The Role Of QRFP & GPR103 In Human Prostate Cancer

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DECLARATION

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

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ABBREVIATIONS

AI	androgen-insensitive
AR	androgen receptor
BMI	body mass index
BPH	benign prostatic hypertrophy
BSA	bovine serum albumin
DHT	Dihydroxytestosterone
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbentassay
ERK	extracellular signal related kinase
ECM	extracellular matrix
FBS	fetal bovine serum
GPR103	G-protein coupled receptor 103
H2O2	hydrogen peroxide
JNK	c-Jun N-terminal kinase.
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
M-MuLV	moloneymurineleukemia virus
mRNA	messenger ribonucleic acid

p38	protein 38
AGR 2	anterior gradient protein 2 homolog
PBS	phosphate buffered saline
PI3K	phosphatidylinositide 3-kinases
PCa	Prostate cancer
PCR	Polymerase Chain Reaction
PSA	prostate specific antigen
PVDF	Polyvinylidenedifluoride
QRFP	Pyroglutamylated RFamide Peptide
RIPA	radioimmunoprecipitationlysis buffer
RNA	ribonucleic acid17
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TEMED	N, N, N', N'- tetramethylethylenediamine
Tris	tris (Sonn et al.) aminomethane
Tris-EDTA	tris (Sonn et al.) aminomethane, pH adjusted with HCl

ABSTRACT

Prostate cancer (Choudhury et al., 2014) is the leading cause of non-cutaneous malignancy and is the second most commonly diagnosed cancer among men after lung cancer. QRFP is a secreted protein that is extensively expressed in the brain with highest expression levels in the medulla, pituitary gland, cerebellum, retina, and vestibular nucleus; in the periphery it is expressed in the adipose tissue prostate gland, bladder, colon, testis and in the parathyroid and thyroid gland. QRFP is a member of the RF amide neuropeptide family. This family might be implicated in extensive array of biological activities for instance, food intake, cardiovascular functioning, blood pressure, analgesia, aldosterone secretion and locomotor activity, resulting in orexinergic activities. It is suggested that obesity is one of the contributing factors for aggressive form of prostate cancer. There is a strong association between adipokines and aggressive form of various cancers. QRFP has been recently described as an adipokine that exerts its functions via activation of the G protein coupled receptor GPR103. At present the role of the QRFP and GPR103 in prostate cancer has not been explored in detail. I studied the potential role of the adipokine QRFP in prostate cancer. The three prostate cancer cell lines: PC3, DU145 (Orbetzova, 2012) and LNCaP (androgen-sensitive) were used as models for my studies. Nevertheless, no studies to date have investigated the effects of QRFP and GPR103 in prostate cancer. This

prompted me to examine the expression and function of QRFP and GPR103 in the human prostate cells. Also I sought to identify the role of QRFP and GPR103 in prostate cancer. The novel data presented in this study demonstrates that QRFP & GPR103 genes and protein are expressed in both human prostate tissue and prostate cancer cell lines. Expression of both QRFP and GPR103 were higher in PCa tissues compared to controls. Moreover, an ELISA detected circulate QRFP serum levels were lower in human cancer patients compared to benign and healthy group. Stimulation with QRFP induced MAPK signalling cascades, AKT, AGR-2 expression, MMP 2, Caspase-3 and AMPK in PC3 & DU145 cells. QRFP also increased PC3 & DU145 cells migration and invasion, nonetheless QRFP showed induced suppression cell proliferation in PC3, DU145 and LNCaP cells.

CHAPTER 1

GENERAL INTRODUCTION

1 BACKGROUND

1.1 THE NORMAL PROSTATE GLAND

The prostate gland is a part of the male reproductive system involved in production of seminal fluid, an alkaline liquid which is rich in spermine, cholesterol, fibrinogens, fibrinolysin, zinc and acid phosphatase, phospholipids, citric acid and some proteins, however the exact function of the prostate gland still remains unclear. This gland is important in protecting the lower urinary tract from infection and inflammation, and for men fertility. The growth and activity of prostate is dependent on male hormones; androgens (Crawford, 2005). Anatomically prostate gland is situated deep in the pelvis surrounding the proximal urethra, beneath the urinary bladder and facade the rectum. The size of prostate gland differs by age. Normal adult human prostate is typically slightly larger than a walnut and weighs around 20-25 grams. The prostate is comprises of four essential zones based on the glandular components, embryonic origins, and form a patho-physiological basis of benign prostatic hyperplasia (BPH) and prostate cancer (Alshaker et al.).

- The peripheral zone (PZ): Extends along the posterior and lateral surfaces of the gland, consists of 70 % of prostatic volume and is more prone to develop cancer.

- The central zone (Leongamornlert et al.): The area surrounding the utricle and contains the ejaculatory ducts. This constitutes up to 25% of the normal prostate volume, CZ has more smooth muscle than the PZ, which makes this zone comparatively resistant to inflammation and hyperplasia.
- The transition zone (Venkateswaran and Klotz): This covered only 5% of the total prostatic volume. It surrounds the proximal urethra and is the state of the prostate, contains sub- mucosal and mucosal glands that grows throughout life and is responsible for the benign prostatic hypertrophy (BPH).
- The anterior fibro-muscular zone: situated anteriorly and contains no glandular tissue and is made up of only muscle and fibrous tissue. Therefore it does not become hyperplastic (Lee et al., 2011).
- PZ and CZ are referred to as external prostate while TZ and anterior fibro-muscular zone are termed as interior prostate. These zones contain essentially no glandular tissue (Iremashvili et al., 2012b).

Histologically the prostate is divided into two parts glandular tissues and duct, glandular tissue is involved in secretion of fluid and ducts, which consist of 7 % of the gland and the remaining 30 % non-glandular tissue, which is fibromuscularstroma. The fibromuscularstroma covers prostate gland, which is also referred as capsule and is shaped by tubuloacinar

glands and connective tissue comprising smooth muscle. The gland padded essentially with two main forms of epithelial cells: Basal cells and secretory cells within various cells types mediated among basal cells and secretory cells. Basal cells are located at the exterior of the gland under the secretory cells. Secretory cells are cuboid formed with pale to clear cytoplasm. This deep coat is a main feature, as it is missing in malignancy (Iremashvili et al., 2012a) & (Reese, 2016).

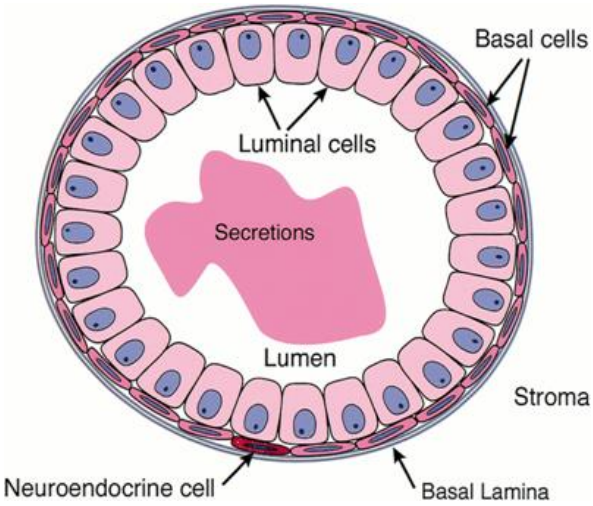

Figure 1.1 Diagram view of the cell types in the interior a human prostatic duct from (Abate-Shen and Shen, 2000).

1.1.1 ANDROGENS:

Testosterone is the main androgen steroid hormone secreted by the testicular leydig cells which is controlled by a complex chain of signals referred to as the hypothalamic-pituitary-gonadal axis. This accounts for the majority of circulating androgen response to luteinizing hormone (Hoogland et al.) about 94 %- 97 %; the remaining androgen production arises essentially from the adrenals and peripheral conversion of estrogen by aromatase (Hiort et al., 1998). Testosterone that circulates in the plasma is essentially bound to one of two kinds of plasma protein sex-hormone binding globulin (SHBGs) or with albumin and only testosterone set free can enters into prostate cells through diffusion. However, the mechanism for testosterone carrying from the leydig cell to the blood stream or lymph is not completely identified (Rove et al., 2012). Testosterone is irreversibly converted into an active metabolic 5 α-dihydrotestosterone (DHT), via enzyme 5- α. After being formed, DHT has a higher binding affinity to the androgen receptor (AR) to control prostatic cellular proliferation and survival, or might be additional metabolized along a number of alternative pathways. The transformation of testosterone to DHT is depending on organs, for instance in prostrate, since DHT is the major biological active androgen. Hydroxysteroid dehydrogenases control ligand the binding of AR in the human prostate (Godoy et al., 2011) & (Amaral et al., 2013).

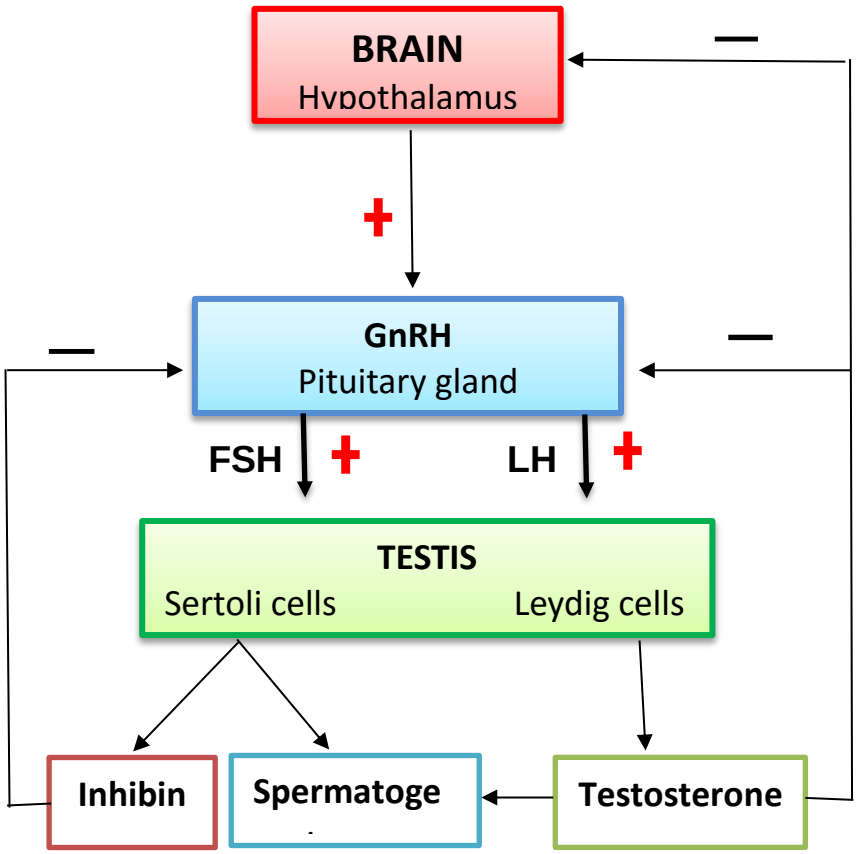

Figure 1.2 Hormonal regulation of the male reproductive system

At the hypothalamic pituitary gonadal axis, GnRH stimulates the release of follicle-stimulating hormone (FSH) which act on the testes to begin spermatogenesis and luteinizing hormone (LH) that secrete testosterone. In turn, the testes production of testosterone and the hormone inhibin prevent the release of GnRH, FSH, and LH in a negative feedback.

Furthermore, testosterone aromatization into estrogen act as a main role in prostate growth by actin interaction with oestrogen receptor B and androgen are essential to regulate the proliferative and apoptosis change that happen throughout normal prostate growth and differentiation. Concentrations of estrogen in prostate stromal tissue are evidently increased in cases of benign prostate hyperplasia (BPH) (Green et al.,

2012). The main role of androgen in normal prostate is to stimulate differentiation of luminal epithelial cells and to control the transcription of genes encoding proteins required for prostate function, for instance prostate specific antigen (PSA). Measuring PSA levels commonly employed for detection and diagnosis of neoplastic malignancies of prostate tissues and response to therapeutic intervention (Green et al., 2012) & (Crawford, 2005). Conversely, PSA is not specific for PCa. Several other conditions can be associated with an elevated PSA level, including benign prostatic hyperplasia (BPH) and prostatitis. Though a PSA level higher than 4.0 ng/mL is generally used as an indication for prostate biopsy, the sensitivity and specificity of the PSA test limits its use as a prostate cancer screening method (Ochiai et al., 2005). In prostate cancer the main function of AR is less obvious, though possibly AR activity modulates the expression of genes related with cell cycle regulation, growth and survival. Irrespective of the mechanism, it is strongly observed that AR signalling plays a critical role in the growth and progression of PCa (Balk and Knudsen, 2008) & (Green et al., 2012). AR ablation therapy has been recently used as an approach to treatment advanced prostate cancer (PCa) and is initially effective in reducing the development of the disease. Nevertheless, it has been observed frequently, PCa appears again as an androgen- tumour with a related life expectancy of only15-20 months (Parimi et al., 2014).

1.2 PROSTATE CANCER (PCa)

1.2.1 EPIDEMIOLOGY

PCa is the leading cause of non-cutaneous malignancy and the second most common diagnosed cancer among men after lung cancer, and is the fifth most frequent cancer globally (rsted et al., 2011). Worldwide around 1,111,689 new cases of PCa were diagnosed in 2012 (Van Rij et al., 2016).The high rates of prostate cancer are recorded in developed countries approximately 3/4 of the cases appear in Australia, New Zealand, Western and Northern Europe and Canada. In 2009 has documented that approximately 192,280 new cases of PCa diagnosed and approximately 27,360 recorded died in Northern America which becomes the highest incidence and significant death (Rosenthal and Sandler, 2010). There are evident differences between the incidences of clinically significant disease universally, nevertheless incident non-clinically pertinent disease is still equal. There are significant variance among northern and southern countries in Europe, with PCa incidence and death being higher in England and Wales and lower in Mediterranean countries (Van Rij et al., 2016). The occurrence rates are comparatively high in certain developing regions such as the Caribbean, South America and sub-Saharan Africa countries. Conversely the lowest incidence is expected in south Asian countries (Sankaranarayanan et al., 2010). The rise in incidence between the developed countries is attributable to numerous

factors. Since the routine examination of transurethral prostatectomy (TURP) histology specimens and practice of serum analysis for prostate specific antigen (PSA) as a sign for PCa has more role to play in the increased incidence mainly in the more developed (Djavan et al., 2011) & (Giles, 2014). However, around 258,000 deaths in 2008, and the sixth leading cause of cancer death. The figure of deaths from prostate cancer is nearly similar in developed and developing regions. Death rates are mostly higher in mostly black populated regions such as Caribbean and sub-Saharan Africa, in contrast it is very lower in Asia. The PCa globally is expected to grow at 1.7 million new cases and 499 000 new deaths in 2030 (Center et al., 2012). Nevertheless, lots of men have prostate cancer with no symptoms therefore does not undergo any specific treatment and finally die of further causes. PCa shares a figure of lineaments with benign prostatic hyperplasia (BPH) and the putative signs of cancer, prostatic intraepithelial neoplasia (Martin et al.). All rise with in spread by men age, all need androgens for proliferate and development, and all react to androgen prevention treatment (Bostwick et al., 2004). The high-grade PIN and early stage cancer is categorized by, aberrations in markers of secretory differentiation, rising nuclear, progressive basal cell layer disorder and nuclear variations, increasing cell proliferation, variation in DNA sequent, and increasing genetic instability. A number of biomarkers demonstrate up-regulation or increase in the development from benign prostatic epithelium to high-grade PIN and cancer, while others are down-

regulated or missing. Present data show that more biomarkers are up regulated; however, the comparative importance of each is unknown. There is a considerable increase in micro-vessel intensity in PIN and carcinoma comparison with normal prostate tissue (Jemal et al., 2003).

1.2.2 ETIOLOGY

The main causes of PCa are still unidentified, however there are several risk factors related with the development of cancers and PCa is no exclusion to this and risk factors are divided into two sections: Endogenous risk factors including (family history, hormones and race) and exogenous risk factors including (diet, environmental agents and other factors) are all involved (Venkateswaran and Klotz, 2010) & (Bostwick et al., 2004).

1.2.3 AGE

Age is one of the significant risk factors; there is a strong association between incidences and mortality of PCa with old age. Appearance of PCa in men under 50 years old is very unusual, with about 60% of cases diagnosed in men over 70 years of age (Macefield et al., 2009). Recent studies confirmed that PCa has a long induction period, and that many men carry the diseases in their 20s and 30s (Gann, 2002).

1.2.4 ETHNICITY

The highest incidence rates of PCa in the USA are in black American men. African American men presented highest incidence rates for PCa than

white American men. Asia is the continent with the lowest incidence universally; Countries for instance China and Japan have comparatively low rates of PCa in native people (Bostwick et al., 2004). The causes for this rates 60% higher risk within African Americans and 38% lower risk within Asian Americans in compare to white Caucasians are still unclear (Ferris-i-Tortajada et al., 2011).

1.2.5 GENETIC FACTORS

Prostate cancer like other cancers is a genetic disorder that begin by the accumulation of chromosomal alterations produced by the clone selection of aggressive cells; about 5-10% of PCa are hereditary. Recent studies, have identified around 100 genetic variants which linked with prostate cancer disease (Stegeman et al., 2015). Some of these genes such as BRCA1, BRCA2, or HOXB13 genes provide proteins that suppression tumor. Inherited alterations in these specific genes, have been linked with increased possibilities of many types of cancers including hereditary prostate cancer (Leongamornlert et al., 2014). Prostate cancer family history increase risk 2 to 3 times for men with first-degree relative by PCa, families with 3 consecutive affected generations, or families with two relations below the 55 ages would be considered for recommendation surgery. It is evident that genetics play a significant role in prostate cancer aetiology (Guy, 2009). On Caucasian families, found that seven prospective genes are recognized implicated hereditary PCa for instance

HPC1, PCAP, CAPB on (chromosome1), HPC2 on (chromosome17), and HPCX on (chromosome 8 and X chromosome) and HPC20 on (chromosome20) (Gronberg, 2003), (Lange, 2010) & (Goh et al., 2012).

1.2.6 ENDOGENOUS HORMONE PROFILE

Androgens have long been involved in prostate carcinogenesis, elevated levels of circulating androgen were found in African-American men compared to white American men. This would make a compelling hypothesis, conversely, the relationship between circulating and intraprostatic levels of androgens and PCa is still under debate (Wirén and Stattin, 2008). Insulin-like growth factor (IGF-1) which was found to have mutagenic and anti-apoptotic effects, are strongly associated with PCa risk (Crowe et al., 2008). Hormone levels might be affected both by exogenous factors; exposure to environmental chemicals and endogenous factors such as genetics can affect hormone activity (Bostwick et al., 2004).

1.2.7 DIET

Diverse dietary factors have been associated in the development of PCa according to epidemiologic literature of migrant populations and geographic variations (Wolk, 2005). However, some prospective studies found no clear association between dietary fat consumption and risk PCa (Crowe et al., 2008) & (Bosire et al., 2013). Many dietary factors and some dietary patterns hold potential in prevent or/ enhance progression risk of PCa which are corresponding with recent dietary guidelines for Americans

(Lin et al., 2015). Several mechanisms have been proposed to explain which dietary fat intake may be possibly linked with PCa: Pro-inflammatory fatty acid metabolites, IGF-1 levels in the serum, free radicals, variation of serum androgens. The high concentration of zinc in normal prostate reduced >90% risk of PCa, but the relation of dietary intake of zinc on PCa is uncertain. An antioxidant found in tomatoes, broccoli, and asparagus, vitamin E, selenium, and green tea regular intake might decrease risk of PCa (Sonn et al., 2005).

1.2.8 PATHOLOGIC MARKERS

95% of the prostate tumors are adenocarcinomas, only around 4% of cases have transitional cell morphology and are thought to arise from the epithelial lining of the prostatic urethra. Few cases have neuroendocrine morphology. These cells are believed to arise from the neuroendocrine stem cells normally present in the prostate (Dan Theodorescu et al., 2009). PCa is different in clinical staging, histopathological tumor growth forms, and survival. Thus, individual assessment of a tumor's aggressive potential is critical for clinical decision-making in men with prostate cancer. Sufficient therapy of PCa fundamentally depends on their single histological kind, stage and grade of malignancy. High grades are related with early and rapid tumor development and consequent metastasis (Hoogland et al., 2014). Low grade and low stage cancers can be treated locally by curative intention such by external or internal radiation, radical prostatectomy, or

subjected to an Active Surveillance (AS) strategy (Böcking et al., 2014). However, the relationship between biopsy and radical prostatectomy specimens still remains a difficult, particularly and notably the under-grading of the biopsy specimen. Grading the malignant prospective of different cancers of the prostate presently is conducted according to the modified Gleason-score according to the International Society for Urologic Pathology (ISUP), on histological sections of biopsies or resected tissue (Fine et al., 2012).

Staging of PCa: Staging of prostate cancer divided into two parts pathological and clinical staging. The tumor, node, metastasis staging (TNM) is widely used staging system for prostate cancer to clinically describe the anatomical range of cancer spread. It divided on 3 main sections of information: T- assesses the tumour, N- spread the malignancy to region and lymph nodes and secondary and M-cancer metastases outside of prostate gland (Cheng et al., 2012).

Gleason Score: The Gleason system is the most prevalent applied pathological grading for PCa as an essential prognostic factor in predicting detections in biochemical failure, radical prostatectomy, lymph node local recurrences, or distant metastasis in patients without treatment, radiation therapy and other therapies, including high-intensity focal ultrasound therapy and cryo-therapy. Moreover, using hematoxylin-eosin (H&E) staining to describe the histological pattern of carcinoma cells (Martin et al., 2011) & (Montironi et al., 2010).

The Gleason score is calculated based on the dominant histologic grades, from grade 1 to grade 5. Adding the two most prevalent pattern grades, yielding a score ranging from 2 to 10, derives the typical score (Veltri et al., 2012). Since there is some indication that the least-differentiated element of the specimen may provide independent prognostic information, the score is normally provided by its separate components such Gleason scores 3 + 4 = 7; or 4 + 3 = 7. Needle Biopsy, first pattern with the highest grade must be recorded and ignoring secondary pattern. Radical Prostatectomy, Gleason score must be depending the primary and secondary patterns with a statement on the tertiary pattern (Blume-Jensen et al., 2015).

1.2.9 TUMOURIGENESIS; CELL PROLIFERATION, THE CELL CYCLE

The complicated balance conserved between cell growth and proliferation factors and apoptosis promoting factors are essential to the regulation of prostate growth. Disturbances in this homeostasis commonly cause the absence of apoptosis and the over activation of factors promoting cell survival and proliferation (Hanahan and Weinberg, 2011). The unavoidable consequence is a dysfunctional signaling pathway leading to tumorigenesis and cancer. Furthermore, cancer cells increase the capability to migration and invasion heterotopic tissues, through bone being the common site of human PCa metastasis (Bononi et al., 2011). Growth and proliferate of normal and cancer prostate cells are controlled

by many growth factor pathways which classified into three distinct groups; insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF) both are mostly stimulators of cell proliferation, survival, differentiation, migration and invasiveness and transforming growth factor-β (TGF-β) responsible apoptosis which is inhibitor prostate growth (Dasgupta et al., 2012). The Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/ mTOR signaling pathways have been shown to play significant roles in the transmission of proliferative signals from membrane bound receptors (McCubrey et al., 2008). Mutations may appear in the genes encoding pathway components including (RAF, RAS, PTEN, PIK3CA, TSC2, AKT, TSC1) or in upstream receptors that activate these pathways. These pathways spread this information by interactions with many other proteins to the nucleus to regulator gene expression (Steelman et al., 2011). Cell proliferation is induced and initiated by the act of growth factors. Cell division comprises of two consequent processes essentially characterized by DNA replication and isolation of replicated chromosomes into two separate cells called cell cycle (Williams and Stoeber, 2012). The cell cycle consists of four distinct phases: G1 phase in which the cell grows and prepares to synthesize DNA, S phase (DNA synthesis), G2 phase (premyotic) proteins are synthesized in preparation for mitosis, and M phase (mitosis) in which cell division occurs. Quiescent phase or G0 is a resting phase where the cell has left the cycle and has stopped dividing (Słonina et al.). Replication of the genomic DNA must be completed before the start of mitosis and is

achieved once in each cell cycle. Genetic disorders in the control of the cell cycle are occurrence in almost human cancers. Main molecules that are implicated cell cycle regulation and apoptosis in PCa cells such as cyclin-depenent kinases (CDKs), p53, p21 and p27 (Koljonen et al., 2006). Through proliferation a cell needs to grow and replicate its genome prior to dividing to generate two daughter cells. Though in cancer cells is essentially a disease in which cells have absent their normal checks on cell proliferation. Cancer cells in order to satisfy the increased supplies of proliferation, often display essential changes in pathways of energy metabolism and nutrient uptake. Many distinct oncogenes such as (AKT src, c-Myc, and H-ras) promot anabolic metabolism in transformed cells (Jones and Thompson, 2009). Prostate cancer initiates when the semen-secreting prostate organ cells transform into cancer cells, proliferating at higher mitotic stages. Originally, the prostate cells begin to proliferate leading to tumor development in the peripheral zone of the prostate gland (da Silva et al., 2013). By the time these cancer cells finally proliferate to further invade neighboring organs, such as the, bladder, seminal vesicles, rectum, and urethra. Through the early metastatic stages, malignant cells from the initial tumor separate from their original location and migrate through blood and lymphatic vessels. In the advanced stages, PCa cells eventually spread to more terminal organs, including bones, liver, and lung. Metastasis is one of the main causes of death in cancer. Metastasis of PCa is consistence of multiple steps, including migration, invasion,

intravasation, circulation and extravasation of tumor cells and then angiogenesis (Jin et al., 2011). Furthermore, metastasis to bone is the most common prostate cancer metastatic (Altieri et al., 2009). Cell migration is crucial steps in several biological processes, including tissue repair and regeneration, inflammation, immune surveillance and embryonic morphogenesis (Condeelis et al., 2005). Abnormal regulation of cell migration leads to progression of several diseases, such as cancer invasion and metastasis (Yamaguchi and Condeelis, 2007). Invasive carcinoma cells gain a migratory phenotype related with increased activation of several genes implicated in cell motility (Bozzuto et al., 2010). Thus, understanding the essential mechanisms of cell migration and invasion are critical for comprehension of both essential biology and the pathology of disease (Yamaguchi et al., 2005). Cancer cells invade throughout the basement membrane and extracellular matrix is the feature of the metastatic process. Each stage in the metastatic process requires distinct molecular interactions between the tumor cells, the cells of the stroma and the extracellular matrix (ECM) (Bozzuto et al., 2010). The interface between normal prostate epithelial cells and the ECM is critical for migration cell proliferation and survival (Jin et al., 2011). Cell migration and invasion are stimulated via a number of chemo attractants, which stimulate intracellular signaling pathways that control reorganization of the actin cytoskeleton. Fundamental to this process is the structural and signaling association complexes between the cytoskeleton and ECM that

are identified as focal adhesions (Chen et al., 2013c). Matrix metalloproteinases (MMPs) have been known act to degrade the ECM. The level of ratios of MMP-2/-9 to tissue inhibitor of metalloproteinases-1 (TIMP-1) In primary tumor prostrate tissues is increased relative to normal prostate tissues (Brehmer et al., 2003) & (Clarke et al., 2009). However, loss of TIMP-1 was associated with up-regulation of MMPs in malignant prostate cancer tissues. Additional, has been found that high concentrations of MMP-2/9 have been detected in plasma patients with metastatic disease (Morgia et al., 2005). Binding of FN to integrin results in MMP-2/9 over-activation through the FAK/ILK/ERK/PI3K/NF-κB pathways, and thus driving to ECM degradation and cancer invasion. Moreover, there are several intracellular molecules including Notch, Sonic Hedgehog, Wnt, NF-κB, Ras/Raf/MEK/MAPK, in addition the ERK/AKT pathways that regulator each side of each of the stages of cancer invasion (Stivarou and Patsavoudi, 2015). Several chemotactic such as EGF influences have been shown to stimulate intracellular signaling pathways that drive to thorny end formation through these mechanisms. Focal adhesion kinase (FAK) is a crucial mechanism of focal adhesion turnover, and is lead to increasing cell migration. A high expresser of FAK and EGFR in prostate cancer PC3 cell lines also associates with increased metastatic possible (Chen et al., 2007) & (Tremblay et al., 1996). PI3K, Rho and Ras activated by Src/ FAK-mediated lead to increased cell proliferation, angiogenesis and migration (Jin, 2011). Activation of Src stmulates cell

migration by different molecules including interleukin 8 (IL-8). Inhibition of Src blocked IL-8 prompted migration in prostate cancer LNCaP cells (Lee et al., 2004). There is an accumulation data describing the function of hepatocyte growth factor (HGF) and its receptor c-Met in PCa development. Interaction of HGF with its receptor and other factors including TGF and TG-b have been demonstrated to modulate tumor cell interaction, cell proliferation, cell migration, cell matrix adhesion, cell invasion, and angiogenesis in PCa cells (Nagle and Cress, 2011). In the regulation of cancer invasion, it has been stated that neuropeptides such as bombesin and vasoactive intestinal polypeptide (VIP) increased the invasive capability of prostate cancer cells PC 3 and LNCaP (Hoosein et al., 1993). However, the mechanism of the influence of these neuropeptides on tumor invasion remains ambiguous. Though, several neuropeptides other than VIP and bombesin are existent in the prostate gland, their influence on cancer invasion has yet to be specified (Nagakawa et al., 1998).

