3 genomic gain in primary, CRPC and metastatic PCa. More importantly, this gain is localized to a genomic region that maps to *PDPK1* gene, is more enriched in advanced samples compared to primary ones, and is associated with high Gleason score and high preoperative PSA levels. From a clinical perspective, the imperfection of the available prognostic tools remains a considerable obstacle for the proper clinical management of PCa. Our work presents the 16p13.3 gain as a potential marker for lethal metastatic disease, which may be used within a panel of other markers. More importantly, we have shown that this alteration can be detected in archived formalin-fixed paraffin-embedded primary PCa specimens prepared routinely in clinics. FISH is a specific detection tool that is established and commonly used in clinical setting. Further experiments on a larger sample size thus seem be promising in establishing the gain as a prognostic marker of

clinical utility.

To support the biological relevance of 16p13.3 gain, we have shown *in vitro* that PDK1 is involved in regulating PCa cells motility –a hallmark of metastasis- across different cell lines, and seems to be occurring independent of AKT phosphorylation status. Further work has to be done in order to better characterize PDK1's involvement in PCa cell motility in terms of mechanism and potential partners, as well as PDK1's role in other phenotypes leading to metastasis, such as invasion. A better understanding of PDK1's role in promoting aggressive disease might not only serve as a prognostic marker, but will also make PDK1 a potential therapeutic target for the subtypes of patients with aggressive PCa. In fact, several groups have attempted to develop a PDK1 inhibitor as a cancer therapy (reviewed in (259)) and such an inhibitor might hopefully be effective in treating at least a subset of metastatic PCa, currently incurable and lethal.

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