1.2.10 APOPTOSIS AND ITS REGULATION

One of the highly significant advances has been the detection that resistance to cell death, specifically apoptosis cell death, is critical aspect of both tumorgenesis and progress of resistance anti-cancer drug (Johnstone et al., 2008). Although, different apparatuses such as oncogene overexpression, genetic instability, epigenetic modifications,

tumor suppressor down-regulation, loss of cell cycle control and influence of tumor microenvironment result in progress of tumor resistance to cell death (apoptosis) (Hanahan and Weinberg, 2011). Kerr, Wyllie, and Currie in 1972 were first used the term of apoptosis to define a morphologically distinct form of cell death, however some components of the apoptosis notion had been clearly described many years before (Elmore, 2007). Apoptosis known as a programmed cell death, which occurs through the activation of cell-intrinsic or extrinsic dependent mechanism, e.g. exposure to irradiation or treatment with cytotoxic drugs, by DNA damage, or by development death signals (Hassan et al., 2014). Apoptosis happens routinely during growth and aged and as a homeostatic mechanism to preserve cell an inhabitants in tissues (Ouyang et al., 2012). Many morphological changes happens during apoptosis and are characterized by membrane cell shrinking, membrane blebbing, nuclear fragmentation, chromatin aggregation, and degradation of DNA fragmentation (Elmore, 2007). The mechanisms of apoptosis and the associated signalling pathways are extremely complicated which can be initiated in a cell through activation of intrinsic pathway or the extrinsic pathway. The extrinsic pathway is initiated via the activation of the transmembrane death receptors on the objective cell to stimulate apoptosis, for example the ligands of CD95/TNF family induce apoptosis through multi-protein complexes and involves activation of the multiple cascade pathways and are quite well comprehend in molecular terms (Marsters et al., 1998).

In contrast, the intrinsic pathway mediated apoptosis involves mitochondria which is an essential player in the integration of variety of apoptotic signals, containing loss of hypoxia, growth factors, DNA damage, and oxidative stress (Spencer and Sorger, 2011). Caspases are the main initiators and executioners of the apoptosis pathway. So far 14 members of caspases have been discovered and classified into two groups, based on the lengths of the pro-domains. The initiator caspases (caspase-1, -2, -4, -5, -8, -9 and -10) have long pro-domains and function are involved in targeting and regulation activation (Jager and Zwacka, 2010). While executioner caspases (including caspase-3, 6 and 7) have short domains and are responsible to role more downstream in targeting and regulating apoptosis pathway cleaving critical substrates (Parrish et al., 2013).

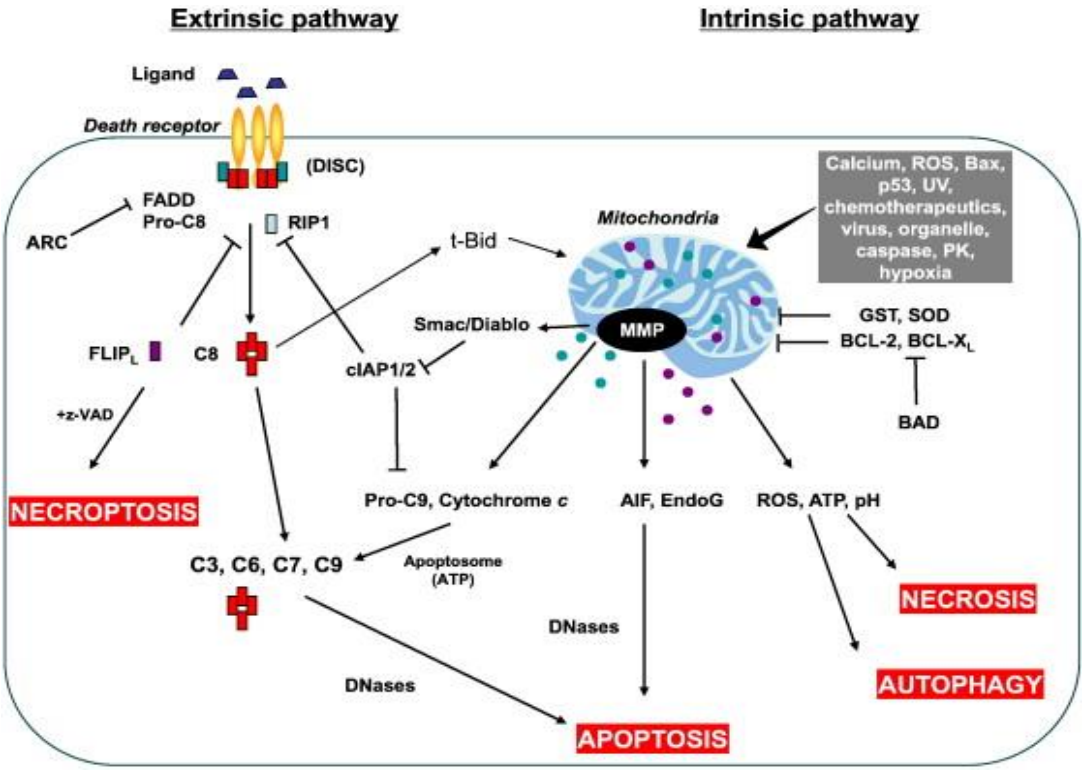

Figure 1.3 The extrinsic and intrinsic pathways of apoptosis: Death receptor pathway (left) is initiated by the ligation of the ligands to their respective surface receptors (Indran et al., 2011).

The two main families of proteins that regulate apoptosis are P53 and BCL-2 family members and are found to have abnormal function and expression in PCa. The main member of apoptotic regulators is transforming growth factor beta (TGF-β) family members that also include BCL2. TGF-β signalling is critical for normal prostate epithelial cell proliferation and induction of apoptosis it acts as a tumour suppressor in initial tumorogenesis, however it promotes tumour growth in later stages of tumour progression (Rentsch et al., 2006). The BCL2 family is one of the main genes known to be associated with prostate carcinogenesis.

1.2.11 BCL-2

The BCL-2 gene family have highlighted the role of proteins as essential regulators of the apoptotic pathway in variety of cells, which are classified based on structural and functional features as either pro-apoptotic group (BAX, BAK, BAD and BID) or anti-apoptotic group (BCL2, BCL-XL, BCL-W, and MCL-1 proteins) (Tzifi et al., 2012). However, BAX families' activation of mitochondria consequence that release of apoptogenic factors including cytochrome C, endonuclease G, Same/Diablo, and apoptosis including factor from the mitochondrial intermembrane space (Du et al., 2000). In prostatic tissues both in normal and hyperplastic, Bcl-2 protein is expressed in the cytoplasm of basal epithelial cells. The occurrence of Bcl-2 overexpression is lesser in localized PCa compared with hormone refractory PCa. The Over-expression of Bcl-2 may assist the PCa cells to live in an androgen-deprived environment, and to accord resistance to antiandrogen therapy (Yoshino et al., 2006).

1.3 MITOGEN ACTIVATED PROTEIN KINASES (MAPK)

To understand the complicated pathogenesis of prostate cancer, the interweave of signalling pathways and cell signal transmissions such as cell survival, proliferation, migration invasion, inflammation and apoptosis, might give a new understanding of the development, progression and inhibition of cancers (Chen et al., 2014a). An extensive different stimuli include growth factors, hormones, cytokines and environmental stresses stimuli cell, through activation of receptor tyrosine kinases (RTKs), by activation of subsequent signalling cascades which play essential roles in cellular processes, such as proliferation, differentiation, migration, apoptosis, cell metabolism, and cell cycle control (Osaki and Gama, 2013). One of main signalling pathway induced by members of RTK family, mediated through; mitogen- activated protein kinase (MAPK) (Pritchard and Hayward, 2013). Mitogen activated protein kinases (MAPKs) family consists of three distinguished group include; extracellular signal-regulated kinase (ERK1/2), p38 isoforms (α/ β/γ/ /δ) and c-jun N-terminal or stress-activated protein kinases (JNK/SAPK) (Correa and Eales, 2012).

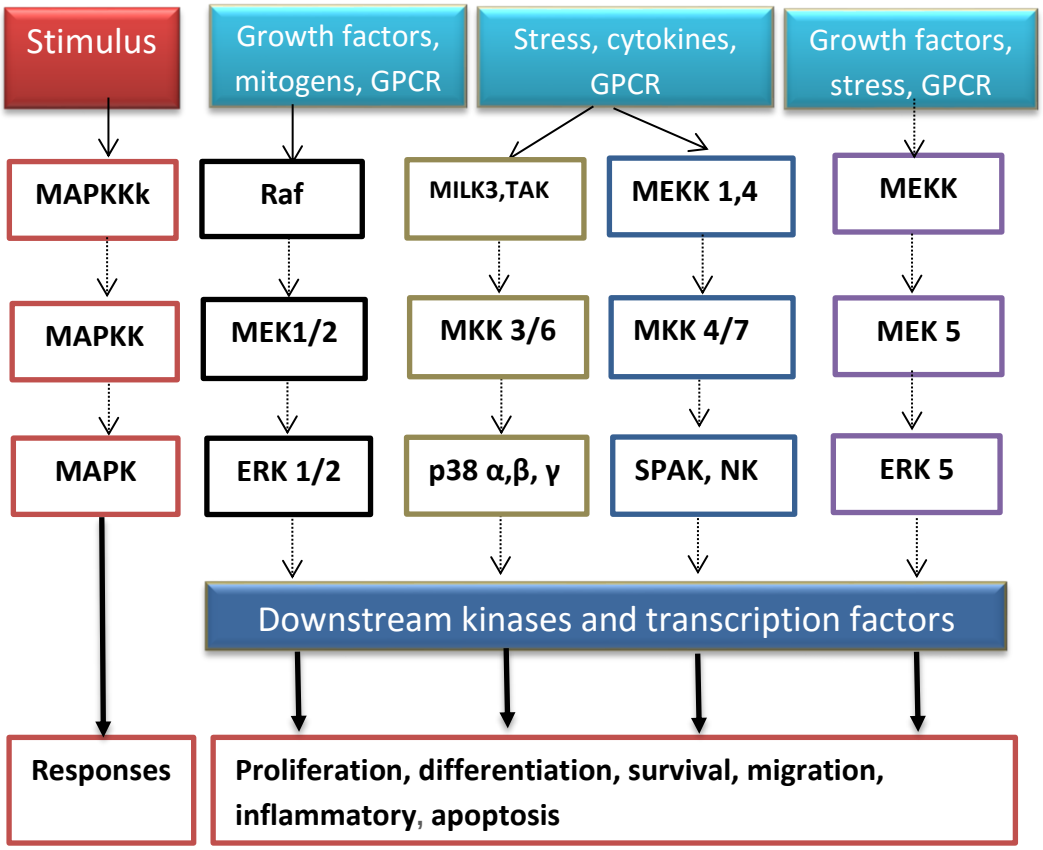

Figure 1.4 Schematic representation of the MAPK cascade activation and potential cross talk signals. MAPK signaling pathways mediate intracellular signalinginitiated by extracellular or intracellular stimuli. Activated MAPKs phosphorylate many substrate proteins including transcription factors, resulting in regulation of a diversity of cellular activities including cell proliferation, differentiation, migration, inflammatory responses, and apoptosis (Osaki and Gama, 2013).

1.3.1 EXTRACELLULAR SIGNAL-REGULATED PROTEIN KINASES (ERK1/2)

The extracellular regulated kinases (ERK1/2) MAPKs are 44/42 kDa serine/threonine kinases, has been strongly related to cell growth, survival, proliferation, cell cycle, invasion, migration, and differentiation in both normal and cancer cells (Saini et al., 2013). Significantly, activation of factors of this pathway is assumed to contribute to tumorigenesis, tumor progression and metastatic disease in a diversity of solid tumors (Manzo-Merino et al., 2014). Nevertheless, ERK can also promote apoptotic regulatory molecules counting bcl-2 family members and caspase-9 (Thakur et al., 2009). The ERK pathway consists of (A-Raf, B-Raf, Raf-1, MEK1/2 and ERK1/2) are strongly activated mostly in response to growth factors, serum, mitogens, ligands for G protein-coupled receptors (GPCRs) and in solid tumors e.g. breast and prostate cancer (Pearson et al., 2001). However, the ERK signaling pathway plays a role in several steps of tumor development. Majority of the signals activating the ERK pathway are initiated by receptor-mediated activation of the small G-protein, Ras -Raf family (Junttila et al., 2008).

1.3.2 p38 MAPKs

The p38 MAPKs contains four members, p38α, p38β, p38γ, and p38δ (Roux and Blenis, 2004). These kinases play roles in cell differentiation, growth inhibition, cell survival, proliferation, and apoptosis (Risco and Cuenda, 2012). Mainly, all p38MAPKs are strongly activated in cells in response to verity of environmental and stress signals, or by pro-inflammatory (TNFα, IL-6 or IL-1) or anti-inflammatory (EGF, TGF-β) cytokines and are weakly activated by growth factors or serum. Activated p38 phosphorylates and controls various transcription factors such as (p53, Elk-1, ATF-2, NF-κB, MEF-2, Max, Mac, or Stat1) and apoptotic mediators as Cdc25A and Bcl-2 (Thornton and Rincon, 2009). p38 has been revealed to promote cell survival in response to stress stimuli, such as in response to DNA damage. Nevertheless, p38α may also have oncogenic functions that are mediated by its involvement in key processes of cancer progression, such as invasion, inflammation and angiogenesis. Several propose that p38 play significant role in several cancers such as prostate, lung leukemia and lymphomas (Whyte et al., 2009).

1.3.3 c-Jun N-TERMINAL KINASE OR STRESS-ACTIVATED PROTEIN KINASES (JNK/SAPK)

The first three mammalian JNK genes were discovered from rat livers injected with cycloheximide JNK1, JNK2, and JNK3 also known as (SAPK-γ, SAPK-α, and SAPK-β) respectively within implicate in growth, morphogenesis, and cell differentiation (Bogoyevitch et al., 2010). JNK proteins, share a threonine-proline-tyrosine (TPY) motif, are activated in response to a diversity of extracellular stimuli, counting, growth factor deprivation, inflammation cytokines (IL-6, IL-1, and TNF), UV irradiation, DNA-damaging agents and mitogens (Pritchard and Hayward, 2013). Depending on the cellular stimulus, JNKs phosphorylate varied substrates, counting transcription factors (p53, AP-1, ATF-2, c-Myc, Elk-1, MLK2) and certain members of the Bcl-2 family (Bode and Dong, 2007). Several studies propose that JNK activity is chronically altered in many cancer types include breast, prostate, lymphoma pancreas and lung cancer (Zhang and Selim, 2012). Nevertheless, studies into the role of JNK in human prostate tissues are rare (Takahashi et al., 2010). Depending on cellular stimulus and types or even JNK isoform, JNKs have opposite function. So, JNKs can induce apoptosis by reduced tumor formation and malignant progression, however also may promote cell survival and proliferation, also involved in regulation of the cell cycle (Rodriguez-Berriguete et al., 2012) & (Lee et al., 2010a).

1.3.4 AKT/PKB KINASE

The serine/threonine kinase Akt also named protein kinase B (PKB) has three isoforms revealed in mammals (Akt1/2/3 -2) encoded by genes (Werden and McFadden, 2010). Akt is a main regulator of multiple cellular processes; include glucose metabolism, proliferation, cell survival, and protein synthesis (Zheng et al., 2012). Therefore many studies proposed that AKT might associate with tumorigenesis by activated phosphatidylinositol 3-kinase (PI3K), which transmits signals from cytokines, growth factors and oncoproteins (da Silva et al., 2013). Furthermore, Akt pathway activated in PCa cells acts as contributing to cancer progression by both stimulation of cellular proliferation, migration, invasion and inhibit apoptosis pathway by inactivate pro-apoptotic factors as Bad and caspase-9 (She, 2013).

1.4 OBESITY RELATED TO PROSTATE CANCER

Obesity is currently a major worldwide concern. It is estimated that above of 2.1 billion people around 30% of world population with overweight or obese and 3.4 million deaths each year worldwide. If its incidence stays on its current curve, approximately 1/2 of adult population global will be overweight or obese by 2030. In Western Europe UK comes behind only Iceland of the highest levels of obesity or overweight 74% of men and 61% of female are either overweight or obese. There are several countries worldwide, including Arabic countries (Libya, Kuwait, Qatar and Egypt) where more than half the female population is obese (Musaiger et al., 2011) & (Musaiger, 2011). Body mass index (BMI) is predictably used measurement in clinical medicine like obesity and population health. However, the issues related to weight gain beyond the accurate capacity of other markers to determine the incidence of obesity have been suggested with body fat percentage being one of the most practical due to its superior capability to stratify patients according to their cardiovascular risks and metabolic (Campbell, 2014). Obesity leads to increases the risk of developing various diseases. Type 2-diabetes, certain types of cancers and cardiovascular disease risk increases due to overweight elevating hypertension and dyslipidemia. 90 % of people with type-2 diabetes are overweight and patients with diabetes are leads to cause of early death, stroke, kidney disease, and blindness (Deshpande et al., 2008). Obesity is

considered one of the main significant features in the progress of insulin resistance, and insulin resistance might lead to type 2-diabetes. Though several studies have reported associations between BMI and risk of individual cancers including that of the prostate, pancreatic, ovarian, breast and colon cancer. There is strong evidence the obesity is an important cause of unnecessary suffering and mortality from many forms of cancer. Also obesity is related with worse prognostic and malignant transformation of epithelial cells (Campbell, 2014) & (Spangler et al., 2007). There are some of biological processes common that could lead to the relation BMI and aggressive PCa. Several hormones, adipokines signaling and androgens implicated in obesity moreover play a role in the initiation and promotion of cancer both at systemic level and a cellular paracrine (Freedland and Aronson, 2004). Although, other studies have found no association between BMI and PCa (Schuurman et al., 2000). To understand the link between obesity and PCa are complicated since obesity is associated not only with excess BMI, however also with altered serum levels of many hormones, as estrogen, testosterone, insulin and insulin-like growth factor (IGF)-1, all of which have to some degree been related to PCa. Furthermore, obesity is highly associated with dietary intake in terms of the quantity of calories in addition to the amount of dietary fat, both of which have been linked to prostate cancer. Obesity might effect on sex hormone binding globulin, testosterone and estrogen, as are hormone levels and growth insulin (Wooding and

Rehman, 2014). High levels of metabolic factors, individually or combined, are not associated to the development of PCa, however are associated to an increased risk of disease progression, although with no proof of synergy between the metabolic factors (Haggstrom et al., 2012). Several studies suggested obese men have lower PSA values. Some men with obese produce less PSA than normal weight men and thus could have late diagnosis of PCa (Kim et al., 2013). However, some studies concluded that BMI is not affected the capability of the PSA level for predict adverse pathologic features, such as seminal vesicle invasion, extracapsular extension, and positive surgical margins across increasing BMI (Banez et al., 2009). Otherwise, obesity has a significant positive associated with prostate volume (PV) in several study populations. Furthermore, obesity is essentially produced by an excess accumulation of adipocyte tissue, and adipocytes are specified for the storage and synthesis of fatty acids (Kwan et al., 2015). Two main types of adipose tissue are known, white and brown, with different origins and functions. White adipose tissue (WAT) plays a role that related to maintaining energy homeostasis through storing triglycerides and releasing fatty acids for energy synthesis, also controls a wide range of functions including glucose and lipid homeostasis, food intake control, immune and inflammatory regulation, or metabolism by secreting a great number of adipokines (Falcao-Pires et al., 2012). The main role of brown adipose tissue BAT is particularly in thermogenesis, the produce of heat essentially mediated by the uncoupling protein-1 (UCP-1),

which produces heat via uncoupling mitochondrial respiration for ATP synthesis (Nieman et al., 2011). Actually, a growing body of evidence has showed the pathogenic roles of adipocytes in the cancer microenvironment, meaning that tumorigenesis implicates constant communication between tumor cells and neighboring normal cells e.g. adipocytes (Nieman et al., 2013). Anew study presented that cancer cells such as breast cancer cell did not yield fatty acids that were unlike from those derived from exogenous basis and both exogenous supplied fatty acids an endogenous synthesized were esterified to the same lipid and phospholipid classes in the same proportions. Might be that fatty acids arising from lipolysis in the peripheral adipose tissue are transferred to the cancer cells for growing (Bing and Trayhurn, 2008). Several studies proposed that cancer cells might use lipids directly from adipocytes as energy source, promoting tumor to grow. It has found that, patients with malignant melanoma, the melanoma cells are found in subcutaneous adipose tissues. PCa cells are exposed to adipose tissue when they invade the retro-pubic fat layer or metastasis to lymph nodes or bone marrow. The extra capsular extension of PCa cells has been stated to metastasis into the per-prostatic adipose tissue. Even other cancers e.g. breast, colon, renal, ovarian, pancreatic and gastric cancer, as well grow in the vicinity of adipose tissues (Nieman et al., 2013). Different mechanisms might obesity promotes cancer development and progressions have been suggested,

including the obesity related low-grade inflammation, insulin resistance, endocrine alterations, and hypoxia–angiogenesis processes.

1.4.1 ROLE OF DYSFUNCTIONAL ADIPOSE TISSUE

Adipose tissue dysfunction result of the interaction between adipocytes and immune cells are linked diseases including insulin resistance, inflammation and cancer (van Kruijsdijk et al., 2009). The role of these factors, including insulin resistance, chronic inflammation, increased levels of plasminogen activator inhibitor-1 (PAI-1), endogenous sex steroids, leptin, and visfatin decreased levels of adiponectin, have been indicated to effect tumor promotion and cancer progression (Prieto-Hontoria et al., 2011).

1.5 ADIPOKINES AND PTOSTATE CANCER

Obesity might increase, many paracrine factors and hormones, which motivate the steroid hormones products in cancer tissue by interact between epithelial compartments and stromal in tissues. Over than 50 hormones and cytokines, known as adipokines and adipocytokines are controlled and secreted by adipose tissue (Dalamaga et al., 2012). One of the paracrine factors is cytokines secreted through both fat cells and white blood cells (WBC). Predominantly there is association between obesity and increased circulating levels of cytokines, and these levels are reduced by weight loss. Several cytokines involved in the progression of tumors to malignancy including growth factors, and transcription factors. Adipose tissue acts as a significant endocrine organ through the synthesis of several adipokines, which regulate insulin sensitivity, control of energy intake, lipolysis and inflammatory processes (Catalan et al., 2013). Adipokines are associated to tumor development and progression due to their many activities on different cell types, essentially by exerting their effects throughout inflammatory pathways. Adiponectin and leptin, visfatin and chemerin are of the most important adipocyte-specific adipokines (Catalan et al., 2012).

1.5.1 LEPTIN:

Leptin, a 16 kDa, 167-amino acid peptide, is produced mainly by adipocytes, which participated to the hypothesis of adipocyte and adipose tissue as an endocrine cell and an endocrine organ. Leptin is one of adipokines that controls metabolism (Pittas et al., 2004). Mainly high levels of endogenous hormones related with obesity, include insulin, insulin-like growth factor I, sex steroids, leptin and have been known as possible mechanisms relating overweight to PCa. In specific, the fat hormone leptin is showed positively associated with PCa. Moreover, leptin levels in the circulate blood appeared to relate directly with BMI and can increase from levels of non-obese subjects was 1–3 ng/mL to e.g. high as 100 ng/mL in obese individuals (Hoda et al., 2012b). However, some studies have suggested that serum leptin levels are higher in obese patients (Tewari et al., 2013). Leptin has a role in the regulation of body weight and influences cellular differentiation and PCa progression. It has been observed that leptin levels are higher in serum obese patients and that leads to increase the risk of influences cellular differentiation and PCa progression. Many latest reports have indicated that leptin acts as a mitogenic factor found in several of human tissues including heart, placenta, kidney, lung, pancreas, prostate, testes, and ovary (Hoda et al., 2012b). The isoforms leptin receptors (ObRa and ObRb through ObRf) have been shown to occur on varied cancer cells (Choi et al., 2005). In vitro, it has been indicated that human PCa express the leptin receptor

and leptin is capable to influence growth of some prostate cancer cell lines (Hoda and Popken, 2008). In vitro studies using PCa cell lines DU145 and PC-3 have proposed that leptin could be involved in PCa cell migration that can influence tumour growth and progression. The MAPK signaling pathway is identified to be significant in cancer cells survival and growing. Therefore, the possible involvement of MAPK pathway in mediating the effects of leptin in prostate cell lines was studied by evaluating the phosphorylation of its downstream ERK1/2. It might be showed that leptin induces ERK1/2 activation in synchronism with increased cell proliferation in prostate cell lines (Samuel-Mendelsohn et al., 2011). Some studies showed that leptin enhance cell proliferation particularly in androgen-independent DU145 and PC-3 prostate cancer cells but not in androgen-dependent LNCaP-FGC cells. However, the effect of leptin on growth of PCa cells is different depending to the androgen sensitivity of the assessed cells (Li et al., 2010a). In contrast one study published that leptin can evidently induce apoptosis in human PCa cell lines. Leptin-induced apoptosis is possibly mediated through balanced activation of p38 MAPK, JAK2/STAT3, and PKC pathways in PCa cells. Exposure prostate PC3, DU145, and LNCaP cells to leptin caused weak, however statistically significant, increase e in the phosphorylation of part signaling pathways involved in cell proliferation and apoptosis including STAT3, JAK2, ERK1/2, and additionally PI3K-Akt (Samuel-Mendelsohn et al., 2011). Furthermore leptin increased cell proliferation, by activation JNK,

and induce inhibition of apoptosis, promoting tumor cell development, invasion and metastasis, supporting the role of this adipokine on PCa (Garofalo and Surmacz, 2006).

1.5.2 ADEPONECTIN:

Adiponectin, was discovered independently by different labs and has four alternative names as Acrp30, apM1, ADIPOQ and GBP28 mainly secreted from adipocytes (Nagaraju et al., 2015). Several studies have suggested the expression of adiponectin in various tissues such as liver, cardiac tissue, fetal tissue, breast milk and placenta, might indicate a probable paracrine/autocrine complementary role for adiponectin (Dalamaga et al., 2012). Adiponectin expression might be increased in many methods most importantly by exercise, calorie restriction, and bodyweight loss (Kadowaki et al., 2014). Compared to other polypeptide hormones, adiponectin circulates at extremely high concentration levels, increasing probability that a smaller cleaved consequence mediates its action on different tissues. The association between adiponectin, inflammation and carcinogenesis is unclear, although one study proposes that adiponectin inhibits transplanted fibrosarcoma growth and infusion inhibits endothelial proliferation (Gialamas et al., 2011). Serum levels of adiponectin and insulin resistance, in compare with other adipokines, are reduced in response to some metabolic dysfunction, such as dyslipidemia, type-2 diabetes, hypertension and abnormal obesity. This proposes that obesity

related diseases might be moderately inverted by weight loss, though latest reports suggest that these changes are relatively small unless there are extreme weight changes, e.g. severe calorie constraint (Roshanaei-Moghaddam et al., 2009). In contrast to most adipokines, hence diponectin is evidentially influence reduces obesity and increase with long fasting and losing weight. Low adiponectin levels and insulin resistance have been associated to several obesity-related disease entities, such as type 2 diabetes mellitus, hypertension, and atherosclerosis. The main role of adipokines is to assistance maintain metabolic homeostasis, however extended roles for adipokines have demonstrated their capability to modulate inflammation, proliferation angiogenesis, and apoptosis (Vansaun, 2013). Two isoforms of adiponectin receptors were known as Adipo R1 receptors are mainly expressed in muscle and Adipo R2 receptors are mostly expressed in the liver (Izadi et al., 2012). Furthermore, it has found that PCa cells express mRNA of AdipoR1 and AdipoR2, cell surface receptors for adiponectin. These results lead to a suggestion that adiponectin inhibits growth PCa growth (Miyazaki et al., 2005). AdipoR1 and AdipoR2, are activated many intracellular signaling pathway such as 5’-adenosine monophosphate-activated protein kinase (AMPK), nuclear factor-κB, peroxisome proliferator-activated receptor-α, and p38 mitogen-activated protein kinase pathways (Wanninger et al., 2009). Adiponectin at physiological concentrations inhibits growth of PCa cell lines androgen-independent (DU145, PC3) and androgen-sensitive

(LNCaP) by stimulate phosphorylation and activation of the AMPK pathway (Bub et al., 2006). The hormone inhibits acts as a direct endogenous inhibitor of inflammation and angiogenesis and reduces cancer invasiveness by inhibiting the activity of matured phagocytic macrophages that have been linked to PCa. Obesity increases insulin secretion and insulin resistance. Furthermore, it can decrease adiponectin levels and AMPK activity that increase the risk of PCa (Kim et al., 2009) & (Izadi et al., 2012). The obesity -related up-regulation of insulin secretion and down-regulation of adiponectin and, as a result, down-regulation of AMPK, are sensible mechanisms whereby obesity directly or indirectly impacts PCa progression (Li et al., 2010a). Adiponectin inhibits cell proliferation and induces apoptosis. Significantly, adiponectin is a mighty inhibitor of PI3K/AKT/mTOR that is able to decrease growth cancer cells exerted through insulin and growth factor-induced signaling (Hebbard and Ranscht, 2014). Additionally, adiponectin can stimulate JNK activation in PCa cells (PC-3, DU145, and LNCaP). Additionally, extremely suppress constitutive STAT-3 activation in DU145 and HepG2 cells (Jarde et al., 2009).

1.5.3 VISFATIN:

Visfatin also known as nicotinamide phosphor ribosyltranferase (NAMPT) or pre-B-cell colony-enhancing factor 1 (PBEF1) was identified as an adipokine produced by visceral and subcutaneous fat tissues, monocytes,

hepatocytes, and macrophages (Friebe et al., 2011) & (Laudes et al., 2010). Thus, several studies presented increased levels of circulating visfatin in obesity, cardiovascular disease and diabetes mellitus. However, other studies presented lower levels of visfatin in these diseases (Prieto-Hontoria et al., 2011). Regrettably only limited studies have researched the effect of visfatin on cancer cells in vitro and in vivo. Increased expression of Visfatin/Nampt/PBEF was discovered in various types of cancers including ovarian cancer, malignant astrocytoma, the human PCa cell lines, androgen-insensitive (PC3) and androgen-sensitive (LNCaP) as well as in human PCa, among specific relevance and emphasis in an obese people (Patel et al., 2010).

1.6 QRFP:

Of growing significance is illuminating the role of neuropeptides in the androgen-independent progress of prostate cancer. However, neuropeptides are secreted by neuroendocrine (NE) cells. The possible contribution of the prostate microenvironment to cancer progress becomes significant. Neuropeptides are mediators of neuronal signaling in nervous systems that have essential functions in regulation of physiological processes and human behavior (Nassel and Winther, 2010). A main challenge in neurobiology is to comprehend the evolutionary and functional significance of neuropeptide in human (Mirabeau and Joly, 2013). Almost all of the biologically active peptides that end with the sequence RFamide. However, neuropeptides described by a joint carboxy-terminal arginine (R) with an amidated phenylalanine (F) designated C-terminal Arg–Phe–NH2 (RFamide) motif have been identified in vertebrates. Presently five types of RFamide peptides recognized in mammals can be subdivided into five groups which are derived from various precursor protein: (Sven Cnattingius) Gonadotropin-inhibitory (GnIH) group, (2) Neuropeptide FF (NPFF) group, (3) Pyroglutamated RFamide peptide (QRFP) group, (4) kisspeptin/metastin group and (5) Prolactin-releasing peptide (PrRP) group (Elphick and Mirabeau, 2014).

1.6.1 RFamide PEPTIDE RECEPTORS

Several orphan receptors become clear to respond to various members of the RFamide family. The PrRP receptor was the first known RFamide receptor that was recognized and originally described as GPR10. Later, the putative receptors for NPFF and NPAF were recognized and termed NPFF1/GPR147 and NPFF2/GPR 74. The kisspeptin receptor was previously revealed as orphan G-protein coupled receptor GPR54 (Fukusumi et al., 2003). Several peptides termed QRFP (26), QRFP (43), P518 or P550 that have similarities with the C-terminal Arg-Phe-NH2 peptide family (RFamide), Individually, two types might expression that both, 26RFa and 43RFa, act like natural ligands of the orphan receptor GPR103 (Kampe et al., 2006). Following studies have discovered that 26RFa and the longer form of 26RFa, named 43RFa (QRFP) family, were isolated from the human hypothalamus and spinal cord (Bruzzone et al., 2006). QRFP is a secreted protein that is extensively expressed in the brain with highest expression levels in the, medulla, pituitary hormone regulation, cerebellum, retina, vestibular nucleus and, as well expressed in the prostate gland, bladder, colon, artery, coronary, testis, thyroid and parathyroid (Findeisen et al.) & (Bruzzone et al., 2006). However, QRFP might be implicated in extensive array of biological activities for instance food intake, cardiovascular functioning, blood pressure, analgesia, aldosterone secretion by the adrenal gland, stimulating metabolic rate, locomotor activity and cancer metastasis. Moreover, 26RFa is proposed to

stimulate bone formation (Baribault et al., 2006). The molecular weight of QRFP is about 15 kDa. The QRFP receptor is an orphan G-protein coupled receptor and has been initially cloned in human brain cDNAs (Primeaux, 2011).

1.6.2 QRFP PEPTIDE RECEPTOR (GPR103)

Several receptors are main goals for regulating hypothalamic–HNS and -HPA axis activity (Gloriam et al., 2007). The central nervous system (CNS) are consists four main groups (A) Ionotropic receptors as the excitatory glutamate N-methyl-d-aspartate (NMDA), or inhibitory GABAA receptors that produce a membrane pore to permit the stream of ions, nuclear receptors counting sex steroid, glucocorticoid and thyroid hormone receptors that control transcriptional activation, (C) receptor tyrosine kinases as tyrosine kinase receptor type and the epidermal growth factor receptor (EGFR), which stimulate intracellular signaling networks as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway and (E) G-protein-coupled receptors (GPCRs) (Hazell et al., 2012). G-protein-coupled receptors (GPCRs) are involved in many diseases such as, obesity, diabetes, hypercalcemia, hypertension, psychotic disorders hypothyroidism, retinitis pigmentosa and cancer, and are therapeutic targets for about half of all for the pharmaceutical used in humans (Vilardaga et al., 2010). It is also one of the major and most functionally and structurally varied in the human genome. Many studies

published that around 880-1000 known GPCRs are identified in the human genome (Suga and Haga, 2007). Furthermore, the 7TM receptors can be clustered into five families: class A (rhodopsin family), class B (adhesion family), class C glutamate family, class D (frizzled/taste family) and class E (Secretin family) (Katritch et al., 2013). Predominantly of GPCRs includes receptors for signals and small molecules such as light, hormones, amino acids, neurotransmitters, odorants, and change in size from small molecules tpeptides to big proteins (Baribault et al., 2006). The common structural feature of GPCRs is seven transmembrane 7-TM domains joined by alternating intracellular and extracellular loops through interaction by heterotrimeric G-proteins (α, β and γ subunits) as single monomers (Kuszak et al., 2009). Two main signal transduction pathways are involving with G protein–coupled receptors the cyclic AMP and phosphatidylinositol. Additionally activated GPCRs interact by cytosolic arrestins (B-arrestin-1/2), which organise uncoupling G-protein receptor in space and time, stimulate receptor internalization and modulate different distal signaling responses such as the MAP kinase cascades (Kamal et al., 2011). However, great number of GPCRs still known as “orphan receptors” whose cognate ligands has not yet been identified. Identification of ligands for orphan GPCRs provides a foundation for comprehending the physiological roles of those GPCRs and their ligands, which can implicate the central nervous, metabolic systems, cardiovascular, reproductive, endocrine, immune, inflammatory and digestive. Initial data indicate that

GPCRs play main roles in the pathogenesis of cancers. Some GPCRs are over- substantial activated in many cancers, and their abnormal signaling is complicated in various stages of tumorigenesis and metastasis (Lee et al., 2001a) & (Takayasu et al., 2006). GPR103 also named (QRFPR, AQ27, SP9155), originally recognized in 2001, which is part of GPCRs class (A) and activated via the (QRFPs) (Xiao et al., 2014). GPR103 demonstrates similarities with orexin, neuropeptide FF, and cholecystokinin receptors. Its mRNA has been discovered mainly in the brain including the, thalamus, hypothalamus, pituitary, basal forebrain, cerebral cortex, and pons in humans. The long form of GPR103 was cloned from hypothalamus cDNA library and a genomic library (Jiang et al., 2003). GPR103 encoded a 455 amino acid protein and phylogenetic analysis showed that the orphan receptor shares characters with peptide-binding receptors. In human, 26RFa/QRFP has been discovered to be a high-affinity endogenous ligand for the earlier identified orphan GPCR, GPR103 (QRFPR) and commonly activates the G_q/G_i signal transduction pathway (Ukena et al., 2014). Considered a ligand for the G- protein coupled receptor GPR103, QRFP may possibly have orexigenic act, thereby acting as an appetite stimulant (Ukena et al., 2010). Orexins originally considered, as neuropeptide appear to be expressed in peripheral tissues. However, newly neuropeptides Orexins acting at the GPCR orexin receptor 1 (OX1R) have indicated to suppress the progress of colon cancer cells via stimulating apoptosis throughout

cytochrome C release from mitochondria and Caspase activation (Laburthe and Voisin, 2012).

1.7 HYPOTH ESIS

This interesting discovery of regulation of neuropeptides on cancer, suggest that play a role in cancer pathophysiology either directly or indirectly. In addition, with obesity playing a role in cancer pathogenesis it is timely that peptides that regulate energy homeostasis may also play a role in caner biology. This leads me to investigate the role of other neuropeptides, such as QRFP on prostate cancer.

1.8 AIMS

- To identify and explore the potential expression and function of QRFP and its receptor GPR103 in human prostate cancer tissue and cell lines (PC3, DU145 and LNCaP).
- To identify the functional effects of QRFP on prostate cancer cell lines.
- To study the effects of QRFP on intracellular signalling pathways in PCa cells.
- To demonstrate the regulation of QRFP and GPR103 by adipokines and hormones on prostate cancer cells.
- To measurement the QRFP levels in human serum blood.

CHAPTER 2

MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 EQUIPMENT

‘Mr. Frosty’ cryo-freezing unit: Fisher Scientific, Loughborough, UK.

Fuji medical X-ray film: Fuji Photo Film Company, Tokyo, Japan.

NanoDrop spectrophotometer: Labtech International, Ringmer, UK.

Spectrophotometer (Beckman DU640): Beckman Instruments Inc, California, and USA.

2.1.2 BIOCHEMICAL REAGENTS AND KITS:

Bovine serum albumin (BSA): Sigma-Aldrich, Gillingham, UK.

CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit: Promega, UK.

Ethidium Bromide: Sigma-Aldrich, Gwellingham, UK.

Ammonium persulphate (APS): Sigma-Aldrich, Gwellingham, UK.

10X protein running buffer: Department of Biological Sciences, University of Warwick – Media Preparation.

Protein gel running buffer: Department of Biological Sciences, University of Warwick – Media Preparation.

10X protein transfer buffer: Department of Biological Sciences, University of Warwick – Media Preparation.

Polyvinylidene difluoride (PVDF) membranes: Millipore, Billerica, MA, USA

N, N, N', N'- tetramethylethylenediamine (TEMED): Sigma-Aldrich, Gwellingham, UK.

30% Acrylamide: Bis Acrylamide (37.5:1): GENE FLOW, Fradley, UK.

PH 7.8 Tris-EDTA buffer: Sigma-Aldrich, Gwellingham, UK.

Halt™ Protease Inhibitor Single-Use Cocktail (100X). Life Technologies, a Thermo Fisher Scientific brand, UK.

PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa. Life Technologies, a Thermo Fisher Scientific brand, UK.

PrimerDesign Ltd Precision Reverse Transcription kit: Primer Design Ltd, Southampton.

Radio-immunoprecipitation Assay or lysis buffer (RIPA): Sigma-Aldrich, St. Louis, MO, USA.

TBE agarose gel running buffer- Department of Biological Sciences, University of Warwick – Media Preparation.

TE (Tris-HCL/EDTA) buffer- Department of Biological Sciences, University of Warwick – Media Preparation.

Tris Buffered Saline (TBS) containing 0.1% TIen-20: Department of Biological Sciences, University of Warwick – Media Preparation.

Tris-HCL [tris (hydroxymethyl) aminomethane, pH adjusted with HCl]: Sigma-Aldrich, Gwellingham, UK.

YO-PRO® (Life Technologies, Carlsbad, CA, USA).

Hoechst® stain (Invitrogen, Eugene, OR, USA).

β-mercaptoethanol: Sigma-Aldrich, Gillingham, UK.

SYBR® Select Master Mix, Life Technologies, a Thermo Fisher Scientific brand, UK.

Trypsin solution: Department of Biological Sciences, University of Warwick – Media Preparation.

Camptothesin: BioVision, California, USA.

GenElute™Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA).

Bis-chorionic Acid (BCA), Sigma Aldrich, Dorset, UK.

Anti-GPR103 antibody (Acris Antibodies, Herford, Germany).

Anti-QRFP-43 antibody (Phoenix Pharmaceuticals, Belmont, CA).

2.1.3 CELL CULTURE MEDIA AND CELL LINES

HAMS-F12 and RPMI-1640 culture media was purchased from Sigma-Aldrich (Gillingham,UK) and from Invitrogen (Paisley, UK) respectively.

Fetal Calf Serum (FCS) was from (Fisher Scientific, UK).

Phosphate Buffered Solution (PBS): Department of Biological Sciences, University of Warwick – Media Preparation.

Becton Dickinson (BD) Falcon™ 25, 75 and 175cm2 cell culture flasks were used for all cell cultures as appropriate.

Antibiotic-Antimycotic (100X) from (Gibco, Life Technologies).

Human prostate cancer cell lines (PC3, DU145 and LNCaP) were obtained from the American Type Tissue Association (ATCC, Manassas, VA, USA).

2.2 METHODS

2.2.1 COLLECTION OF HUMAN SAMPLES

Human prostate tissue was obtained from men undergoing prostate procedures (e.g. radical retro-pubic prostatectomy (RRP), transurethral resection of the prostate (TURP) and transrectal ultrasound (TRUS) and prostate biopsy) at University Hospital, Coventry (University hospitals Coventry & Warwickshire (UHCW) NHS Trust). A favourable ethical opinion was obtained from the NHS National Research Ethics Service (North West 3 Research Ethics Committee - Liverpool East) prior to tissue collection. Local Research & Development UHCW NHS Trust) approval was also obtained prior to the commencement of tissue collection at University Hospital, Coventry. Men undergoing prostate surgery for either benign or malignant conditions or undergoing a prostate biopsy for suspected cancer either with an elevated age-specific PSA or with an abnormal prostate investigation were approached for potential inclusion into the study. Patients were given a full explanation of the nature of the study including the potential benefits and risks of taking part. Potential participants were also given an information sheet (see Appendix A) with which to make an informed decision about their inclusion in the study. Patients who were willing to be recruited for inclusion in the study were asked to sign a consent form. Patients received a signed copy of the consent form (see Appendix B) and a further signed copy of the consent

form was subsequently filed in the patient records. In some cases, patients had catheters in situ and so this was noted at the time of recruitment. The patients' medical and drug history was taken as well as any prior treatment, if any, for prostate cancer. During TURP procedures and TRUS & prostate biopsies prostate tissue was collected and immediately snap frozen in liquid nitrogen and stored at -80 ^{o}C until use. Radical prostatectomy specimens were removed en-bloc and were formalin-fixed-paraffin-embedded as per standard hospital practice. Tissue was available for use after the hospital pathologist had issued a final pathology report of the specimen for grading/staging purposes.

2.2.2 CELL CULTURE

Prostate cancer cell lines (PC3, DU145 and LNCaP) cells were cultured, as per manufacturer's instructions, in Hams-F12, and RPMI media respectively, supplemented with 10 % fatal calf serum (FCS), 5 ml of 100x Antibiotic-Antimycotic and cultured in 75 cm^2cell culture flasks. Flasks were incubated in a humidified incubator at 37°C in 5 % CO2 and routinely passaged at approximately 70-80 % confluence (examined by microscopic inspection). Prior to each experiment in all tests, cells were used between the third and twenty-five passages only

2.2.3 SUBCULTURE OF ADHERENT CELLS:

Cells were washed with PBS. 2-3 ml of Trypsin EDTA solution were added to the flask (T75 cm^2) and incubated at 37 ^{o}C for 3-5 minutes until the cells

detached. 8-10 ml of fresh medium was transferred into the flask to block further trypsinization. After centrifugation at 1000 rpm for 5 min, the cells were re-suspended with fresh medium. A part of the cell solution was taken and transferred into a new flask containing 15 ml of growth media for further culturing. Every 2 –3 days the media was replaced with fresh media.

2.2.4 RESERVATION OF CELLS:

Cells were prepared as described previously for splitting and then centrifuged at 1000rpm for 5 minutes. The cells were re-suspended in cryo-protective medium (5% dimethyl sulfoxides DMSO, 95 % FBS). 1ml of the cell suspension was dispensed into a cryogenic vial and then stored in a cryo-freezing container at -80 ^{o}C. After 48 hours, the cells were transferred into liquid nitrogen.

2.2.5 CELL COUNTS USING HEMOCYTOMETER:

Haemocytometer was used to determine the cell density (cells/ ml) of medium and hence the appropriate volume of cell suspension to use when freezing cells or plating cells out prior to experiments. The haemocytometer was cleaned by using 70 % ethanol solution and the cover-slip placed in the correct position on the glass slide. The appearance of Newton's rings, rainbow-like interference patterns, which are visible on holding the haemocytometer up to the light, indicated that the cover slip was in the right position. The cell suspension was mixed and 50 µl removed and mixed with an equal volume of Trypan Blue solution that penetrates the

membrane of nonviable cells, staining them blue. The haemocytometer chamber was filled by placing a Gilson's tip containing the cell suspension and Trypan Blue mixture at the junction of the cover-slip and the counting chamber. The haemocytometer was then viewed under a microscope. The scored area was brought into focus and under low magnification. Generally, cells in the left-hand and top grid markings were included while those in the right hand and bottom markings were excluded. The total number of viable cells was then calculated as follows:

Total number of viable cells **=** Total cell volume **x** 2 (dilution factor in Trypan Blue) **x** mean number of unstained cells (unstained count/2) **x** 10^4. If the cell count was low, more than two $1mm^2$ areas were counted and averaged.

2.3 TOTAL RNA EXTRACTION AND COMPLEMENTARY DEOXYRIBONUCLIC ACID (cDNA) SYNTHESIS

Total RNA was extracted from human prostate cancer tissue and cell lines PC3, DU145 and LNCaP using Qiagen RNeasy plus Mini Kit. RNA samples were treated by RNase-free DNase to eliminate genomic DNA contamination and the concentration quantified. The purity and quantity of the extracted RNA was measured by a Nano-Drop spectrophotometer. Generally, 1 microgram of total RNA was reverse transcribed into cDNA, by using Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase and random hexamers as primers.

2.3.1 REAL TIME QUANTITATIVE PCR

The sequences for the sense and antisense primers respectively are listed in (Table 1). SYBR Green® real-time PCR was performed on an ABI 7500 fast real time PCR using the primers listed in table 1. PCR was performed using 4 µl (1:5 diluted) cDNA in 10 µl Master Mix, 4 µl water and 1 µl sense and antisense primers. A series of three dilutions for each cDNA was used to ensure linear amplification and to measure primer efficiency. For analysis, expression of genes of interest were normalised against the expression of the housekeeping gene 18s RNA or GAPDH. Negative controls for all the reactions included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. The relative mRNA levels were expressed as a ratio using the "2-∆ct method" for comparing relative expression results between treatments in real-time PCR. All gene expressions were measured by real-time PCR, using 1 µg total RNA and oligo-dT primers as RT primers.

Table 2.1 Primer Sequences of the sense and anti-sense primers.

Gene / product size	Forward primer	Reverse primer
hQRFP (136bp)	5'-AGGCAGGACGAAGGCAGTGA -3'	5'-GACCGAAGCGGAAGCTGAAG-3'
hGPR103 (140bp)	5'-CCAGTCTACCGCTGTTGTGA-3'	5'-GCCAGACCACACCTAGCATT-3'
GAPDH (190bp)	5'-TGC ACC ACC AAC TGC TTA G-3'	5'-GAT GCA GGG ATG ATG TTC-3'

2.3.2 SEQUENCE ANALYSIS

The PCR products samples were purified from the 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). PCR products were then sequenced in an automated DNA sequencer, and the sequence data were analysed using Blast Nucleic Acid Database Searches from the National Centre for Biotechnology Information, confirming the identity of the products.

2.4 WESTERN BLOTTING

2.4.1 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentrations were determined calorimetrically using the BCA (bicinchoninic acid) protein assay kit.

2.4.2 MAKING: SDS-PAGE gel

Using Bio-Rad mini Protean II Gel system 8 % or 10 % SDS-PAGE gel was made up, respectively, according to manufacturer's protocol. First the resolving gel was prepared by adding 30 % Acrylamide: Bisacrylamide (37.5:1),1.5M Tris-HCL tris (hydroxymethyl) amino-methane, pH adjusted with HCl], pH 8.0), pH 8.8, 10% SDS, deionised water, 10% ammonium persulfate (APS) and N, N, N', N'- Tetramethylethylenediamine (TEMED). The mixture was poured into the casting system to gain the length of gel required, leaving sufficient space for the stacking gel and then allowed to set at 20 ^{o}C for 30 minutes. Apart from 1.5 M Tris-HCl, pH 8.8 (substituted by 0.5 M Tris-HCl, pH 6.8), the stacking gel was prepared using all of the reagents as described above. The mixture was subsequently applied on top of the pre-set resolving gel and a comb was placed to allow Well formation. The gel was allowed to set at 20 ^{o}C for 20 minutes. The gel was located into the electrophoresis running system and adequate running buffer was added to the tank.

2.4.3 WESTERN BLOTTING OF CELL LYSATES AND CONDITIONED MEDIA

Sample preparation

Western blotting was used to assess protein levels in cell lysates and conditioned media from both treated and untreated prostate cancer cell lines. Protein lysates were prepared by adding equal amounts of radio-immunoprecipitation lysis buffer 1x RIPA buffer to disrupt cells & tissues, centrifuged at 8,000 rpm for 5 minutes and resulting protein lysates were quantified using a BCA (bicinchoninic acid) protein assay kit. 5 µl of each cell supernatant or standard was added to 96 well plate and a 195 µl of a mixture of CuSO4, PBS and BCA solution, was added to each well (4 µl CuSO4, 5 µl PBS and 200 µl of BCA solution) each well or sample. Followed by incubation for 20-30 minutes at 37°C and the absorbance was measured using a plate reader (Multiskan Ascent 96/384 Plate Reader) at 570 nm. Samples were adjusted to equal amounts using standard 2X Laemmli buffer and placed in a boiling water bath for 5 min, and then allowed to cool at room temperature. The proteins were separated by SDS-PAGE and transferred to poly vinylidene difluoride (PVDF) membranes at 100 V for one hour in a transfer buffer containing 20 mM tris, 150 mM glycine, and 20 % methanol. PVDF membranes were blocked in tris buffered saline (TBS) containing 0.1 % TIen-20 and 5 % BSA for one or two hours. The PVDF membranes were incubated with the relevant

primary antibodies overnight at 4°C. On the following day, membranes were washed thoroughly four times in 60min with TBS-0.1% Tween before incubation with the appropriate secondary antibody for one hour at room temperature. Antibody complexes were visualized using chemiluminescence ECL^{+}. The appropriate positive and negative controls were utilised. The densities were measured using a scanning densitometer coupled to scanning software ImageQuant™.

Table 2.2 Table demonstrating antibodies used for western blotting with concentrations and relevant secondary antibodies.

Primary antibody	Concentration	Secondary antibody	Concentration
Phospho – AKT	**1: 1000**	**Anti – rabbit**	**1: 5000**
Phospho –QRFP	**1: 4000**	**Anti – rabbit**	**1: 12000**
Phospho – GPR103	**1 :3000**	**Anti – rabbit**	**1: 8000**
Phospho – P38	**1: 1000**	**Anti – rabbit**	**1: 2000**
Phospho – JNK	**1 :1000**	**Anti – rabbit**	**1: 2000**
Phospho – AGR2	**1: 1000**	**Anti – sheep**	**1: 4000**
Phospho– Csapease-3	**1: 1000**	**Anti – rabbit**	**1: 1000**
B-actin	**1: 15 000**	**Anti – rabbit**	**1: 10 000**

2.4.4 MAPK- (ERK1/2, p38 and JNK1/2 MAPK) AND AKT ACTIVATION ANALYSIS

Prostate cancer cell line PC3 and DU145 cells were serum starved overnight seeded on to 6 well plates., and then treated with or without QRFP 100 nM for different time pointe (0 = Basal, 5, 15, 30 and 60 minutes) for MAPK ERK, P38, JNK and AKT studies and incubation with QRFP for 24 hours to detect downstream signalling pathways. Cells were then lysed with RIPA buffer and adjusted to equal amounts using Laemmli buffer, mixed, sonicated, boiled, centrifuged (5,000 rpm for 3 minutes), and stored at -20 ºC until use. 15-25 µg total protein per well was loaded onto 10 % SDS-PAGE gel. Following this, proteins were transferred onto PVDF membranes, blocked in tris buffered saline (TBS) containing 0.1% TIen-20 and 5 % BSA for one hour. The PVDF membranes were incubated with primary antibody for phosphorylated proteins (ERK/ p38/ JNKs/ Akt) overnight at 4°C respectively (table 2.2). Membranes were washed thoroughly for 60min with TBS-0.1% Tween before incubation with the secondary antibody for one hour at room temperature. Antibody complexes were visualized using chemiluminescence ECL prime. The densities were measured using a scanning densitometer coupled to scanning software Image Quant™.

2.4.5 STRIPOING AND RE-PROBING MEMBRANES WITH TOTAL ANTIBODIES

DENSITOMETRY ANALYSES OF WESTERN BLOTS

The membranes were submerged in stripping buffer 10 % SDS (1M Tris-HCl, pH 6.8, β-mercaptoethanol) and incubated at 50oC for 30 minutes with occasional gentle agitation followed by washing with TBS-0.1 % Tween at room temperature 10 minutes twice. PVDF membranes were blocked in tris buffered saline (TBS) containing 0.1 % TIen-20 and 5% BSA for one hour at room temperature. The PVDF membranes were re-probed with primary antibody for total ERK, p38, JNK, Akt and GAPDH 1:1000 dilution overnight at 4°C. On the following day, membranes were washed thoroughly for 60min with TBS-0.1 % Tween before incubation with the secondary antibody for one hour at room temperature. Antibody complexes were visualized using chemiluminescence ECL prime. The densities were measured using a scanning densitometer coupled to scanning software Image Quant™.

2.5 PHOSPHO-KINASE ARRAY

The Human Phospho-Kinase Array was achieved to analyzing profiling protein phosphorylation at the cellular level, which is fundamental to understand how the intracellular signaling pathways respond to changes external and internal stimulations. This array allows for the simultaneous detect of the relative phosphorylation of 43 proteins.

2.5.1 SAMPLES PREOARARTION

Twenty -four hours after culture PC3 and DU145 Cells in T75 cm^2 flask, cells were starved for 24 hours and then stimulated with QRFP (basal, 1, 10 and100nM) for different time point 5, 15, 30 and 60 minutes. Cells were washed with PBS and lysed by Lysis Buffer provided with the kit (R&D Systems) mixed with protease & phosphatase Inhibitor cocktail and then rocked gently for 30 min at 4 °C. The supernatant lysate is collected by microcentrifugation at 14,000 × g for 5 min and the protein concentrations were quantified using a BCA (bicinchoninic acid) protein assay kit.

2.5.2 ARRAY PROCEDURE

The Human Phospho-Kinase Array was separated into 2 parts of membranes (A & B) and the nitrocellulose membranes were blocked with the given blocking array buffer. These were later incubated with cell lysate overnight at 4 °C on a rocking platform shaker. Later membranes were washed three times with 1x wash buffer for 10 minutes on a shaker to remove boundless proteins. Membranes (A&B) were incubated with antibody A and B cocktail respectively for 2 hours then followed by further washing with 1x wash buffer x3. Then membranes were incubated with a mixture of biotinylated detection antibodies and streptavidin HRP secondary antibodies. Chemiluminescent ECL Prime exposure reagents were applied to detect spot densities. Blot array images were examined by using the GeneTools image analysis software (Syngene). The averaged

density of duplicated spots representative each phosphorylated kinase protein was defined and used for the relative alterations in phosphorylated kinase.

2.6 PROLIFERATION, MIGRATION AND INVASION ASSAY

Several techniques and kits are available to use to measure many aspects of cell proliferation, cell viability such as MTS assay. The tetrazolium compound MTS assay is a quantitative colorimetric assay and highly sensitive method for assaying cell proliferation and survival in mammalian. Cell migration and invasion in addition to proliferation, are significant facets of many critical biological processes. Monitoring of cell migration and invasion is critical in biomedical research, in order to know the pathways regulating migration and invasion, and to develop agents that control these processes. Some reports require credible assays for monitoring migration, and invasion and several are available, including the “scratch assay” and the “transwell assay.

Similarly, the xCELLigence CIM assay might be used to investigate possible therapeutics and study the fundamental biology of invasive and migratory processes. Nevertheless, in contrast to classic transwell assays, this CIM assay may follow migration and invasion in real-time at minute or second intervals automatically with no need for exogenous labels. This technique has been shown to be extremely positive when working with

primary cells. It has been recommended that the CIM assay eliminates the variability and provide flexibility, accuracy, fast, high quality results for less time and clear data presentation. The migratory cells requirement attaches to the microelectrode to be identified, thus the CIM assay is not suitable with non-adherent cells or cells grown in over each other as LNCaP cells. Though, coating the membrane with ECM proteins or using certain chemokine to increase cell attachments might overcome this (Iqbal et al., 2013). Real time cell proliferation, migration and invasion experiments were performed using the 'xCELLigence' system (ACEA, Biosciences, San Diego, CA, USA), which comprise of the Real Time Cell Analyzer Dual Purpose (RTCA-DP) instrument was placed in a humidified incubator maintained at 5 % CO2 and at 37°C, E-plate 16 for proliferation while CIM plates (Cell migration & invasion) (Roche Diagnostics GmbH). As cells attached to the bottom of the well and grow over, the RTCA software (Roche) monitors and reports as change in Cell Index (Rove et al.2012). CI is a measure of cell number, cell attachment, and cell increasing.

2.6.1 MTS PROLIFERATION ASSAY

Cell proliferation was determined with CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit. PC3 and LNCaP cells were trypsinized and cultured on 96-well plates overnight in a humidified incubator at 37˚C in 5% CO2 environment. The media was aspirated and replaced with 100µL of serum-free media HAMS-F12 and RPMI-1640 medium containing

1% FCS respectively and treated with different concentrations of QRFP 1 nM, 10 nM and 100 nM for 24 and 48 hours. Media supplemented with 10% FCS was used as a positive control. Following QRFP treatment, 20 µl MTS reagent was added to plate containing 100 µl of culture medium in each well. The absorbance at 490 nm was recorded using an ELISA plate reader (EL800, Bio-Tek Instruments, Inc, Winooski, VT, USA). The percentage of the absorbance was calculated against untreated cells.

2.6.2 xCELLigence PROLIFERATION ASSAY

For proliferation assay 100 µl of media was added to each well of the E-plate 16 and scanned to check all connections are ok. Background measurement was taken before plating the cells. PC3 and DU145 cells were split as described above and counted on hematocytometer. Aroun100 µl of cell suspension (6x103) cells were seeded in each well. Plate was left for 30 mints at room temp under the hood for the cells to settle down before being put on the SP/DP system. After 24 hours' incubation, cells were treated with QRFP 0 nM=basal, 1 nM, 10 nM, 100 nM and 10 % FCS as positive control (5 µl). Plate was put on the plate reader, until the end of experiment, which was in total 24 and 48 hours.

2.6.3 xCELLigence INVASION ASSAY

For invasion assays, most the stuffs including; Eppendorf tubes, CIM plate assembly tool, matrigel and tips, were placed in the fridge, 24 hours before the experiments and all experiments were carried on ice. Complete media

was changed with serum free media at least 4 hours before starting the experiment. CIM plate was scanned to inspect connections were satisfactory. Matrigel was diluted at 1/15 using cold serum free media. 50 µl of matrigel was added to create a 3D biomatrix film in each well prior to cell seeding of the upper chamber of the CIM plate and the excess 30 µl coat of matrigel was removed and plate was left for 4 hours in the hood. 160 µl pre-warmed media with QRFP (0 nM, 1 nM, 10 nM and 100 nM) also EGF 50 ng/ml as positive control were added to the lower chamber of the plate. Both the upper and bottom chambers were carefully placed together. Additional 30 ul of serum free media was added to the top chamber. Plate was loaded on RTCA, connections were checked and left to equilibrate inside the chamber. Background measurement was taken after 1-3 hours. Plates were taken out and 100 ul of PC3 and DU145 cells were added to upper chamber and left in the hood for 30 mints to settle down the cells. Plate was loaded on the RTCA for 8 hours.

2.6.4 xCELLigence MIGRATION ASSAY

For migration assays, the same method was followed without Matrigel steps.

2.7 APOPTOSIS ASSAY

Many critical methods of apoptosis markers include chromatin condensation which uses YO-PRO-1 dye stain to distinguish between normal and apoptotic cells from a cell population. Propidium iodide and Hoechst 33342 are often used together for flow cytometric or fluorescence imaging analysis of the stages of apoptosis and live cells. Other apoptosis appears in the cell nucleus, such inter-nucleosomal DNA fragmentation and finally chromatin condensation. Several of the assays used to detect apoptosis investigate the characteristic DNA fragmentation that occurs through apoptosis. DNA fragmentation ELISA assay was used as a biochemical hallmark of late stage apoptosis to illuminate apoptosis in both the extrinsic and intrinsic apoptotic pathways (Hooker et al., 2012).

2.7.1 Vybrant® APOPTOSIS ASSAY

PC3 and LNCaP cells were harvested and plated in 24-well plates and incubated 24 hours for the apoptosis assy. Cells were replaced and incubated in serum-free media for at least 4 hours prior to commencement of cell treatments with 0nM= (basal), 1 nM, 10 nM and 100 nM QRFP, with Hydrogen peroxide (1:200 000) used as positive control and 100 nM leptin as negative control. After the individual time period had elapsed cells all cells were labelled by Hoechst 33342 ® stain (Invitrogen, Eugene, OR, USA) for 30 minutes before to the adding of the nucleic acid stain YO-PRO® (Life Technologies, Carlsbad, CA, USA). Two independent time-

points were used for the apoptosis assay: 24 and 48 hours. YO-PRO® is an iodine-based stain that only labels cells that are identifying apoptotic cells and does not label living cells wile Hoechst for live cells, thus allowing detection of cells undergoing apoptosis using (fluorescent microscopy). YO-PRO® allows assessment of apoptosis without interfering with cell viability. Cells were labelled for an additional 30 minutes with YOPRO® and were washed twice later with PBS warmed solution. Plates were imaged using the GE healthcare IN Cell Analyzer 1000. Number of apoptotic cells was analysed using the formula below:

$$\frac{\text{YO-PRO® - labelled (apoptotic) cells}}{\text{Hoechst® - labelled (total) cells}} \times 100 = \% \text{ rate of apoptosis}$$

2.7.2 DNA RAGMENTATION ELISA

This kit detects BrdU-labelled fragments released from degraded cells in a sandwich ELISA format. Apoptosis levels were measured using Photometric Enzyme-Linked ImmunoSorbent Assay (ELISA) for detection of 5-bromo-2′-deoxy-uridine (BrdU) labelled DNA fragmentation in cell lysates. About of 10,000 of PC3, DU145 and LNCaP cells were cultured in 96 well plates for 24 hours independently. 10 µM of BrdU reagent was added to label the cells overnight following this cells were incubated with QRFP (1, 10 and 100 nM) for 24 and 48 hours. Camptothecin, DNase and H2O2 (2 µM) were used as positive control. 100 µl of anti-DNA coating solution was added to each 96 well plate and

left to incubate at 37 ºC for 1 hour. The plate was then washed three times with wash solution. The final wash solution was then left in situ prior to irradiation in a microwave oven for 5 minute to allow denaturing of the immunocomplex BrdU-labeled DNA-fragments. 100 µl of Anti-BrdU antibody peroxidase conjugate (Anti-BrdU-POD) was then added and incubated for 90 minutes which interacts with the BrdU-labeled DNA to form an immunocomplex. After washing the plate 100 µl of substrate solution was added and incubated in the dark for 20 minutes before adding 20 µl of stop solution and incubating for an additional 5 minutes and then measured the plate at 450 nm. The results were calculated as values comparative to basal and analyzed by Graphpad software for statistical significance.

Table 2.3 The Human Phospho-Kinase Array coordinates.

List of phosphokinase and phosphorylation site used in assay		
Akt 1/2/3 (S473)	Akt 1/2/3 (T308)	Fgr (Y412)
AMPK alpha1 (T183)	AMPK alpha2 (T172	Fyn (Y420)
beta-Catenin	Chk-2 (T68)	GSK-3 alpha/beta (S21/S9)
c-Jun (S63)	CREB (S133)	Hck (Y411)
EGF R (Y1086)	eNOS (S1177)	HSP27 (S78/S82)
ERK1/2 (T202/Y204, T185/Y187)	FAK (Y397)	HSP60
JNK 1/2/3 (T183/Y185, T221/Y223)	Lck (Y394)	Lyn (Y397)
MSK1/2 (S376/S360)	p27 (T198)	p38 alpha (T180/Y182)
p53 (S15)	p53 (S392)	p70S6 Kinase (T421/S424)
PDGF R beta (Y751)	PLC gamma-1 (Y783)	PRAS40 (T246)
Pyk2 (Y402)	RSK1/2/3 (S380)	Src (Y419)
STAT2 (Y689)	STAT3 (S727)	STAT3 (Y705)
STAT5a (Y699)	STAT5a/b (Y699)	STAT5b (Y699)
STAT6 (Y641)	WNK-1 (T60)	TOR (S2448)
Yes (Y426)	GSK-3 alpha/beta (S21/S9)	PLC gamma-1 (Y783)

2.8 SERUM COLLECTION

Human blood samples (serum) were collected following ethical approval from 3 patient groups: healthy controls, benign prostatic hypertrophy (BPH) and prostate adenocarcinoma (PCa). Samples were stored at -80 ^{o}C and were thawed slowly on ice. Circulating levels of human QRFP were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using the QRFP-26 (Human) - EK-048-76 EIA Kit (Phoenix Pharmaceuticals, Inc USA) and employed as per manufacturer's protocol. The assay employs a competitively enzyme immunoassay technique.

Five Serial dilutions were prepared giving concentrations ranging from 100 ng/ml- 0.01 ng/ml. Air was used as blank. 50 µl of undiluted samples were added to each well of standards, positive control, 25 µl primary antibody and 25 µl biotinylated peptide. All samples were measured in duplicate and incubated for 2 hours at room temperature. The plate was washed 4 times with 350 µl/well of 1×assay buffer. 100 µl/well of SA-HRP solution were added and incubated for 1 hour at room temperature. Following plate second washing 100 µl/well of TMB substrate solution was added and incubated at room temperature for 1 hour. Terminate reaction with 100 µl/well of stop solution (2N HCI) was then added directly and mixed thoroughly using a plate rocker. Absorbance O.D. was then measured by using a micro-plate reader (Tecan Group Ltd. Männedorf, Switzerland) at 450 nm and analyse results.

2.9 STATISTICAL ANALYSIS

All results presented are expressed as the mean ± S.D. A student's t-test was used to difference between two groups. Comparisons between more than two groups were analysed by ANOVA (non-parametric) analysis followed by post hoc Tukey's multiple comparison test. Values were considered to be statistical significance set at either *, $p < 0.05$, **, $p < 0.01$ or ***, $p < 0.001$. All statistical analyses were performed using Graph Pad software (La Jolla, CA, USA).

CHAPTER 3

THE EXPRESSION OF QRFP AND ITS RECEPTOR (GPR103) IN PROSTATE CANCER CELL LINES AND TISSUE

3 INTRODUCTION

The QRFP is a member of RFamide family of neuropeptides, characterized by their common protein C-terminus consisting of an arginine (R) and an amidated phenylalanine (Crawford). QRFP exerts its functional effects through activation of Gprotein coupled receptor GPR103. The human QRFP gene was discovered using a search of the DNA sequence information. To date only one type of GPR103 has been identified in human (GPR103), and two types of receptors GPR103a and b has been identified in rodents. Initial studies using protein quantification and quantitative PCR analysis showed QRFP and its receptor (GPR103) were abundantly expressed in the brain include hypothalamus, cortex and spinal cord. QRFP is strongly conserved hypothalamic neuropeptide that has been characterized in various species. In 2013 a group of researchers demonstrated that GPR103 is also expressed in human prostate cancer tissues and its expression significantly increases with progression of cancer. However, the studies to date have demonstrated conclusively the effect of QRFP on prostate cells.

Appetite is commonly influenced in advanced cancer patients leading to anorexia and consequently insufficient food intake (Dwarkasing et al., 2014). The pathogenesis of anorexia is multifactorial and is associated to disorders in any of these complex interaction including physiologic, gastrointestinal, neuronal system and endocrine mechanisms as well as

nutritional factors (Adams et al., 2009). Furthermore, cytokines, hormones, and chemicals derived from the tumour are found in the circulation and might prompt a various set of neurochemical events causing an imbalance between food intake and energy expenditure in cancer anorexia (Ihnatko et al., 2013). Anorexia in cancer patients is thought to be caused by the inefficiency of food intake regulating systems in the hypothalamus to react adequately to negative energy homeostasis during cancer development (Dwarkasing et al., 2015). Under normal conditions food intake and energy consumption are controlled by complicated, redundant, and distributed neural systems involving thousands of genes and reflecting the essential biological significance of sufficient nutrient supply and energy homeostasis (Lenard and Berthoud, 2008). The hypothalamus controls a number of body functions, including feeding behaviour by different mechanisms, where interconnecting nuclei, particularly the arcuate nucleus (ARC), through interaction of peripheral hormonal and metabolic signals control all processes relating to regular energy homeostasis (Sedlackova et al., 2012). The hypothalamus is the major regulator of feeding behaviour and receives information from peripheral hormones for instance peptide YY, glucagon like peptide-1, ghrelin and adipose signals including leptin and insulin and integrates input from several brain regions (Primeaux et al., 2013). Neuronal pathways through these nuclei are organised into a complicated neural network in which specific orexigenic and anorexigenic peptides circuits affect feeding and energy expenditure (Lenard and

Berthoud, 2008). Thus, it is assumed that several neuropeptides play a significant role in the regulator of appetite and hormonal secretion might be involved in cancer-associated anorexia (Oswiecimska et al., 2005). There are many peptides implicated in the regulation of energy homeostasis, some of which are secreted centrally and others peripherally in the gastrointestinal tract (GI), while some secreted at both locations (Orbetzova, 2012). Disturbances of neuropeptide and neurotransmitter secretion impact the hormonal secretion such as neuropeptide Y (NPY), leptin, ghrelin and Insulin in cancer-associated anorexia (Jatoi et al., 2001). Leptin has been observed to play a main role in body mass regulation by acting in the central nervous system (CNS) to both increase energy expenditure and reduces food intake. Leptin crosses the blood-brain barrier (BBB) in a process that is highly organized and its receptors exist both centrally, in the hypothalamus, and peripherally, in several of organs including skeletal muscle, liver, pancreatic islets, kidney, bone marrow and lung (Kastin and Pan, 2000). Neurons holding anorexigenic neuropeptides are triggered by the adipocyte hormone leptin and stimulate reduced food intake and weight loss (Wisse et al., 2001). Low leptin concentrations in the brain increase the activity of the orexigenic signals for instance neuropeptide Y that stimulate feeding and negative energy expenditure, and reduction the activity of the anorexigenic signals that leads anorexia (Inui, 2001). In patients with advanced cancer, the serum leptin levels might be significantly lower as a result of decreased body fat weight

(Perboni and Inui, 2006). Serum leptin level appear to increase in the presence of anorexia-inducing cytokines, like interleukin-1β (IL-1β), and IL-6 and tumor necrosis factor α (TNFα) (Engineer and Garcia, 2012). Neuropeptide Y (NPY), a neurotransmitter, is the most predominant peptides of the hypothalamus that stimulates food intake and is implicated in the regulation of sympathetic activity, body mass, and energy balance., but also produces various other effects on behaviour and other functions (Inui, 1999) & (Sanchez-de-la-Torre et al., 2011). NPY consists of an interrelated orexigenic network which includes orexin, galanin, agouti-related protein (AgRP), opioid peptides and melanin-concentrating hormone (MCH). Most of these peptides are up-regulated in ob/ob mice that deficiency functional leptin, and their expressions are increased in fasting (Chigr et al., 2014). A number of studies suggest that changes of the NPY regulation play a role in the pathogenesis of anorexia associated cancer. This neuropeptide has been involved in the appetite-motivate effects of megestrol acetate, one of the drugs used to cure cancer associated anorexia. The other supposition is that it stimulates Neuropeptide Y synthesis, transfer and release in the hypothalamus (Perboni and Inui, 2006). Many RFamide-related peptides have been found in vertebrates and invertebrates and affect a range of physiological system including food intake, analgesia, locomotor activity, hormone regulation and blood pressure (Primeaux et al., 2013). Polyglutamylated RFamide peptide (QRFP) is a potent agonist of the G protein-coupled

receptor, GPR103 which is expressed in various tissues, but mainly in the hypothalamus (Jossart et al., 2014). It is hypothesized that QRFP exert actions in different brain areas in the hypothalamus and beyond the hypothalamus (Bruzzone et al., 2007). Some brain regions related to feeding behaviour were considered QRFP binding locations and several also expressed GPR103 (Primeaux et al., 2013). Besides increasing food intake, QRFP increases fat mass, body weight and adipogenesis and reduces thermogenesis (Jossart et al., 2014). The only description of circulating, QRFP is in anorexia women where levels are higher (Galusca et al., 2012). However, circulating levels of QRFP in human prostate cancer have not been described.

3.1 HYPOTHESIS

Human expression and circulating QRFP levels are potentially associated with prostate cancer and can possibly be used as a novel biomarker in prostate cancer.

3.2 AIMS

- The aim of this chapter is to investigate if QRFP & GPR103 are expressed in have the expression in human prostate cancer tissue and prostate cancer cell lines PC3, DU145 and LNCaP cells.
- To determine human serum QRFP levels in prostate cancer.

3.3 RESULTS

3.3.1 EXPRESSION OF QRFP AND GPR103 mRNA IN PROSTATE CANCER CELL LINES AND HUMAN PROSTATE TISSUE

RT-PCR was used to detect, the expression of QRFP peptide & GPR103 receptor mRNA in human benign and malignant prostate cancer tissue and in human prostate cancer cell lines (PC3, DU145 and LNCaP cells). The cells were cultured according to the methods previously described; Total cellular RNA was extracted from cells using 200 µl of a cell lysis buffer mixed with 10 µl/ml β-Mercaptoethanol (BME) per well. Each well was scraped using a 1 ml pipette tip to ensure adequate lysis of the cells. The following cell lysates and buffer was removed and placed in an Eppendorf tube on dry ice immediately for rapid freezing. For prostate cells RNA purification was performed using the GenElute™Mammalian Total RNA Miniprep kit according to the protocol. For human tissue RNA was purified employing a TRIZOL® RNA isolation as described in protocol (Deshpande et al. 2008). RNA was extracted and converted into cDNA. The cDNA was amplified by RT PCR using the primers for QRFP & GPR103 described in table 1. Both GPR103 and QRFP gene expression was observed in PC3 cells, LNCaP cells and in human prostate tissue (Figure 3.1).

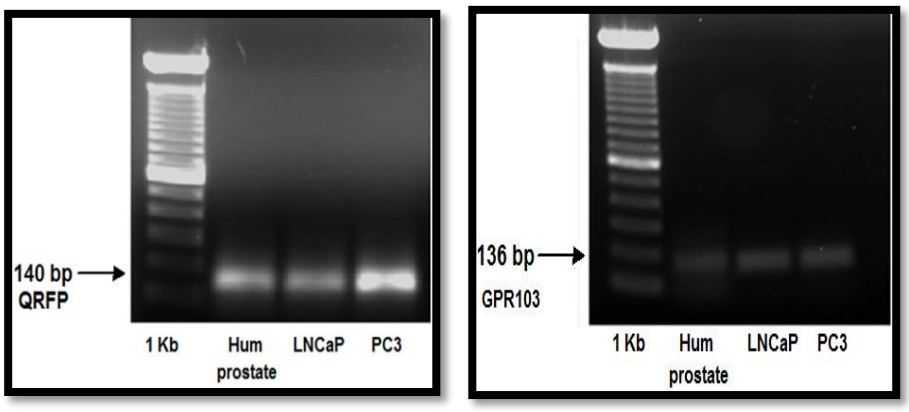

Figure 3.1 RT-PCR analysis for QRFP & GPR103 gene expression in prostate cancer. The mRNA for both QRFP (136 bp) and GPR103 (140 bp) were detected in human prostate tissue also in PCa cell lines PC3 and LNCaP. Bands were excised and purified using QIAquik gel extraction kit (Qiagen) and sequenced to confirm their identity. A no sample negative control was done using water as an alternative to cDNA and RT to emphasize absence of genomic contamination.

3.3.2 QRFP AND GPR103 PROTEIN EXPRESSION IN HUMAN PROSTATE TISSUE AND CELL LINES

Protein was extracted from BPH tissue, LNCaP, PC3 and DU145 cells in order to demonstrate the presence of QRFP and GPR103 protein. Protein levels were identified using western blot using the relevant antibodies Anti-QRFP & GPR103 with secondary anti-goat. Please see (table 2-2) for the relevant antibody concentrations.

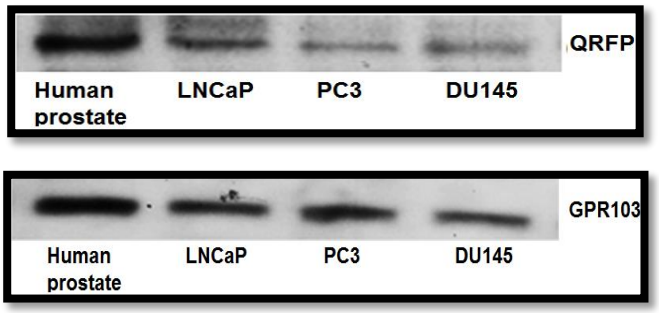

Figure 3.2 Detection protein expression of QRFP & GPR103 in PCa. Western blot analysis revealed protein expression for QRFP (14 kDa) and GPR103 (49 KDa) in the three prostate cancer cell lines (PC3, DU145 and LNCaP), and I was also able to detect expression of GPR103 and QRFP in human prostate tissue.

3.3.3 DETERMINING QUANTITATIVE EXPRESSION OF QRFP & GPR103 IN HUMAN PROTATE CANCER

In order to evaluate if there was any variance between the expression of QRFP & GPR103 mRNA and protein between benign and malignant prostate tissue. Five human prostate tissue samples were used for each group of benign and malignant. QRFP & GPR103 mRNA levels in human prostate tissue were determined by a real-time PCR assay. GAPDH was used as an internal reference gene for each sample. The data showed expression of QRFP & GPR103 were statistically significantly higher in prostate cancer samples compared to the samples from benign prostate hyperplasia (BPH) according to a t-test for QRFP ***P<0.001 and GPR103 ***P<0.001 (Figure 3.3/3.4). The explanations for this are possibly several and will be discussed later. As used mRNA for determine any difference between QRFP and GPR103 protein expression in benign and malignant three samples from each group benign and malignant were applied. The resultant western blot is presented below (Figure 3.5/ 3.6/ 3.7).

Quantification of the PCR and western blot were achieved. The resultant graphs and blot are shown consistent data. The expression levels of both QRFP and GPR103 are significantly higher in the malignant prostate tissues compared to the benign hyperplastic prostate tissues (BPH).

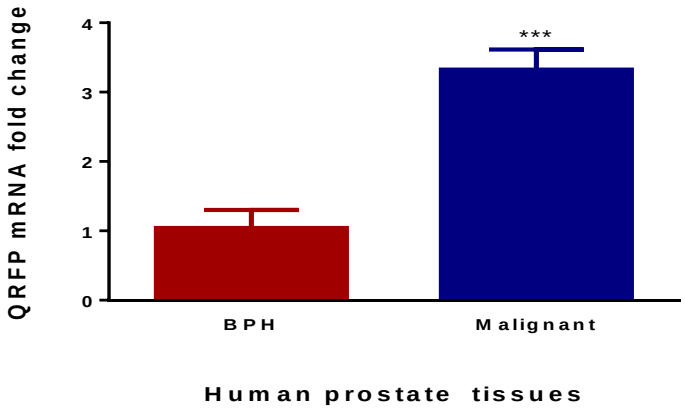

Figure 3.3 Bar chart representing quantification QRFP mRNA expression in benign prostatic hyperplasia and tumour resection, (***P<0.001, for n = 5 per group, mean ± S.D).

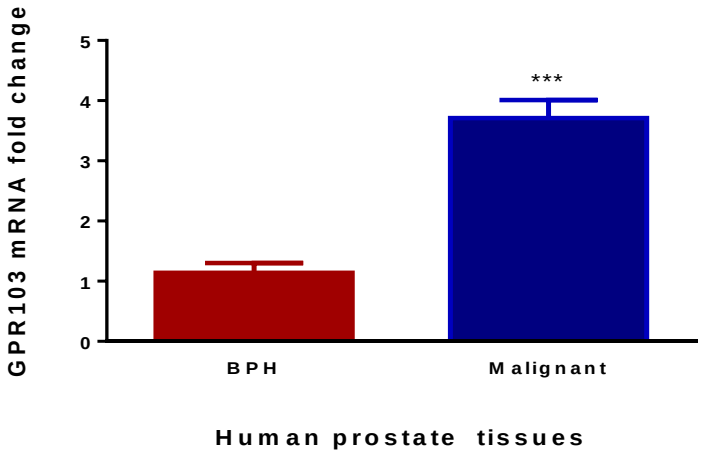

Figure 3.4 Bar chart representing quantification GPR103 mRNA expression in benign prostatic hyperplasia and tumour resection, (***P<0.001, for n = 5 per group, mean ± S.D).

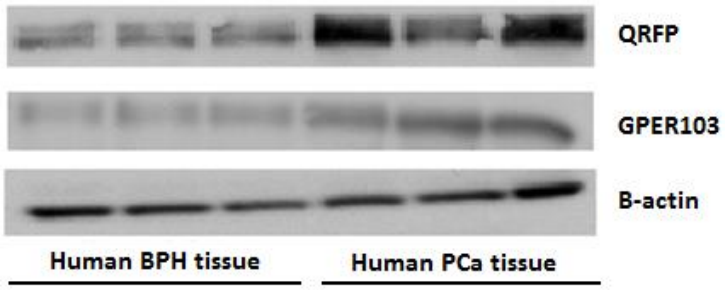

Figure 3.5 Quantification of the western blot showing protein expression levels of QRFP (14 KDa) and GPR103 (49 kDa) in human benign and malignant tissue. B-actin was used as a loading control.

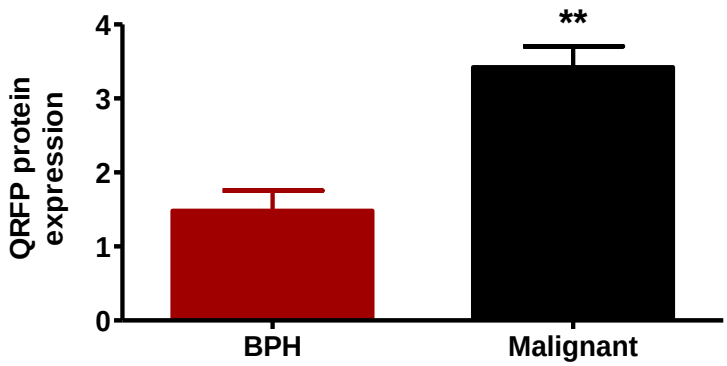

Figure 3.6 Bar chart representing QRFP protein quantification expression in benign and malignant prostate tissue, (**P<0.01 for n = 5 per group, mean ± S.D).

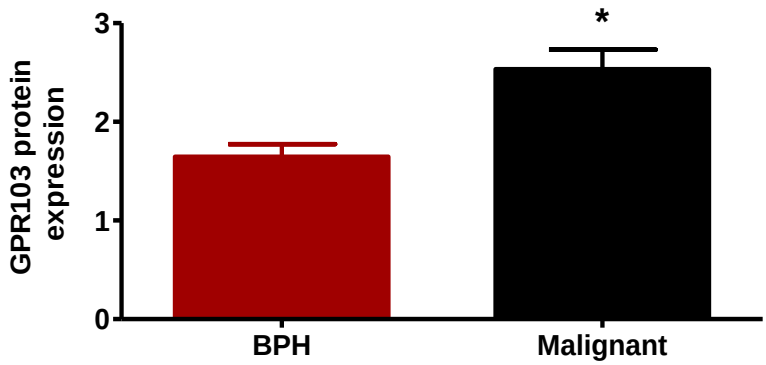

Figure 3.7 Bar chart representing GPR103 protein quantification expression in benign and malignant prostate tissue, (*P<0.05 for n = 5 per group, mean ± S.D).

3.3.4 CIRCULATING QRFP LEVELS IN PROSTATE CANCER

Serum levels of QRFP were measured in a total of 44 men with the age range of 40 – 91.3 years. QRFP serum concentrations were significantly lower in BPH and cancer patients *p < 0.05 and **P<0.01 respectively compare to controls. It was noted the BPH group appeared to have a higher mean level of QRFP as compared to the prostate cancer group (*p < 0.05) Figure 3.8. The intra- assay coefficients of variation for these samples varied between were control 3.1%, benign 3.7% and cancer 3.76%.

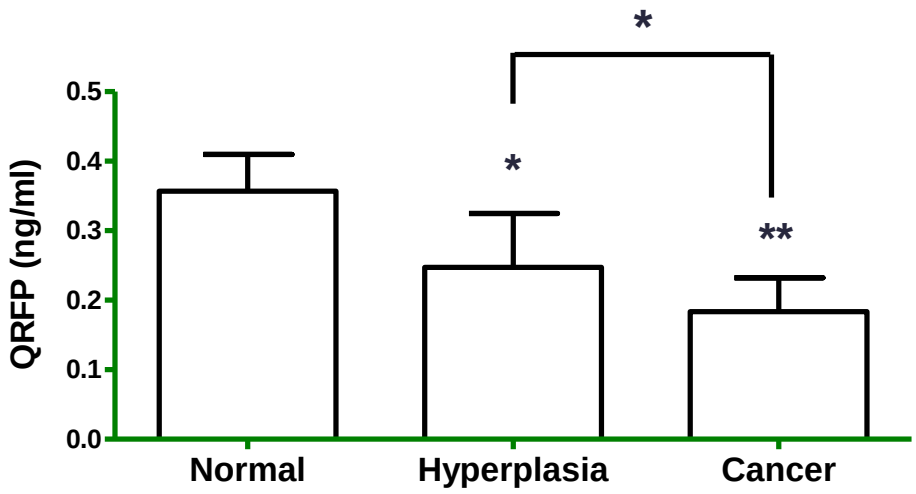

Figure 3.8 Graphical representation of QRFP serum levels in controls and patients. QRFP serum levels (ng/ml) were significantly decreased both in hyperplasia and cancer groups *p < 0.05 and **P<0.01 when compared to the control group and also significantly decreased in cancer compared to hyperplasia group *p < 0.05.

3.4 DISCUSSION

The fundamental starting point of this project was to determine whether the neuropeptide, QRFP and its receptor GPR103 mRNA and protein expressed in human prostate cancer tissue and cell lines. This assist to hypothesis that QRFP may potentially, plays a role in prostate cancer development. Given that QRFP regulates food intake and obesity and obese people have more aggressive from of prostate cancer. This study might possibly provide evidence backing theory QRFP has a mechanism in the link between obesity and prostate cancer.

Results from this study demonstrate that increased mRNA and protein expression of GPR103 and its ligand QRFP in the human prostate tissue and in prostate cancer cell lines PC3, LNCaP and DU145 cells. The purpose of the electrophoresis of the PCR products is simply to demonstrate that QRFP and GPR103 mRNA are present in prostate cancer cells. The data detected from western blot further confirmed protein expression of QRFP and GPR103 in human prostate cancer tissue samples and cell lines (PC3, LNCaP and DU145) cells. The gene level of QRFP and GPR103 mRNA expression were also examined comparing between benign and malignant human prostate tissue. Interestingly, the increase expression level of QRFP & GPR103 were significantly higher in malignant prostate samples compared to benign prostate hyperplasia (BPH). Western blot further confirmed the protein expression levels of

QRFP/GPR103 which was similar to qRT-PCR analysis. QRFP protein expression was also significantly higher in malignant tissue compared with benign tissue. However, the increased expression of QRFP and GPR103 in malignant prostate tissue was based on sample size of n=5. In this studies I did not take into account factors such as weight, age, stage and grade of disease and type of drug treatment. Further studies are required with large sample size to confirm whether there is a difference in gene expression of QRFP and GPR103 between benign and malignant prostate tissue with closely matched patients' situation in terms of treatment received and stage/grade of PCa.

This study supports the hypothesis that increase expression of QRFP/GPR103 in prostate malignant tissue may play an important role in prostate cancer development. It could as well be considered that increased QRFP/GPR103 expression in malignant prostate tissue correlates to a pathological condition and provides cancer cells ability to support its high metabolic rate. The two main drugs commonly used by pharmacological therapy, glucocorticoids and megestrol acetate, however both have limited efficiency. A better grasp of the underlying mechanisms of this disease will assistance in the development of new therapies for patients with cancer-induced anorexia. This study sought to explain a relationship between circulating QRFP and prostate cancer. Thus far, no study has been demonstrated that QRFP has a role in prostate cancer and it the effect

seemed to be mediated by exogenously applied QRFP. The data described here allow us to demonstrate the role of QRFP in pathological feeding behaviour and other role functions in cancer patient specific which associated with anorexia. It was logical thus to examine the levels of circulating serum QRFP to detect if indeed patients with prostate cancer have different levels of QRFP than their non-affected counterparts. To my knowledge, this is the first study to evaluate circulating serum QRFP levels in human cancer patients which showed that there was a significant difference in serum QRFP levels with BPH and cancer groups within the control group (healthy men) provided by the assay kit I used. In the small group of patients in this study, (n = 44) it can be realized that QRFP level in the mean with hyperplasia was significant lower compared with health group. Cancer group appeared significant lower compared with the benign group. These differences in circulating levels support the previously discussed data of differences in mRNA and protein levels in patients with BPH and PCa, at the prostate level, implicating a role of QRFP in prostate cancer. Moreover, cancer groups appeared to have the lowest circulating serum QRFP level. A number of studies published report QRFP as a neuropeptide with orexigenic action, therefore playing a significant role in energy homeostasis and regulation of food intake. The actual expression and signalling characteristics and physiological actives of QRFP and its receptor GPR103 are remain unrevealed (Davies et al., 2015). In these situations, the possibly negative energy balance previously described in

anorexia issues would be partially responsible for the QRFP profile as well to undernutrition affect. Moreover, this finding support the opinion that variations in endogenous QRFP can be a part of a wide adaptive response include other regulators of feeding behaviour. Low circulating QRFP level might be associated with anorexia and other some symptoms noted in cancer patient. It is possible that a tumor may produce factors that inhibit NPY transport, or release through neuronal targets downstream of NPY. In anorectic cancer patients, neuropeptide Y concentration were found to be lesser than controls, which linked with the level of anorexia (Takahashi et al., 2010, Aoyagi et al., 2015). Number of studies have published that NPY immunostaining were higher in PCa than that in benign epithelium. Their studies also suggested that NPY can directly regulate PCa cell development through Y1-R, NPY and the related receptors are overexpressed in PCa and might play a pertinent role in PCa development at both androgen dependent and independent stages. In addition, more aggressive the prostate cancer has lower NPY circulating levels (Liu et al., 2007).

I noted that QRFP mRNA tissue expression higher in the PCa than in BPH which showed QRFP enhanced PCa migration and invasion while human serum level QRFP were circulated lower in PCa than in benign. There is a critical requirement to differentiate those patients with advanced prostate cancer from those with non-advanced ones. These interesting findings

may lead to QRFP as a prognostic biomarker. In an analysis of Gleason grade the trend proposes that there can be a link between QRFP levels and Gleason score. The sample size in the study however were small and were not able to match for BMI, age, type of drug treatment many of our subjects were on. In conclusion the present study suggests that circulating level of QRFP are decreased among cancer patients with advanced disease. This exploratory investigation recommends additional studies are required of QRFP in patients with cancer associated anorexia with full detailed assessment including recent oral intake and age and wild range of subjects covered in the study.

CHAPTER 4

FUNCTIONAL EFFECTS OF QRFP ON PROSTATE CANCER CELL LINES

4 HYPOTHESIS

There has been significant progress in the comprehension of signalling and mechanical mechanisms regulating cell death, growth, adhesion, migration and invasion in prostate cancer. Prostate cancer mortality is caused by the migration of cancer cells from their site of origin within the prostate gland to multiple distant organs in the body. Increased cell invasion shapes a primary cellular property of the metastatic phenotype, and this in transformation comes from enhanced cell migration and/or increased protease activity. (Voll et al., 2014). Understanding these mechanisms is assisting us to obtain a best assessment of the diseased state. Accumulating evidence from different areas of immunology, physiology and psychology has confirmed that the endocrine systems and immune-nervous are functionally connected with each other. This interconnected is thought to be very significant and has been proposed to play a key role in various different diseases, such as cancers (MacKinnon, 2000). Neuropeptides act as effective mitogens for several cancer types, such as small cell lung cancer and prostate cancer (Patrikidou et al., 2012). Neuropeptides play an important role in functional regulation in the microenvironment of the tissues in physiological condition. The normal prostate organ contains many neuropeptides such as neuropeptide Y gastrin-releasing peptide (GRP) and substance P secreted by either the neuroendocrine cells or autonomic and sensory nerve terminals. The

functions of neuropeptides produced by the neuroendocrine cells might be to regulate prostate cancer development (Nagakawa et al., 2001). Certainly, many peptides have been shown to impact prostate cancer cells such as bombesin which enhances the migration and invasion of prostate cancer cells (Sumitomo et al., 2000). QRFP is a neuropeptide involved in energy homeostasis. To my knowledge, this is the first study demonstrating a very high expression of QRFP in malignancy compared to BPH in human prostate. QRFP may have a potential functional effect on prostate cancer cells and these can be demonstrated using in vitro models.

4.1 AIMS

- To determine if QRFP has proliferative effects on PC3, DU145 and LNCaP cell lines.
- To identify if QRFP induce the cellular migration or invasion of prostate cancer cell lines PC3 and DU145 cells.
- To determine if QRFP has an apoptotic effect on PC3, DU145 and LNCaP cell lines.

4.2 RESULTS

4.2.1 REGULATION OF QRFP ON PROSTATE CANCER CELL PROLIFERATION

MTS calorimetric assay was applied to measure cell viability of the prostate cancer cell lines PC3 & LNCaP cells following stimulation with various concentration of QRFP (1, 10 and 100 nM). Around 6000 cells were plated in 96 well plates experiments were performed in quadruplets. Data are demonstrated as the mean ± S.D and are represented relative to basal. QRFP appears to clearly inhibit the proliferation of both human prostate cancer cells lines PC3 and LNCaP cells after 24 hours' incubation (Figures 4.1/4.3). There was no significant effect of QRFP in cell viability in 48 hours treated cells compared to basal (Figures 4.2/4.4).

The data obtained show that there was no functional proliferative outcome of QRFP on prostate cancer cell lines (PC3 & LNCaP). However, further experiments are needed to determine the role of QRFP on prostate cancer cell proliferation.

4.2.2 CELL PROLIFERATION ASSAY USING xCELLigence SYSTEM

Cell proliferation experiments were performed using different concentrations of determine the effect of QRFP different concentrations (1, 10 and 100 nM) on prostate cancer cell lines in 24 hours, using the xCELLigence system. EGF (50 ng/ml) was used as appositive control. There were no significant changes the doubling times in both prostate cancer cells PC3 and DU145 cells with all doses of QRFP (Figures 4.5/4.7). However, there appeared to a slight increase in doubling time in both cell lines at a concentration of 1 nM. Cell proliferation for 24 hours showed similar trend as with MTS assay that is no effect of QRFP cell proliferation. In contrast, 48 hours showed increased doubling times significant effect being at the highest dose of QRFP used (100 nM) *P<0.05 on PC3 cells and DU145 cells, nevertheless were not statistically significant changes on QRFP doses (1 and 10 nM) dependent manner compared to basal, the results are presented in (Figures 4.6/4.8).

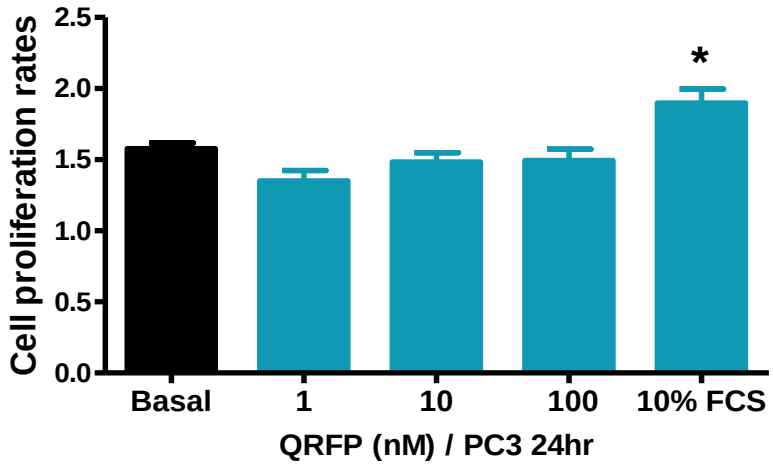

Figure 4.1 MTS assay demonstrating the effects of QRFP on PC3 cell proliferation at 24 hours using concentration dependent (1, 10 and 100 nM), respectively 10% FCS was used as positive control. Data are expressed as rate to basal viability mean ± S.D, (n = 5) *P<0.05.

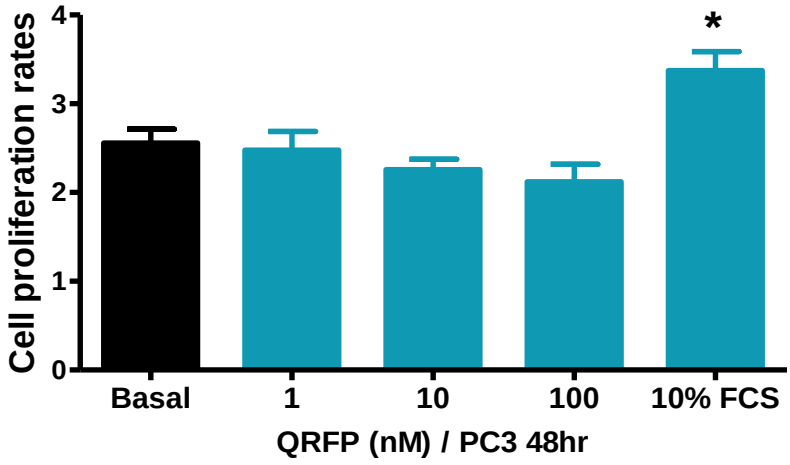

Figure 4.2 MTS assay demonstrating the effects of QRFP on PC3 cell proliferation at 48 hours using concentration dependent (1, 10 and 100 nM), respectively 10% FCS was used as positive control. Data are expressed as rate to basal viability mean ± S.D, (n = 5) *P<0.05.

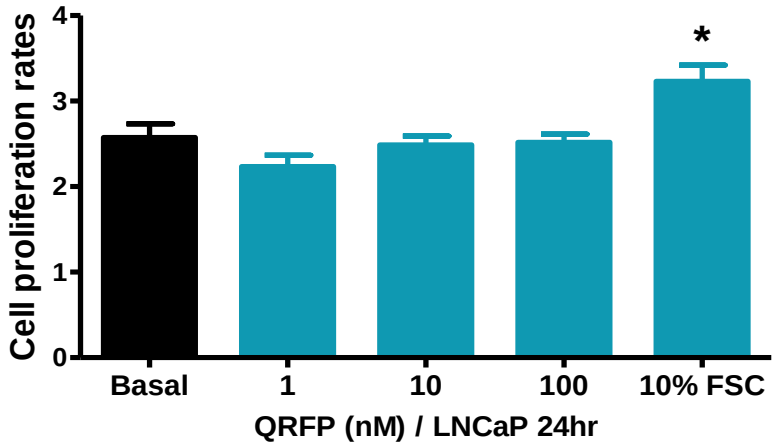

Figure 4.3 MTS assay demonstrating the effects of QRFP on LNCaP cell proliferation at 24 hours using concentration dependent (1, 10 and 100 nM), respectively 10% FCS was used as positive control. Data are expressed as rate to basal viability mean ± S.D. (n = 5) *P<0.05.

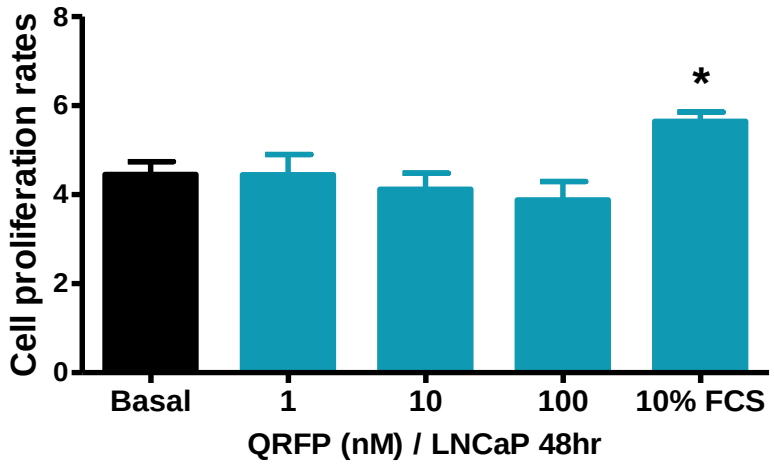

Figure 4.4 MTS assay demonstrating the effects of QRFP on LNCaP cell proliferation at 48 hours using concentration dependent (1, 10 and 100 nM), respectively 10% FCS was used as positive control. Data are expressed as rate to basal viability mean ± S.D, (n = 5) *P<0.05.

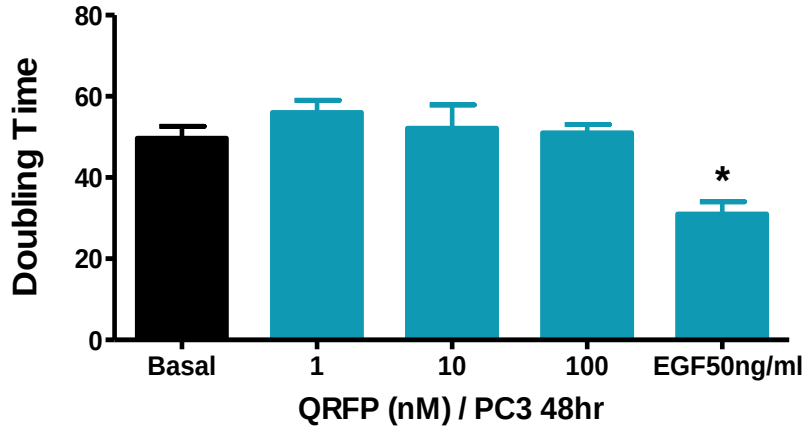

Figure 4.5 The effect of QRFP on PC3 cell proliferation using xCELLigence system. Representative graph the doubling time in hour of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 24 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 5) *P<0.05.

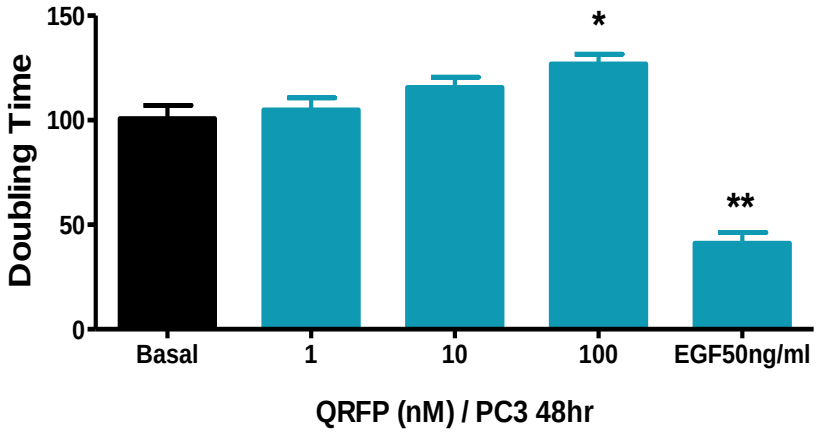

Figure 4.6 The effect of QRFP on PC3 cell proliferation using xCELLigence system. Representative graph the doubling time in hour of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 48 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 5) *P<0.05, **P<0.01.

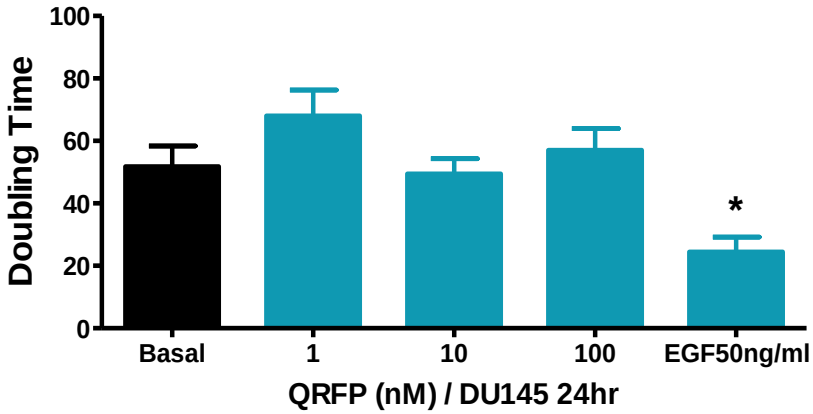

Figure 4.7 The effect of QRFP on DU145 cell proliferation using xCELLigence system. Representative graph the doubling time in hour of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 24 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 4) *P<0.05.

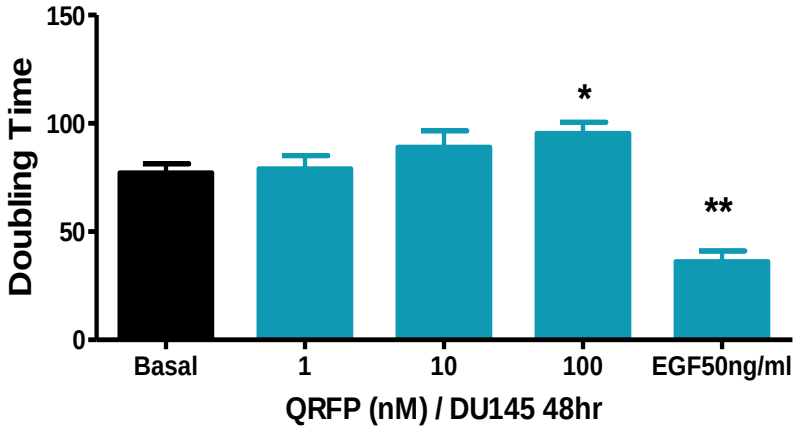

Figure 4.8 The effect of QRFP on DU145 cell proliferation using xCELLigence system. Representative graph the doubling time in hour of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 48 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 4) *P<0.05, **P<0.01.

4.2.3 EFFECTS OF QRFP INCUBATION ON PC3 AND DU145 CELL MIGRATION AND INVASION

xCELLigence system also applied to determine cell migration and invasion assay.

4.2.3.1 CELL MIGRATIO ASSAY

The effect of QRFP treatment on PC3 cell after 8 hours showed there was a significant concentration dependent increase in cell migration (10 nM) *P<0.05 and (100 nM) **P<0.01 (Figure 4.9). In DU145 cells there was a less significant increase in cell migration with different QRFP concentration after 8 hours' treatment compared with PC3 cells. The only statistically significant change in cell migration was QRFP (100 nM) **P<0.01 compared to basal as showed in (Figure 4.10). EGF 50ng/ml was used as a positive control as well lead to a statistically significant increase in cell migration **P<0.01.

4.2.3.2 CELL INVASION ASSAY

To determine whether QRFP contributes to prostate cancer cell line invasion, using the xCELLigence system. Cells were stimulated with either QRFP concentration dependent as described in protocol experiments, respectively. EGF 50 ng/ml used as positive control. Data are demonstrated as mean ± SD of four or five wells processed in parallel. A representative experiment is shown in (Figure 4.11) at the 8 hours QRFP increase invasion of PC3 cell were statistically significant (100 nM)

**P<0.01 and (10 nM) *P<0.05. However, no statistically changes were noted with 1 nM QRFP as compared to basal. QRFP shown less effect on DU145 cells invasion compared to PC3 cells which was statistically changes at dose (100 nM) **P<0.0. In contrast no statistically effect on doses (10 nM) and (1 nM) as compared to basal in (Figure 4.12).

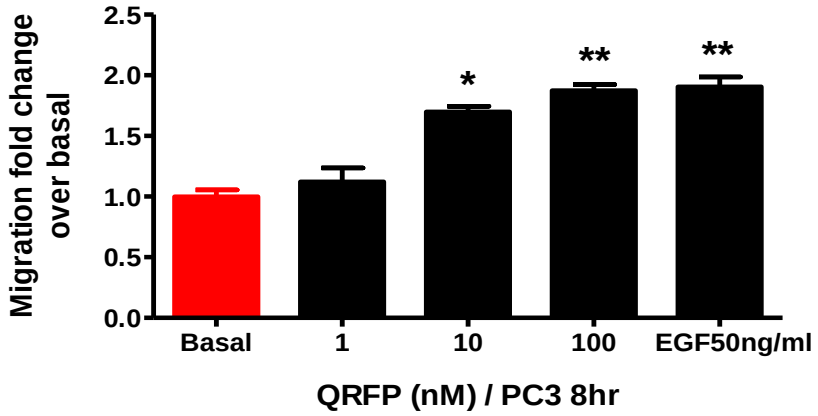

Figure 4.9 The effect of QRFP on PC3 cell migration using xCELLigence system. Representative graph rate of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 8 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 5) *P<0.05, **P<0.01.

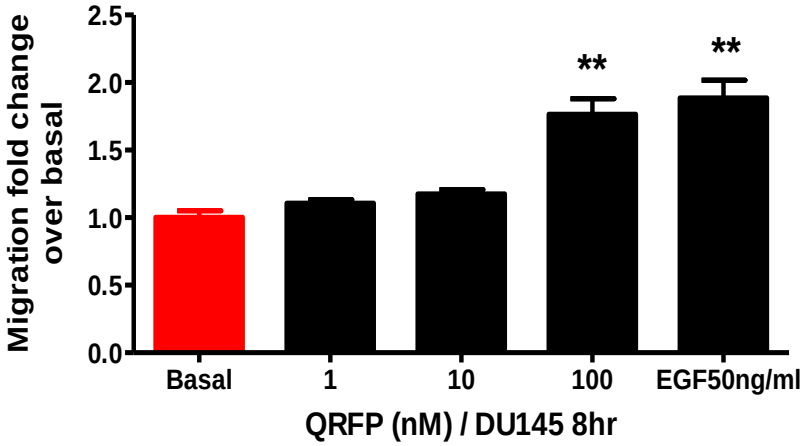

Figure 4.10 The effect of QRFP on DU145 cell migration using xCELLigence system. Representative graph rate of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 8 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 5) **P<0.01.

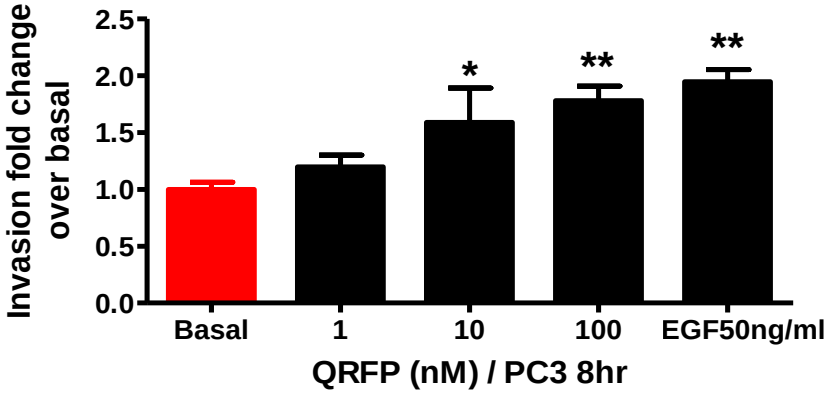

Figure 4.11 The effect of QRFP on PC3 cell invasion using xCELLigence system. Representative graph rate of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 8 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 5) *P<0.05, **P<0.01.

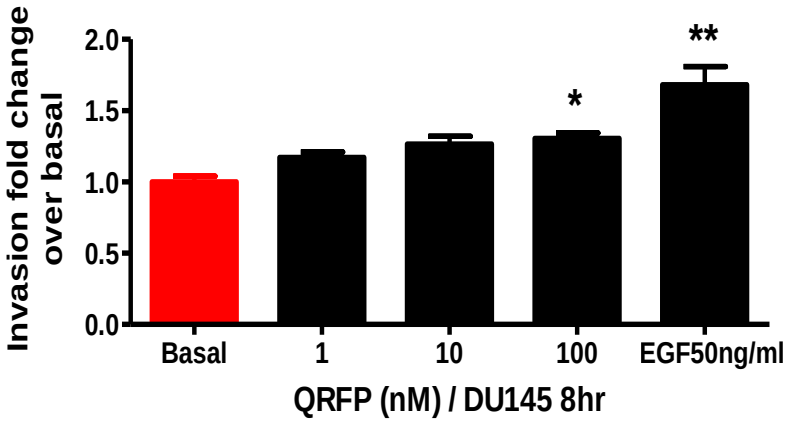

Figure 4.12 T**he effect of QRFP on DU145 cell invasion using xCELLigence system**. Representative graph rate of QRFP concentration dependent (1, 10 and 100 nM) compared to basal cells for 8 hours as described in materials and methods. EGF50 ng/ml representative as positive control. Results were calculated by the xCELLigence RTCA software mean ± S.D, (n = 5) *P<0.05, **P<0.01.

4.2.4 EFFECTS OF QRFP ON APOPTOSIS ASSAYS

The apoptosis assay was carried out using YO-PRO-1 staining as per the protocol described earlier for 2 time points 24 and 48 hours. Results are demonstrated as mean ± S.D and were performed in triplicate. QRFP resulted induced apoptosis after 24 hours' incubation in PC3 cell at lowest QRFP concentration (1nM) *P<0.05, and no statically change have shown at 10 and 100 nM (Figure 4.13). Though, QRFP after 48 hours showed a significantly increase apoptosis at dose (100nM) *P<0.05 compared to basal (Figure 4.14). QRFP appeared to be significantly increase apoptosis in LNCaP cells at (1 nM) *P<0.05 after 24 hours' treatment relative to basal as shown in (Figure 4.15). However, QRFP showed in a significant increase in apoptosis after 48 hours' time point in LNCaP cell compared to basal cells, *P<0.05 at (10 and 100 nM). Extra experiments need achieved to confirm these results (Figure 4.16). Camptothecin 2 mM was used as a positive control and showed a significant increase in apoptosis of PC3 cells and LNCaP cells, **P<0.01 after 24 hours.

DNA fragmentation assay was carried out as per the protocol described earlier for 2 time points 24 and 48 hoursr. Results are demonstrated as mean ± S.D. DNase a known drug to induce apoptosis in several cellular models was used as a positive control to confirm that the assay was successful and showed a significant increase in apoptosis of PC3 cells **P<0.01, at 24 hr and 48 hr. (Figure 4.17) presented QRFP induced

concentration dependent increase in apoptosis of PC3 cells and statistically significant *P<0.05 on dose (1 nM) compared to control basal cells at 24 hours. While no statically changes in doses (10 nM) and (100 nM). For 48 hours QRFP incubation on PC3 cells was statically significant on high dose (100 nM) *P<0.05, and no significant effect on doses (1nM) and (100 nM) compared to basal cells as showed in (Figure 4.18). DU145 cell at 24 hours which presented apoptosis in the lower QRFP dose (1nM) *P<0.05 were no significantly change in doses (10 nM) and (100 nM) compared to basal (Figure 4.19). However, QRFP treatment 48 hours showed significant effect on doses (10 nM) and (100 nM) *P<0.05 relative to basal cells (Figure 4.20). This experiment preformed also on androgen-sensitive LNCaP cells were significant increase apoptosis on doses (1 nM) and (1 0nM) *P<0.05 and no significant change on high dose (100 nM) at 24 hours (Figure 4.21). In LNCaP cells at 48 hours also QRFP showed statically significant increase apoptosis on doses (10 nM) and (100 nM) *P<0.05 were no statically change on dose 1 nM **(**Figure 4.21). DNase was used as a positive control showed a significant increase in apoptosis of LNCaP cells **P<0.01 at 24 hours, however no statically change at 48 hours. Camptothecin (2 mM) induced a significant increase in apoptosis **P<0.01.

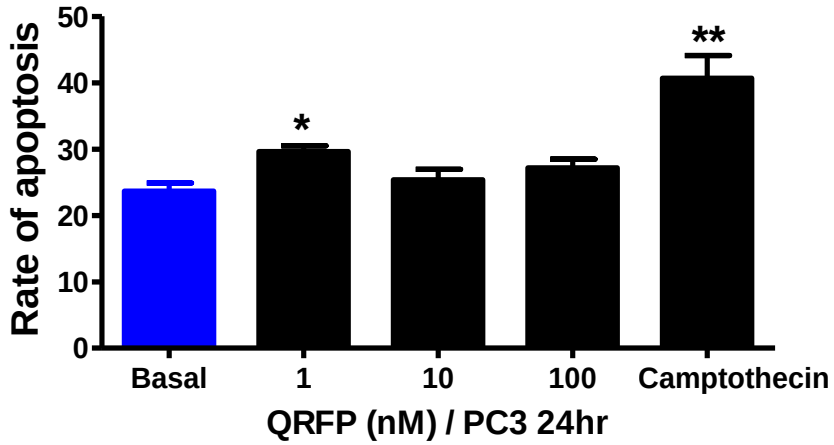

Figure 4.13 Yo pro-1 and propidium iodide used to measurement of PC3 cell apoptosis. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 24 hours, respectively Camptothecin was used as positive control. Data are expressed as rate to basal (n = 4) *P<0.05 and **P<0.01.

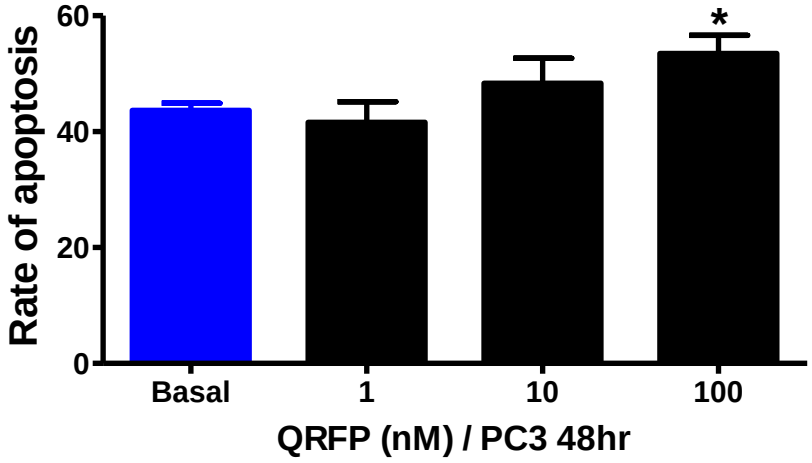

Figure 4.14 Yo pro-1 and propidium iodide used to measurement of PC3 cell apoptosis. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 48 hours. Data are expressed as rate to basal (n = 4) *P<0.05.

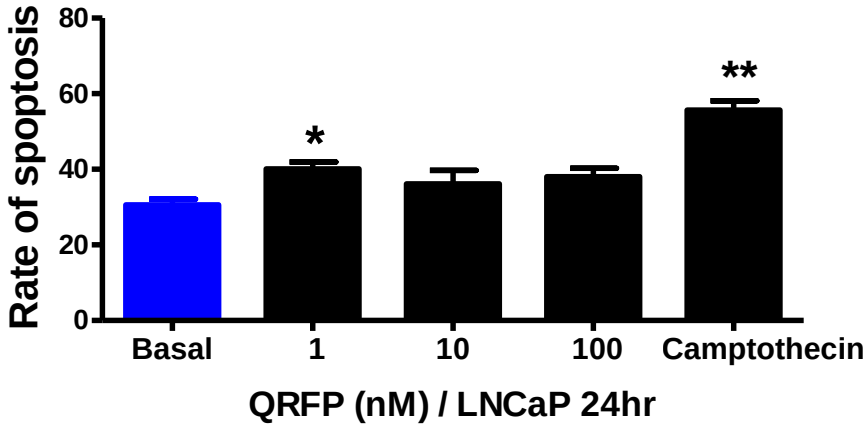

Figure 4.15 Yo pro-1 and propidium iodide used to measurement of LNCaP cell apoptosis. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 24 hours, respectively Camptothecin was used as positive control. Data are expressed as rate to basal (n = 4) *P<0.05 and **P<0.01.

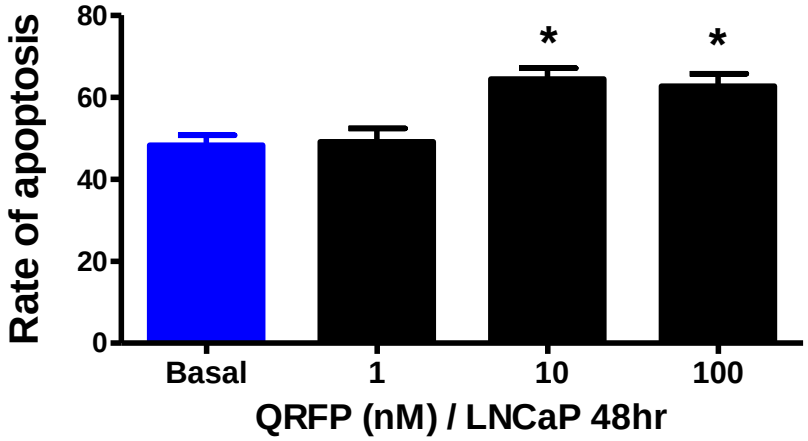

Figure 4.16 Yo pro-1 and propidium iodide used to measurement of LNCaP cell apoptosis. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 48 hours. Data are expressed as rate to basal (n = 4) *P<0.05.

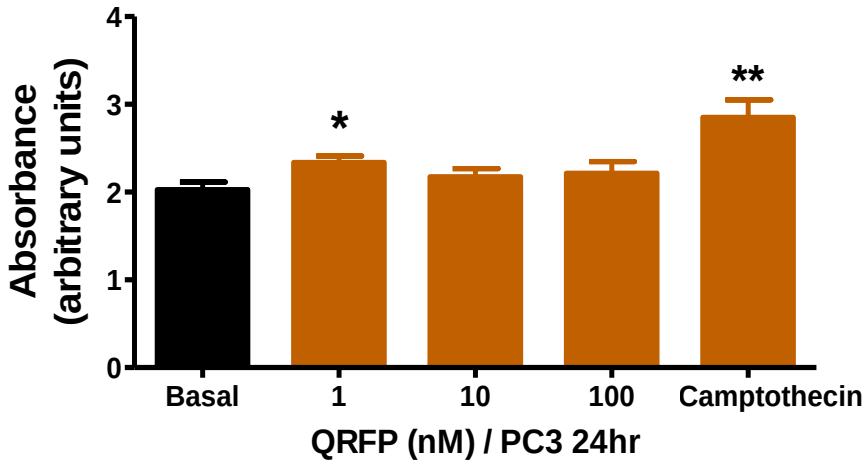

Figure 4.17 Assessment of apoptosis by TUNEL assay on PC3 cells. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 24 hours, respectively Camptothecin was used as positive control. Data are expressed as rate to basal (n = 4) *P<0.05 and **P<0.01.

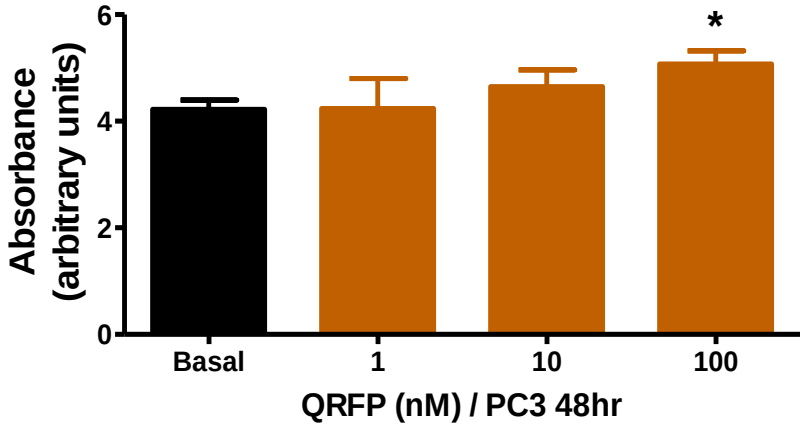

Figure 4.18 Assessment of apoptosis by TUNEL assay on PC3 cells. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 48 hours. Data are expressed as rate to basal (n = 4) *P<0.05.

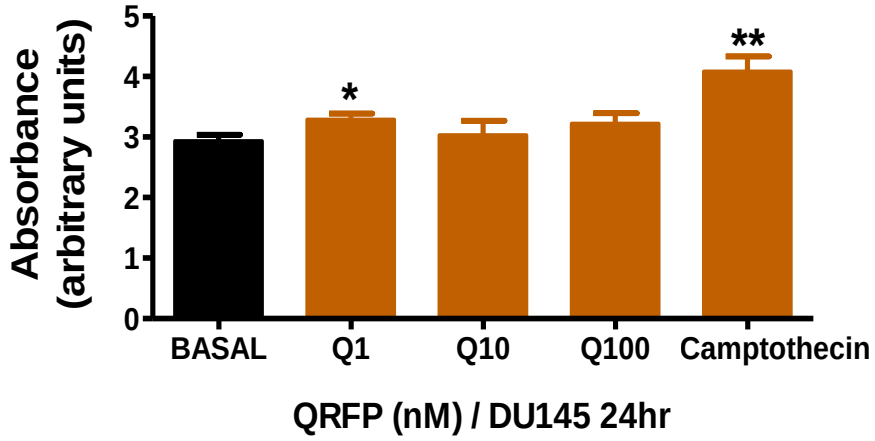

Figure 4.19 Assessment of apoptosis by TUNEL assay on DU145 cells. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 24 hours, respectively Camptothecin was used as positive control. Data are expressed as rate to basal (n = 4) *P<0.05 and **P<0.01.

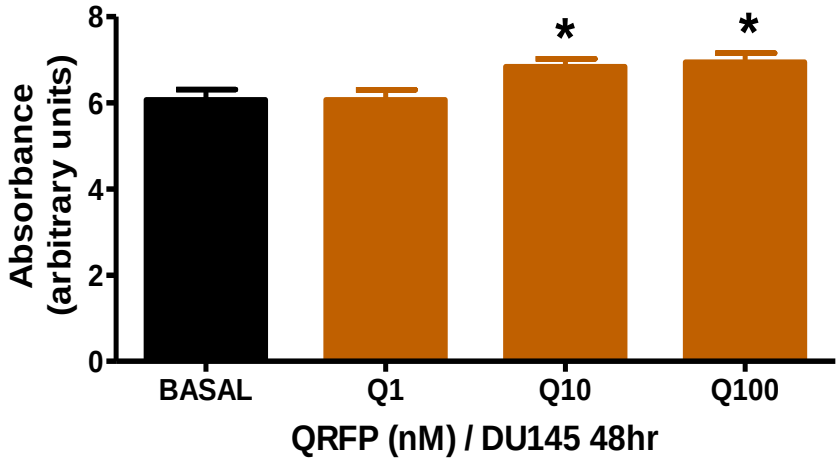

Figure 4.20 Assessment of apoptosis by TUNEL assay on DU145 cells. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 48 hours. Data are expressed as rate to basal (n = 4) *P<0.05.

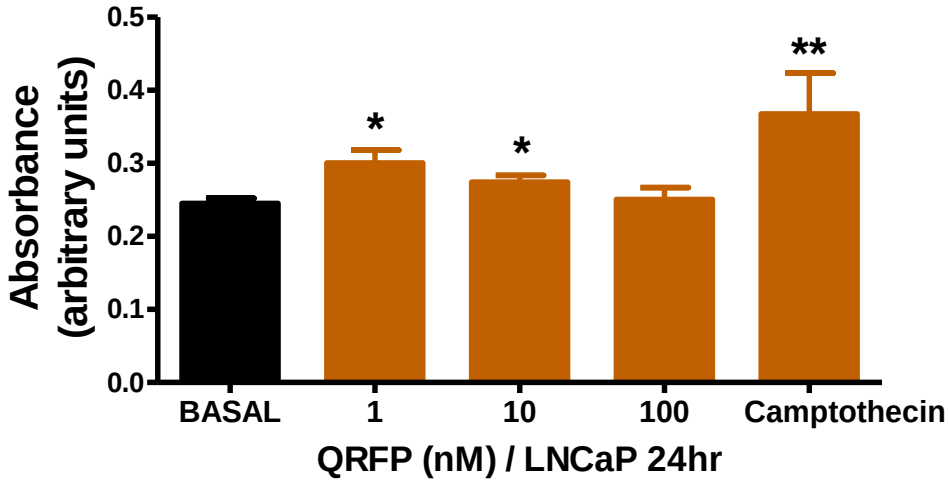

Figure 4.21 Assessment of apoptosis by TUNEL assay on LNCaP cells. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 24 hours, respectively DNase was used as positive control. Data are expressed as rate to basal (n = 4) *P<0.05, **P<0.01.

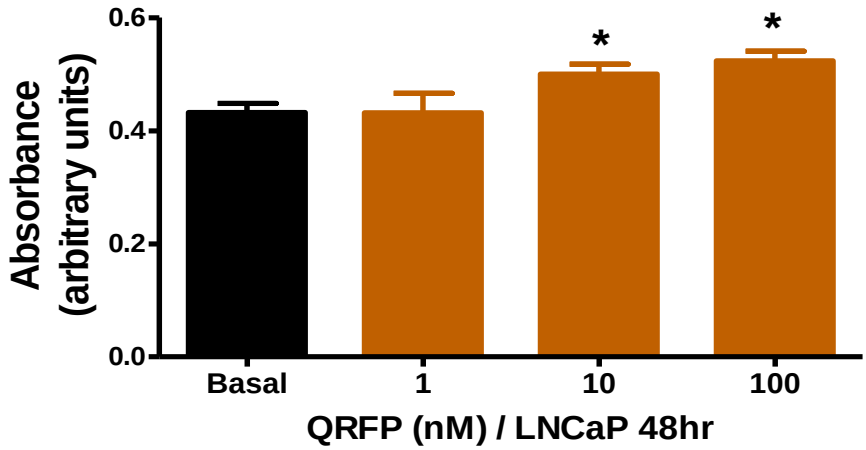

Figure 4.22 Assessment of apoptosis by TUNEL assay on LNCaP cells. Statistical analysis of the rate of apoptosis under different QRFP concentrations (1, 10 and 100 nM) for 48 hours. Data are expressed as rate to basal (n = 4) *P<0.05.

QRFP concentration (nM)	Cell line/ (Hour)					
	PC3		DU145		LNCaP	
	24	48	24	48	24	48
Camptothecin	++		++		+++	
1	+	/	+	/	+	/
10	+/-	+	/	+	+	+
100	/	+	/	+	+/-	+

Table 4.1: Table illustrates the overall effects of QRFP on cellular apoptosis in PC3, DU145 and LNCaP cells. Level of significance of increase/decrease of apoptosis: / = no change, +/-- = change no significant, + = ($p < 0.05$), ++ = ($p < 0.01$) and +++ = ($p < 0.001$).

4.3 DISCUSSION

Reregulation of cellular apoptosis, proliferation, migration, invasion and adhesion are key process involved in cancer development. In fact, many peptides have been shown to impact and modulate prostate cancer cells via regulating cell apoptosis, proliferation, migration, invasion and adhesion. For instance, neuropeptide neurotensin (NT) and bombesin, have been shown to impact on prostate cancer development in experimental models. Neuropeptide Y has also been also shown proliferative or anti-proliferative effects in different cell systems (Lee et al., 2001b). My study present clear evidence that human neuropeptide QRFP plays roles in stimulating key steps in regulation of vital pathogenesis functions in prostate cancer cells. This study included (three) different prostate cancer cell lines to investigate the proliferative effect of QRFP: androgen-independent PC3 cells, DU145 cell cells and androgen-dependent LNCaP cells. Meanwhile, both xCELLigence system and an MTS assay were performed to enhance analytical accuracy. Real time xCELLigence system allows account of the cell lines response to metal toxicity more accurately as compared to typically used end-point assays. The data observed from this system indicated QRFP has no proliferation effect in both androgen-independent PC3 cells and DU145 cells at all-time point 24 and 48 hours. However, longer treatment of QRFP after 48 hours appear to show anti-proliferation effects in both PC3 cells and DU145 cells.

The similar results from MTS assay were observed no proliferation changes at 24 hours' post treatment. Though, there was slight trend decrease proliferation cells at highest QRFP dose in PC3 cells and LNCaP cells after 48 hours' treatment, nevertheless these were not statically significant. Besides, as demonstrated in (figure 4.2/4.4), QRFP appears to induced apoptosis at a high dose, leading to concept that 100nM might be the effective dose on prostate cancer cells.

As shown that QRFP does not regulate proliferation. An additional functional assays were carried out to demonstrate the effect of QRFP on prostate cancer cell lines. Tumour cell migration and invasion are prerequisite for developing to metastasis. In order to monitor the migration, and invasion after QRFP treatment in prostate cancer cell lines the real time cell analysis (RTCA) was also used. Notably there is a limitation of this system that it is unable to count the cells grow on top of each other like LNCaP cells. Also, the disadvantages that prostate cancer cells grow in clusters/aggregates instead of in a monolayer and weak cell surface adhesion will also be a common problem, to investigate cell migration and invasion. Differential effects of tissue culture coating substrates on prostate cancer cell adherence, morphology and behaviour (Liberio et al., 2014). Therefore, LNCaP cells has been omitted for determined If QRFP has metastasis effect on prostate cancer cells. Interestingly, it has been found that QRFP induced migration on two prostate cancer cell lines PC3 cells

and DU145 cells after 8 hours (Figure 4.9/4.10). In the PC3 cells there seems to be a dose-dependent significant increase in cell migration with the highest rate of cell migration being at the highest doses of QRFP used (10 nM &100 nM). The same observation obtained from DU145 cell increase migration, however just in highest QRFP concentration (100 nM). The acquirement of tumor cell invasiveness is an essential aspect of tumor development and a key factor of morbidity and mortality in prostate cancer patient. xCELLigence system applied to monitoring invasion cells using the same protocol in invasion assay above. This system provide Matrigel as extracellular matrix component coated on upper surface of the microporous membranes to enhance invasion cells. Interestingly PC3 cells after 8 hour appears significant increase invasion through matrigle at the two doses of QRFP used 10 nM and 100 nM. Corresponding to the cell migration assay the rate of cell invasion appeared to increase in a dose dependent manner, with the highest rate of invasion being shown in the highest dose of QRFP (100 nM). Also QRFP induced invasion in Du145 cells at high dose (100 nM) individual at 8 hours (Figure 4.11/4.12). In the same set of experiments, EGF 50 ng/ml, used as positive controls.

These two different functions assay migration and invasion presented consistence finding. Treatment of prostate cancer cell lines with different concentration of QRFP or exposure times presented change in migration and invasion level. QRFP show stronger effect in PC3 than in DU145 cells.

It might due to that PC3 cells have characteristic more metastatic potential than DU145 cells which have a moderate metastatic potential, however both cell lines could reflect the characteristic of the most advanced types of prostate cancer. This reveals consistently with the previous studies suggesting that the neuropeptides secreted by neuroendocrine cells play a key role in the progression as well as the spreading of the cancer (Alonzeau et al., 2013). Though the mechanisms of the effect of QRFP on cancer migration and invasion remains investigated.
The surprising findings and interesting research demonstrated that the pro-apoptotic effect of neuropeptide QRFP on prostate cancer cell lines. Besides, only few reports about neuropeptides and receptors that can inhibit development and/or stimulate apoptosis of prostate cancer cells (Alexandre et al., 2014). Only one study showed neuropeptides orexins has apoptotic role in colon cancer, and this finding enhance a new dimension to the biological function of these neuropeptides which may have significant implications of suppression cancers (Rouet-Benzineb et al., 2004). Thus, this is first study presented clearly that neuropeptide can regulate the apoptosis potential of prostate cancer cells. Fluorescent dyes YO-PRO® 1 and PI has shown that 24 hours' treatment of PC3 cells with low dose QRFP (1 nM) produced a significant decrease in cell viability and induced in apoptosis (Figure 4.13). In addition, 48 hours' treatment PC3 cells with QRFP showed induced apoptosis statically change on relatively higher dose (100 nM) and (10 nM) but this was no statically significant

(Figure 4.14). Consistently LNCaP cells after 24 hours showed induced apoptosis and inhibited growth of cells at dose QRFP (1 nM) but no effect with other doses (Figure 4.15). Although, 48 hours' treatment of QRFP at dose (10 nM) and (100 nM) showed an increased level of apoptosis in LNCaP cells (Figure 4.16). The effect of QRFP on apoptosis in both cell lines were found to be dose and time dependent. The similar results were obtained from PC3 cells as well has shown in LNCaP cells, nonetheless LNCaP cells appearance slightly more increase apoptosis and yet this cell line is described as being high sensitive to apoptosis than PC3 cells. TUNEL assay for detection the later stage of apoptosis cells via DNA fragmentation which is another hall mark of apoptosis was used for further determination and confirm of the possible role of QRFP in modulating cell apoptosis. Consistent with previous results, I found that extensive DNA fragmentation was only observed after 24 hours in PC-3 cells and DU145 cells, exposed to QRFP (1nM), produced a significant increase in apoptosis compared to basal cells control (Figure 4.17). It is appearance that after 48 hours' treatments DU145 cells more sensitive to increase apoptosis on doses (10 and 100 nM) compared to PC3 cells were only significantly increase on dose 100 nM (Figure 4.18). Consistence to AI PC3 and DU145 cells found LNCaP cells presented slightly more increase apoptosis at two time points 24 and 48 hours with doses dependent (Figure 4.21/4.22). Eventually in the two apoptosis assays the effect of QRFP on prostate cancer cell lines apparent induced in apoptosis at 24 hours was

statistically significant on dose (1 nM). Whereas at 48 hours' time point or (10 nM) and (100 nM) dose of QRFP was there an actual increase in the rate of apoptosis in either cell line. Camptothecin a known as anti-cancer drug to induce apoptosis in many cellular models was used as a positive control which sensitive to detect by the both assays used. Camptothecin induced a significant increase in apoptosis in the three cell lines (**P<0.01). These data shown that QRFP inhibit cell-proliferation in the three prostate cell lines PC-3 and DU145 cells (androgen resistant) and LNCaP cells (androgen-dependent), indicating that QRFP induced death cells in prostate cancer cell lines is not dependent on androgen receptor expression of the cells.

In conclusion the finding from functional assays to assess the QRFP role on prostate cancer cells were identical. These findings that the QRFP regulates prostate cancer cell lines migration, invasion and apoptosis are novel and surprising, given the prominent role that QRFP have been shown to have in regulating prostate cancer cells development. This leads to hypothesis that QRFP has duality functional effect in prostate cancer cells depend on dose dependent and time point. However, as far I know no studies presented the ambivalence of neuropeptide regulation functions in cancers, therefore, depth studies required to demonstrate and confirm the controversial finding.

CHAPTER 5

THE MECHANISM OF QRFP ON SIGNAL TRANSDUCTION PATHWAYS IN PROSTATE CANCER

5 INTRODUCTION

The molecular information of prostate carcinogenesis has not been fully outlined, nevertheless it is clear that for normal prostate gland cells develop into malignant prostate cancer cells, it requires many transformation steps (Isaacs, 1997). Accumulating evidence suggests that changes of genetic alterations resulting in tumor suppressor genes and oncogenes expression are responsible for the growth and progression of prostate cancer (Shand and Gelmann, 2006). Alterations in cancer genes (such as in prostate cancer) likely result in a large number of molecular events occurring in an erroneous fashion. A main goal for researchers is to understand the potentially altered genes that play important roles in cancer progress (Sarkar et al., 2010). One of the important events is protein phosphorylation, which when changed, can result in system wide disorder and deregulation of the signal transduction pathway (Radivojac et al., 2008). Protein tyrosine phosphorylation and de-phosphorylation are greatly conserved signalling mechanism for regulating proteins, has been discovered in all eukaryotes studied. Protein phosphorylation controls various physiological and pathological processes in the human body such as, metabolism, cell cycle, differentiation, cell survival, mitogenesis and gene transcription. The kinase family is one of the major target families in the human genome. Every kinase and phosphatase targets various substrates. Abnormalities in these kinase pathways have been implicated

in the development of several malignancies, including pancreatic, lung, breast and prostate cancer. Many lines of evidence propose a role for protein kinase in prostate cancer such as p53, EGFR, β-Catenin AMPK, AKT and MAP kinase signal transduction pathways (Markert et al., 2011), (Zadra et al., 2014) & (Edlind and Hsieh, 2014). Erroneous function of tumour suppressors, including PTEN, TP53, ras and RB, CDKN have been linked to the development of prostate cancer (Mulholland et al., 2006). Several of the defining features of cancer, such as apoptosis, proliferation, migration, invasion and metastasis result from disorder of regulatory signalling pathways. Certainly, since their implication in carcinogenesis, kinases remain critical targets for many of the oncological drugs (Harsha and Pandey, 2010) & (Cheng et al., 2011). The use of microarrays for gene expression analysis has led to an increase in the number of reports regarding this subject. These findings have described a variety of changes in gene expression associated with many diseases, such as prostate cancer (Massoner et al., 2013). In several cases, different kinases were implicated in multiple steps involved in the synthesis of a single protein. The study on protein phosphorylation is significant due to the important role of kinases and phosphatases in cellular processes. As several of these cancer cells originated from human tumours, they can lead to functional proteomic studies depending on the experimental design. Examining the proteomes for proteins that hold patterns similar to the optimal phosphorylation action of a protein kinase will aid the identification of

possible physiological substrates of the kinase. The current protein profiling of cultured cells using antibody arrays is allowing in-depth examination of therapeutic targets in cancer researches (Gembitsky et al., 2004). Also using of antibody arrays for high quantity phosphoproteomic profiling of cultured cells is helpful research to assess aberrantly activated signaling transduction networks including MAPK kinase pathways (Harsha and Pandey, 2010). Proteomic protein phosphorylation at the cellular level is required to know the intracellular signaling upon external and internal prompts. To demonstrate signalling pathway essential for the oncogenic function of QRFP, Phospho-Kinase Array were employed in PC3 cells.

5.1 HYPOTHESIS

Intracellular signalling pathways mediated by QRFP

The process of signal transduction through receptor tyrosine kinases is initiated by an extracellular stimulus that activates its designated receptor, thereby initiates downstream signalling mechanisms. This can be established by a sequential phosphorylation of a three-tier kinase cascade, which conveys the signal to the nucleus, where a cellular response can be transcription mounted. The effects of many treatments such as chemical modifiers, growth factors, and androgens on PCa cells, have been termed up to now. However, the network and function of the MAP kinase pathways

in PCa are still unclear (Koul et al., 2013). Utilisation of techniques that incorporate signalling kinases, including the MAP kinases pathway, ERK, p38, JNK and AKT/PKB kinases has increased our understanding of the control of cellular event by growth factors (proliferation, differentiation and development) and also stresses (apoptosis). Western blotting analysis was significant used in research specific in cancer area to identify the protein expression (Liu et al., 2014).

5.2 AIMS

- To demonstrate if QRFP has a role in the protein kinase pathways in prostate cancer cell lines.
- To demonstrate if QRFP activates specific MAPKs pathways ERK, JNK and p38 in PC3 and DU145 cell lines.
- To demonstrate if QRFP regulate phosphor-AKT in PC3 and DU145 cell lines.

5.3 RESULTS

5.3.1 THE EFFECT OF QRFP ON PHOSPHORLATION 43 SIGNALLING MOLUCULES IN PC3 CELLS

Human Phospho-Kinase Array was performed to identify the possible signalling pathway kinases that may be involved in QRFP dose dependent induced activation of PC3 cells. At the same time this allowed for the detection of the relative phosphorylated levels of 46 proteins, as well as MAP Kinases, Src family and JAK/STAT pathways amongst others. Experiments were carried out with different doses of QRFP (1, 10, 100 nM) for 60 minutes in PC3 cells. The samples were collected and analysed as given in the protocol (Materials and methods). Out of the 46 proteins, QRFP affected phosphorylation of 19 proteins.

Interestingly, all QRFP concentrations demonstrated an increase in the phosphorylation in the MAPK proteins, p38 (T180/Y182) and JNK (T183/Y185, T221, and Y223). Additionally, QRFP also increased the phosphorylation of Akt 1/2/3 (S473), STAT2 (Y689), STAT5a/b (Y699), GSK-3a/ß (S21/S9), AMPKa1 (T183), AMPKa2 (T174), EGF R (Y1086), HSP27 (S78/S82), Lyn (Y397), Chk-2 (T68), Fyn (Y420), Yes (Y426), Lck (Y394) and Hck (Y411). In contrast, the three QRFP concentrations showed decreased ERK1/2 (T202/Y204, T185/Y187) and mTOR (S2448) in (Figure 5.1/5.2).

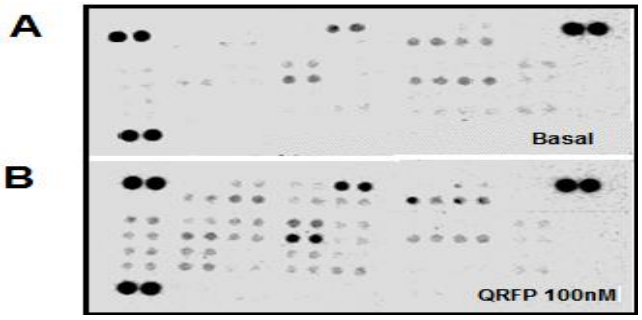

Figure 5.1 The effects of QRFP on phosphorylated signaling proteins in PC3 cells. Representative Immunoblots showing multiplex detection of phosphorylated proteins in PC3 cells treated by dose dependent QRFP (1, 10 and100 nM) at time points 60 minutes of incubation.

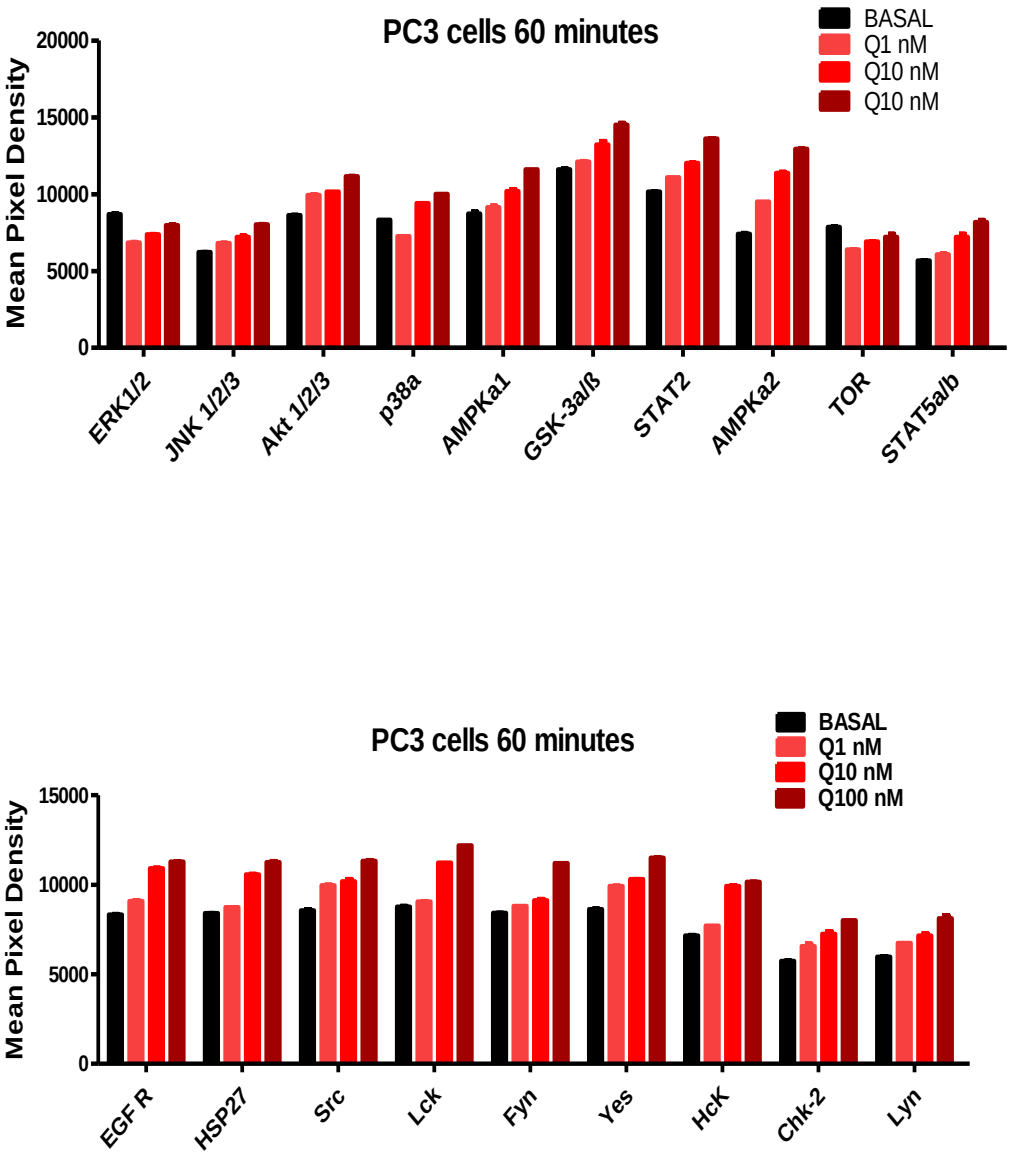

Figure 5.2 The effects of QRFP on phosphorylated signaling proteins in PC3 cells. Graphic representation of the most significant fold changes in protein phosphorylation levels in PC3 cells compared to untreated cells (basal). Data analysis was performed using a one-way ANOVA.

Human Phospho-Kinase Array was performed to identify the possible signalling pathway kinases that can be involved in QRFP at two different time points, namely 5 and 25 minutes. Experiments were carried out using PC3 cells and stimulated by QRFP (100 nM). The samples were collected and analysed as given in the protocol (Materials and methods).

Interestingly, at 5 minutes QRFP 100 nM treatments showed an increased phosphorylation of the MAPK proteins ERK1/2 (T202/Y204, T185/Y187) were showed slight increase in JNK (T183/Y185, T221, and Y223) and Akt 1/2/3 (S473) also increased the phosphorylation EGF R(Y1086). However, some interesting proteins showed decrease phosphorylation p38α (T180/Y182), AMPKα1 (T183), AMPK α2 (T172), mTOR (S2448), Chk-2 (T68) GSK-3a/ß (S21/S9) Figure 5.3.

At 25 minutes of treatment of PC3 cells by QRFP 100 nM, there was a significant increase in the phosphorylation of the MAPK proteins p38α (T180/Y182), JNK (T183/Y185, T221, and Y223) and Akt 1/2/3 (S473), compared to 5 minutes' activation and baseline. In addition to this, the increase in phosphorylation was also present in AMPK α2 (T172), STAT2 (Y689), STAT6 (Y641), STAT5a/b (Y699), Chk-2 (T68) and GSK-3a/ß (S21/S9). In contrast, some interesting proteins exhibited a decrease in phosphorylation, namely ERK1/2 (T202/Y204, T185/Y187), AMPKα1 (T183), EGFR (Y1086) and mTOR (S2448) Figure 5.3.

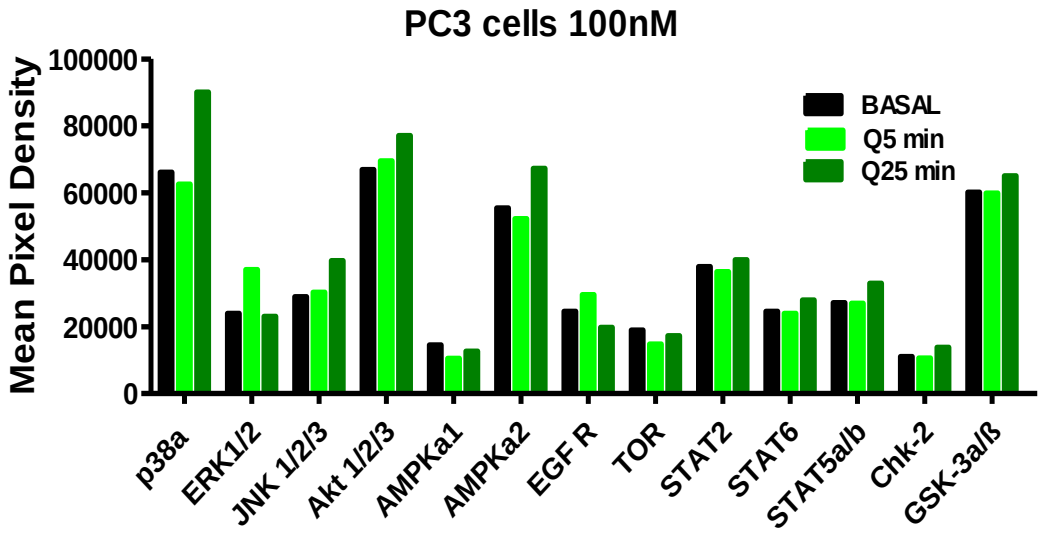

Figure 5.3 The effects of QRFP on phosphorylated signaling proteins in PC3 cells. Representative graphic showing multiplex detection of the most significant fold changes in protein phosphorylation level in PC3 cells treated by 100 nM QRFP for two time points (5 and 25 minutes) of incubation compared to untreated cells (basal). Data analysis was performed using a one-way ANOVA.

5.3.2 THE EFFECT OF QRFP ON MAPK EXPRESSION IN PC3 and DU145 CELLS

To examine the signalling pathways likely to be involved in QRFP, the expression of phosphorylated forms of ERK1/2, p38 kinase, JNK and AKT were analysed by western blotting. PC3 and DU145 cells were treated with QRFP (100 nM) for time duration between 0, 5, 15, 30 and 60 minutes to perform time dependent effects of QRFP on MAPK and AKT phosphorylation. Following stimulation of PC3 cells and DU145 cells with QRFP resulted in a significant activation of ERK 1/2 **P<0.01 and ***P<0.001 respectively at 5 minutes' treatment concentration of 100 nM QRFP, (figure 5.4/5.5). A significant increase in p38 phosphorylation was observed at differing times in the two cell lines. In PC3 cells the greatest activation was at 30 minutes (**P<0.01) and decreased significantly at 15 and 60 minutes *p < 0.05 (Figure 5. 6).

In DU145 cells, activation of p38 increased significantly at about 5 minutes ($p < 0.05$). At 15 minutes, p38 levels appeared to drop back down to near basal levels before increasing again at 30 minutes ($p < 0.05$), with maximum activation occurring at around 60 minutes (P<0.01) compared to basal **(**figure 5.7). In PC3 cells, JNK phosphorylation occurred around 30-60 minutes following stimulation with QRFP $p < 0.05$, (figure 5.8). However, in DU145 cells JNK activation was at its peak around 5 minutes (P<0.01) and appeared to reduce towards basal levels between15-30 minutes,

before increasing again after 60 minutes $p < 0.05$, (figure 5.9). Phosphorylation of AKT also appeared to increase significantly in PC3 cells at about 15 minutes' post-stimulation and the level remained significantly higher even at 60 minutes $P<0.01$, (figure 5.10). Activation of AKT in DU145 cells occurred at around 30 minutes ($p < 0.05$) and increased significantly at about 60 minutes $P<0.01$, (Figure 5.11).

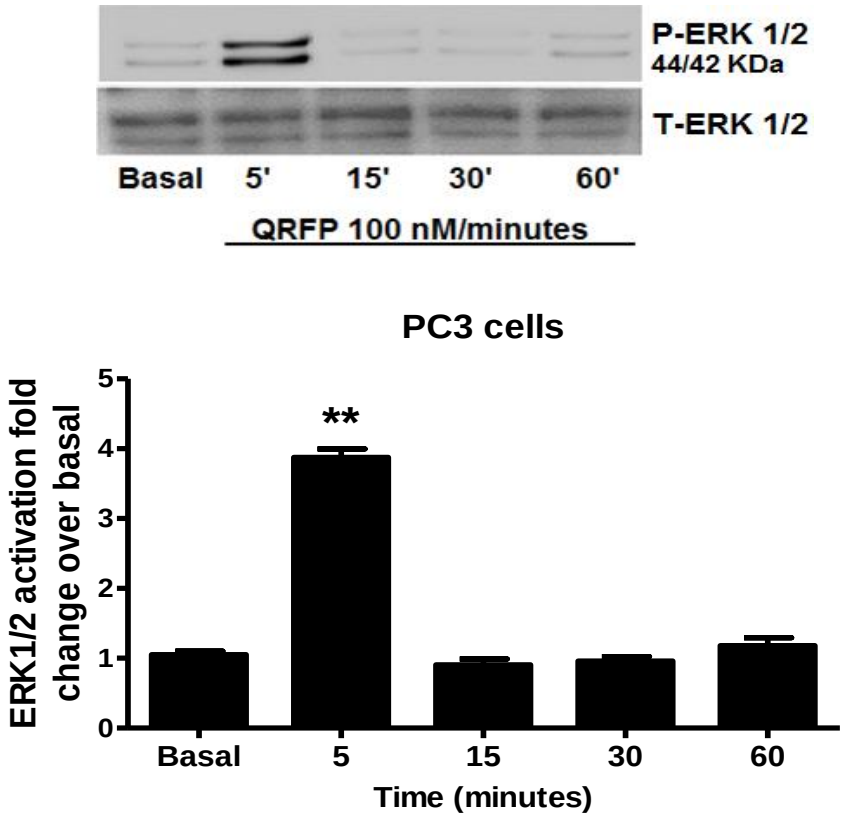

Figure 5.4 The effect of QRFP on (ERK 1/2) MAPK expression in PC3 cells.

Western blot analysis showing changes in ERK1/2 protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in ERK 1/2activation in PC3 cells. Data represents the mean ± S.D from three independent experiments; **P<0.01 compared to basal expression.

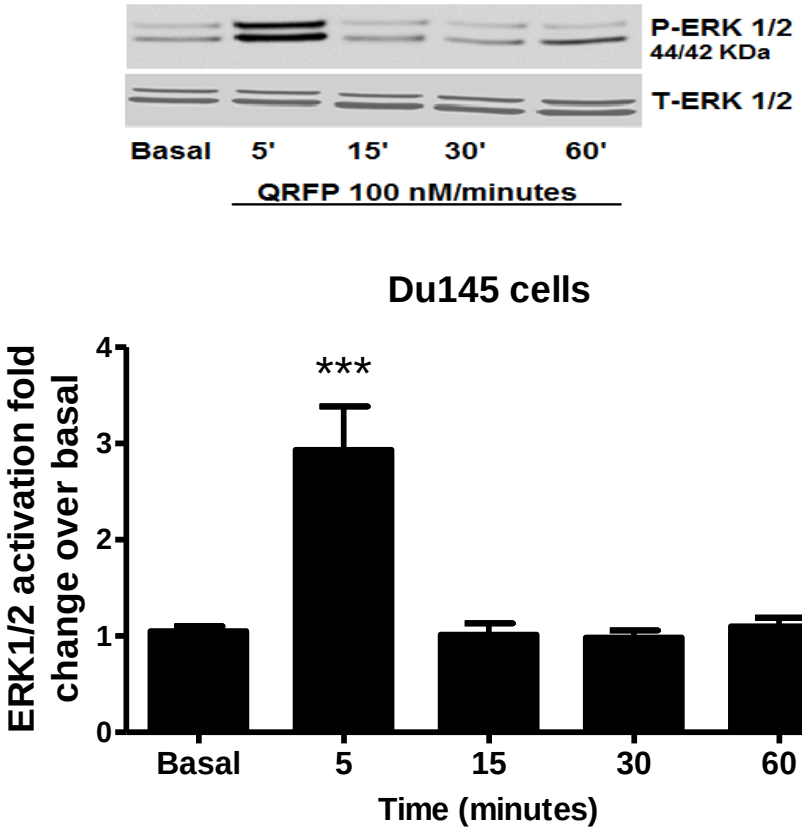

Figure 5.5 The effect of QRFP on (ERK 1/2) MAPK expression in DU145 cells

Western blot analysis showing changes in ERK1/2 protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in ERK 1/2 activation in DU145 cells. Data represent mean ± S.D from three independent experiments; ***P<0.001 compared to basal expression.

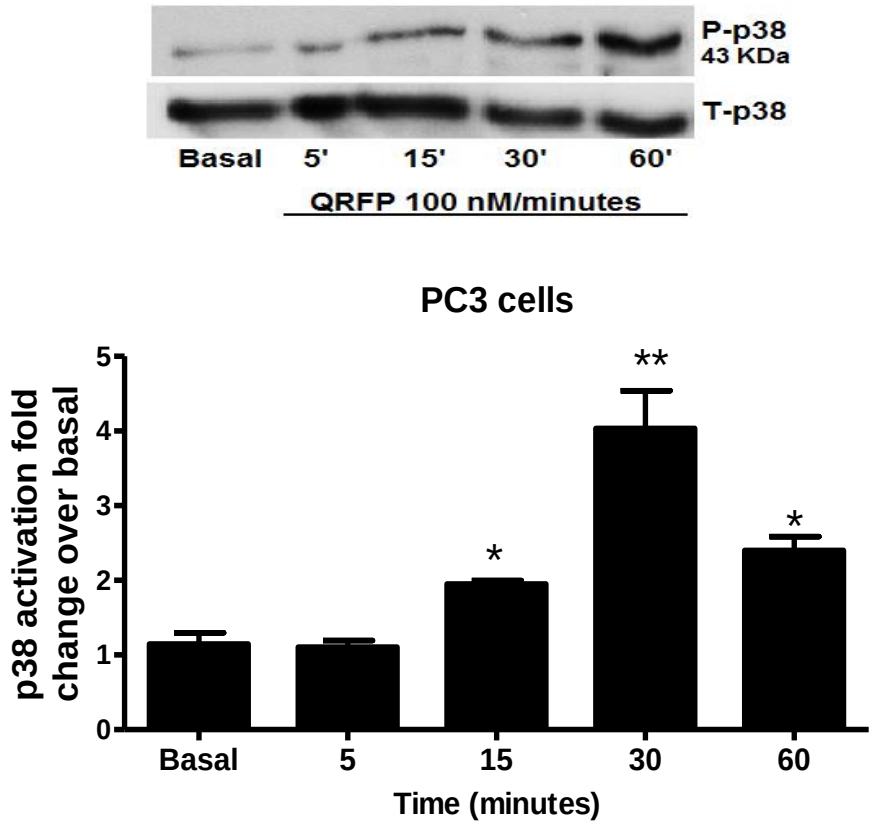

Figure 5.6 The effect of QRFP on (p38) MAPK expression in PC3 cells

Western blot analysis showing changes in p38 protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in p38 activation in PC3 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

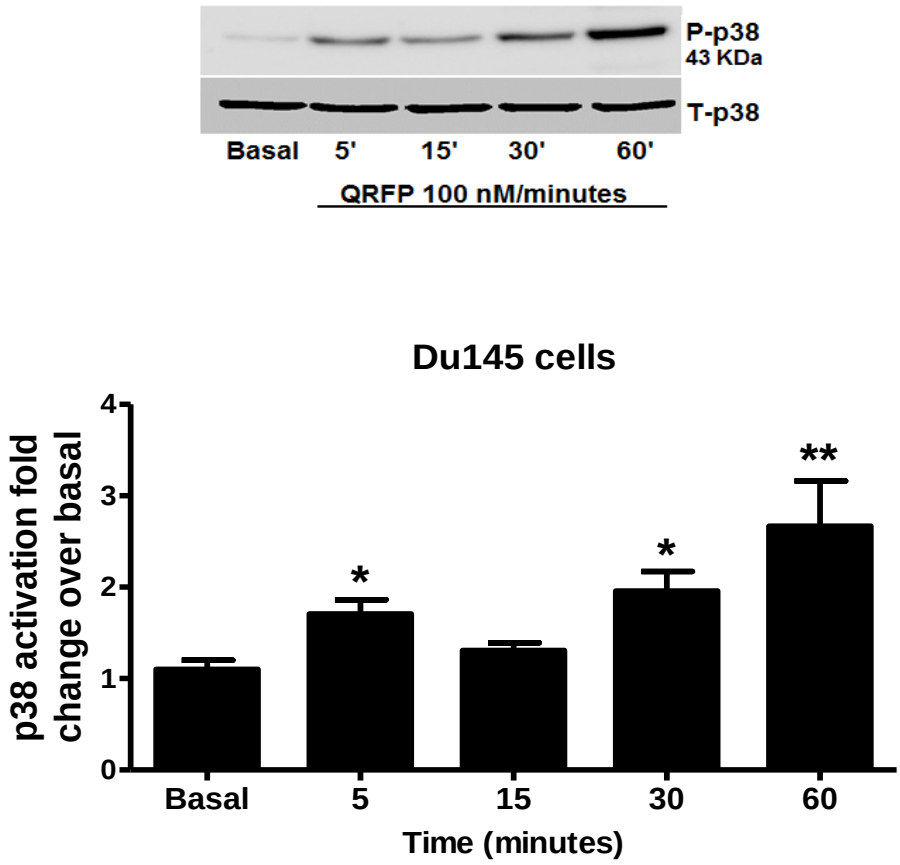

Figure 5.7 The effect of QRFP on (p38) MAPK expression in DU145 cells.

Western blot analysis showing changes in p38 protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in p38 activation in DU145 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

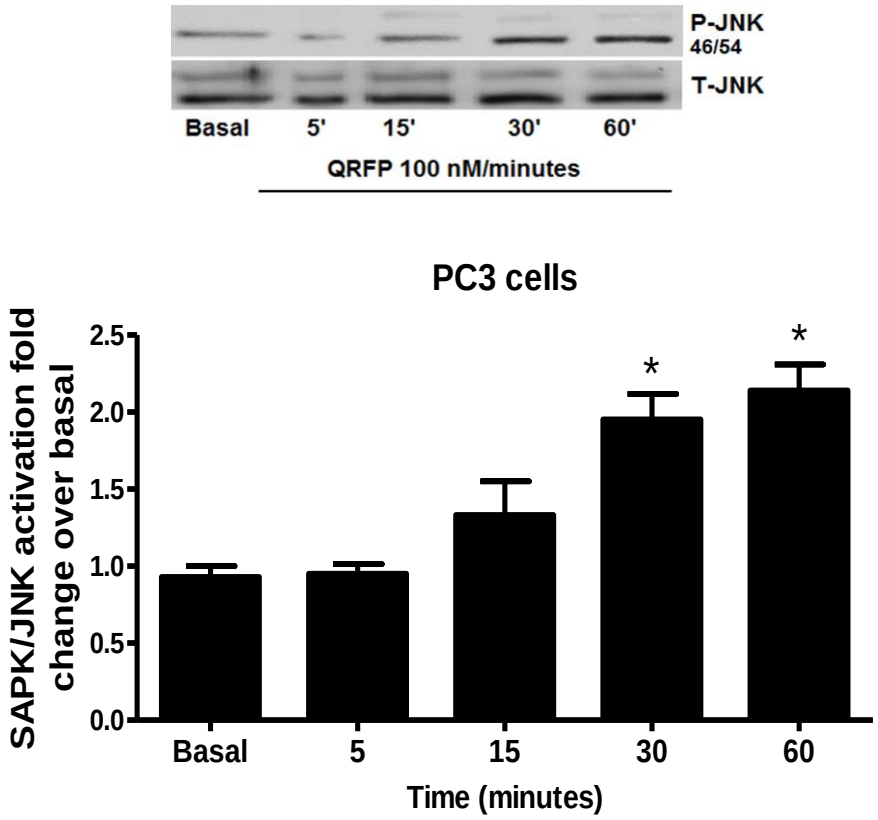

Figure 5.8 The effect of QRFP on (JNK1/2) MAPK expression in PC3 cells.

Western blot analysis showing changes in JNK1/2 protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in JNK1/2 activation in PC3 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 compared to basal expression.

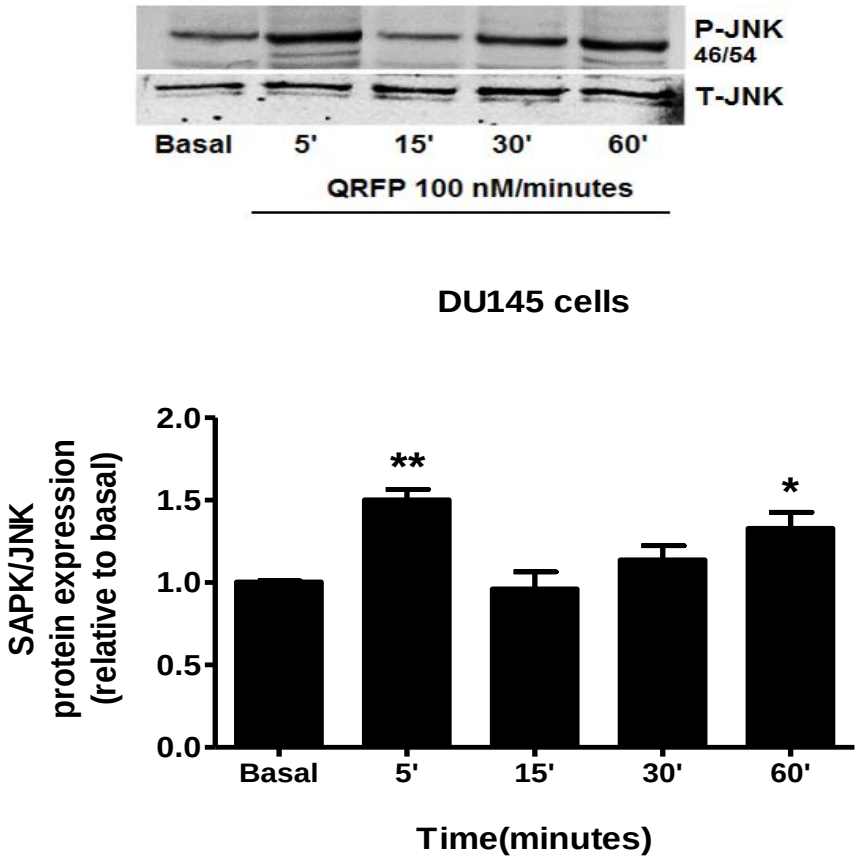

Figure 5.9 The effect of QRFP on (JNK1/2) MAPK expression in DU145 cells.

Western blot analysis showing changes in JNK1/2 protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in JNK1/2 activation in DU145 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

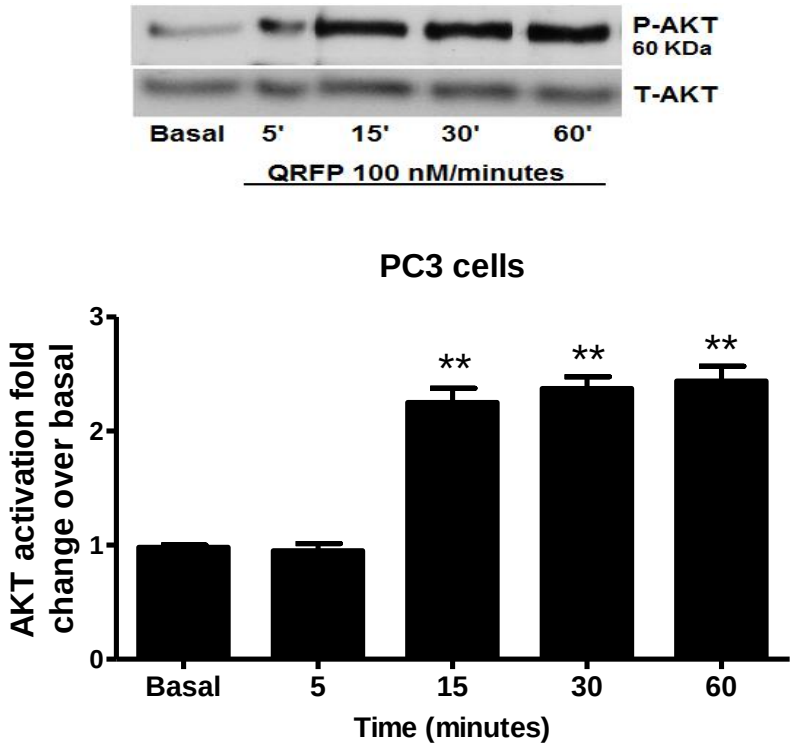

Figure 5.10 The effect of QRFP on (AKT) MAPK expression in PC3 cells

Western blot analysis showing changes in AKT protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in AKT activation in PC3 cells. Data represent mean ± S.D from three independent experiments; **P<0.01 compared to basal expression.

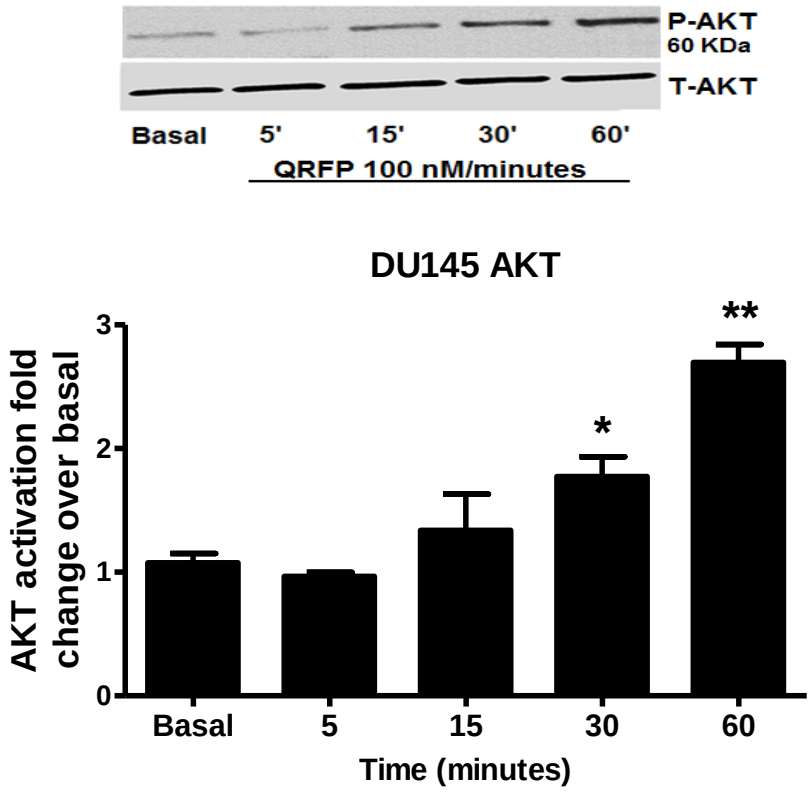

Figure 5.11 The effect of QRFP on (AKT) MAPK expression in DU145 cells

Western blot analysis showing changes in AKT protein phosphorylation and corresponding total protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 5, 15, 30 and 60 minutes) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in AKT activation in DU145 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

5.4 DISCUSSION

It has been shown that in functional assays, exposure of prostate cancer cells to QRFP increased cell migration, invasion and apoptosis in a dependent dose-time manner. Human Phospho-Kinase showed a variation in the effect of the three QRFP doses. The three QRFP doses 1, 10 and 100 nM demonstrated an increase in phosphorylation of the Src kinases member including Lck, fyn, yes, HcK, and lyn as well EGF R for the initial panel of phosphoprotein. Increased activation of Src co-operates with a wide range of proteins such as receptor tyrosine kinases, GPCR, epidermal growth factor receptor (EGF-R) and others regulation of a central pathways, include Ras/Raf/ /ERK, PI3K/PTEN/AKT, focal adhesion kinase (FAK) and STATs (Tatarov and Edwards, 2007) & (Li et al., 2010b). Several studies have indicated that an increased expression of the Src family and EGFR play a significant role in cell invasion, metastases and prostate cancer progression (Varkaris et al., 2014).
In contrast, QRFP decreased ERK1/2 and mTOR activation. Both are associated with prostate cancer proliferation and in line with our findings QRFP inhibits prostate cell proliferation. Activation of Akt 1/2/3, STAT2, STAT5a/b, GSK-3a/ß and HSP27 share many common functions including cell proliferation, migration, invasion and enhancement of prostate progression. Meanwhile, p38, AMPKa1, AMPKa2, and Chk-2 inhibit cell proliferation and induction of cell apoptosis. Cancer development is

associated with increased phosphorylation of Akt and inhibition of apoptosis pathways. The changes in Akt activity leads to the modulation of prostate cancer cell proliferation, survival and tumour growth (Li et al., 2007). The transcription factor STAT5a/b represents a possible therapeutic target for progressive prostate cancer. Recently it has been shown that STAT5a/b activation enhances proliferation but is also involved in the induction of metastatic prostate cancer cells, by the promotion of migration and invasion in PC3 and DU145 cells (Gu et al., 2010). HSP27 has been shown to increase cell migration and invasion upon increase MMP-2 expression and is associated with prostate cancer progression (Shiota et al., 2013) & (Voll et al., 2014). It has been shown that selenium can inhibit the growth of colon cancer cells by activation of AMPK·1 and suppression of mTOR signalling via Akt-dependent or independent pathways. In addition, other studies have indicated that AMPK·1 is required for the inhibition of mTOR and cell cancer proliferation (Lee et al., 2010b). The overexpression of IGF-binding protein-3 (IGFBP-3) activated the down regulation of AMPK, and AMPKα1/2 siRNA induced inhibition of the mTOR pathway and apoptosis in prostate cancer cells (Du et al., 2015). In the present study QRFP activated AMPK, leading to inhibition of the mTOR pathway and suppression of cell proliferation in prostate cancer cell lines. GSK-3 has been shown to be very unique in different signalling pathways, a process which is complex and controversial. Inhibition of GSK-3 activity is expected to be tumour promoting in many cancers. It can inhibit tumours

by supressing the Wnt/beta-catenin pathway (Gao et al., 2015). Chk2 is regulated by upstream ATM pathways, which are frequently activated simultaneously in cells exposed to various genotoxic stresses, such as cytotoxic chemotherapy agents (Smith et al., 2010). Generally, data suggests that alterations in CHEK2 can contribute to prostate cancer risk and that the DNA-damage–signalling pathway could play a significant role in prostate cancer progression (Dong et al., 2003). Time dependence of QRFP effects in PC3 cells results phosphorylation proteins in different manner. Nearby, 5 minutes QRFP100 nM showed increase phosphorylation of the MAPK proteins ERK1/2 for short time while negligible increase activation of JNK and Akt 1/2/3 also increased the phosphorylation EGF. The over-expression of EGF R proteins have been observed in both androgen insensitive PC3 and DU145 cells. Phosphorylation EGFR mediated activation many intracellular signaling cascades including of MAPK kinase MEK/ERK, phosphoinositide 3-kinase (PI3K)–Akt pathways (Gan et al., 2010). Activated ERK and Akt translocate to the nucleus, leads in downstream effects prostate cancer, including cell proliferation, cell apoptosis, angiogenesis, migration and invasion (Huang and Chang, 2011). However, some interesting proteins showed decrease phosphorylation of p38α, AMPKα1, AMPK α2, mTOR, Chk-2 and GSK-3a/ß.

QRFP appears also has a role by increasing phosphorylation of proteins at 25 minutes including of MAPK pathway JNK, p38α and AKT, however ERK showed a decrease. Moreover, the increased phosphorylation appeared in (AMPK α2, STAT2, STAT6, STAT5a/b, Chk-2 and GSK-3a/ß). Phosphorylation p38α has been reported as stimulatory effect on induction pro-apoptosis and inhibit EGF R also negatively regulate with cancer cell progression (Swat et al., 2011a). mTOR observed significant decrease in phosphorylation from dose and time dependency. So far the effects of QRFP on these signalling events in prostate cancer cells remain unclear. Examine the QRFP effect in PC3 cells by using human phosphor array appears spotted various proteins pathways functions. QRFP has been found to exhibit an increase in phosphorylation of a range of proteins including MAPK through a number of cellular signalling pathways. In PC3 cells, I studied QRFP induced signalling pathways, and the role of cell its GPCR (GPR103). MAPK regulates different cellular processes in PCa; a crucial player in cancer pathways seems to be the ERK pathway. The increased expression level of this signal pathway was revealed in human prostate cancer compared with normal prostate tissues (Johnson et al., 2008) & (Chen et al., 2013b).
The data I present demonstrate significant increase in ERK1/2 phosphorylation after 5 mins using the highest concentration of 100 nM QRFP in PC3 and DU145 cells which was determined with the cell migration and invasion studies. ERK1/2 has been shown to be activated

by several pathways depending on the distinct ligand, cell surface receptor, and cell type (Robertson et al., 2010). ERK1/2 is activated by a complex cascade comprising of Ras/Raf/MEK/ERK which regulate gene expression; moreover, this pathway also regulates the activity of several genes implicated in apoptosis (Lu and Xu, 2006). High expression levels of the Ras/Raf/MEK/ERK pathway might be promoted by autocrine and paracrine acting growth factors and has been related with progressive PCa (Johnson et al., 2008). Ras signaling is involved in cancer cell invasion and metastasis. Also it has been shown that the activation of EGFR-ERK1/2 pathway promoted the migration and invasion of prostate cancer cells (Li et al., 2015). However, it has been observed that Ras/Raf/MEK/ERK is expressed at low levels in hormonal- independent prostate cancer lines. Therefore, while it is reasonable to consider that activation of Ras/Raf/MEK/ERK cascade could contribute to prostate cancer development and it could not be that simple as some of these well-known prostate cancer cells such as PC3 and DU145 cells have low expression level of this cascade (Swat et al., 2011b). It has been shown that activating ERK by agents such as Resveratro and Bryostatin in DU145 cells and Phenylethyl isothiocyanate in PC3 cells induced apoptosis; additionally, vitamin D has inhibitory effects on cell proliferation by decreasing activated ERK (Ghosh et al., 2005). Also, a previous study proposed that activation of ERK1/2 signalling contributes to apoptosis via the suppression of the survival signalling AkT in cervical cancer cell (Zhuang and Schnellmann,

2006). This pathway, has been found to be implicated in different of pathways depending on the individual ligand, cell surface receptor and kind of cells (Robertson et al., 2010). The observation that ERK activation responded to QRFP at a short time point, whilst a longer time point led to a reduction in ERK, may be due to the fact that ERK pathway has a low expression level in androgen independent such PC3 and DU145 cells and poor outcome. Otherwise, as previously discussed, this may be a case of demonstrating ERK1/2 activation early related to through use of the correct dose of 100 nM QRFP or time-point which related to migration and invasion functions. Most published the effect of activation ERK pathway in prostate cancer is involved in cell proliferation, cell survival and poorly differentiated metastatic, however recently has been shown activation ERK promotes cell migration and invasion in prostate cancer cells (Rodríguez-Berriguete, 2012) & (Ding et al., 2015). In contrast, some studies have shown activate ERK dependent apoptosis in the three prostate cancer cell lines (Ghosh et al., 2005).This was the first study identified the effect of QRFP on ERK pathway in mediating prostate cancer cell.

Unlike ERK1/2, P38 is weakly activated by mitogens but strongly activated in cells in response to stress events include inflammation, UV radiation, osmotic shock and heat. Activation of p38 in PCa could be a result of up regulated upstream kinases (MKK3/6) and down regulated MAPK phosphatases. Recently, it has been suggested MKK4 may contribute to

activation of p38 MAPK in some conditions (Koul et al., 2013) & (Frank and Miranti, 2013). Phosphorylation p38 has been demonstrated in PCa cell lines when exposure by toxic agents, and their activation is implicated in apoptosis (Chang et al., 2008). p38 MAPK is involved in apoptosis induction by acts to have some roles such as in activating caspases (Maroni et al., 2004). In other studies, TGF-β1 has shown associated with enhanced activation of caspases-8, -9 and -3 in PC3 and DU145 cells and induced apoptosis particularly through activation p38 pathway (Al-Azayzih et al., 2012). Exposure of PC3 cells to arsenic trioxide induced apoptosis through activation of the p38 pathway (Su et al., 2013). Many evidences shown that p53 and PTEN have negative expression in prostate cancer androgen-independent PC3 and DDU145 cells by activation p38 pathway when exposure to some chemicals Berberine and Decitabine (Festuccia et al., 2008). Also TNFα, activates p38 induces apoptosis in LNCaP cells but not in PC3 cells (Frank and Miranti, 2013). Many different groups have been reported to active the p38 by IL-6, EGF, FGF-1/2, TNF-α, keratinocyte-derived growth factor (KGF), vitamin-D, and Neu differentiation factor (NDF) are implicated to prostate cancer (Koul et al., 2013). Hence, p38 activation may contribute to suppression of prostate cancer progress (Shimada et al., 2006). Otherwise, it has been demonstrated that the activation of MMP-2 in prostate cancer cell invasion mediated through the p38 MAPK pathway (Koul et al., 2013). Chen *et a* reported that stimulation of the G protein-coupled receptor, P2Y, induced

PCa cell invasion regulated by the activation of p38 pathway (Chen et al., 2004). Due to dual functions of p38 activation in genotoxic stress the exact effects in prostate cancer still in doubt. The novel data shown significant increased activation p38-phosphorlation in prostate cancer androgen-independent by QRFP dose 100 nM. The literature reviews indeed propose a significant role for activation p38 in prostate cancer cells however the precise role has not been completely elucidated. The data presented here is in line with the literatures and assists only to provide support. Preferably the further investigations required Caspases-3 and MMp-2 expression would help demonstrate and confirm the true significance of this results.

It has been suggested that similar to p38, JNK known as activated protein kinase (SAPK) induction apoptosis in response to several kinds of stress pathway (Wada and Penninger, 2004). JNK has mediated by upstream MKK7 kinase also associated with p38 activated upstream MKK4 (Sui et al., 2014). JNK could activate a range of transcription factors including, c-Jun, c-fos, Elk-1, c-My, ATF-2 and p53. Also, JNK phosphorylation can activate mitochondria protein Bcl-xL and Bcl-2 (Rodriguez-Berriguete et al., 2012). JNK MAPK is activated in response to certain growth factors or stresses such as chemotherapeutic agents, ultraviolet (UV) radiation commonly leads to cell death by activation of the mitochondrial apoptotic pathway in, prostate cancer cells (Gururajan et al., 2005). Phosphorylation of the JNK pathway induces apoptosis by activation of c-Jun, ATF-2, AP-1

and Fas/Fssl mediate the activation of caspeases (Sui et al., 2014). An apoptotic role mediated of JNK signalling has also been presented in apoptosis induced by many chemotherapeutic agents, including doxorubicin, vinblastine, cisplatinum and etoposide (Kuntzen et al., 2005). Shivendra V. *e*t al, reported that Guggulsterone could induced apoptosis in PCa cell lines through activation JNK pathway (Xiao et al., 2011). The objective of this study was to demonstrate whether QRFP has a role effect through activation JNK1/2 pathway on two prostate cancer cell lines PC3 and DU145 cells. However, QRFP mediated activation of JNK pathway in prostate cancer cells and whether this induces apoptosis, or progression has not been studied. This is first study showed that QRFP has increased activation of JNK MAPK in PC3 cells at 30-60 minutes, also has shown increased significantly JNK activity at time point 5-60 minutes in DU145 cells than in PC3 cells, but more increase activation in PC3 cells. This study shows that the death cell prostate cancer cell lines caused by is associated to activation of JNK pathways. The correlation between QRFP and JNK/SAPK activation mediated apoptosis induction is not a cell line specific effect and also not affected by p53 status. The result appears to suggest it induced apoptosis, however, it has not provide any insight into the signalling pathways involving this process.

AKT signaling pathway activated by several growth factors, cytokines and oncogenes (Shukla et al., 2007). They have shown that activation of the PI3K-Akt phosphorylation through some of mechanisms could contribute to induced tumor invasiveness and cancer development. Several reviews highlight the possible role for the PI3K/AKT pathway in PCa progression. Additional finding from current study is that pharmacological inhibition of the Akt pathway significantly affects EGFR induced PCa cell migration (Gan et al., 2010). Due to the high expression of Akt phosphorylation signals in response to external stimuli and is activated in many human cancer, I hypothesized that treated QRFP on prostate cancer cell lines mediated Akt pathway might be has a role in prostate cancer cells. In this study, has shown strong effect of QRFP by increase activation of Akt signalling in human prostate cell lines. PC3 cells indicated increase activation of AKT more than DU145 cell. It possible that PC3 cells has higher-expression phosphorylation of AKT (Guo et al., 2010). The PI3K/Akt/mTOR signaling pathway has been shown to regulate many cellular proses including cell survival, proliferation, metabolism, migration, and angiogenesis. It is well recognised that the activation of the p38 and JNK and pathways has been associated with events leading to apoptosis. Furthermore, the ERK and P13/AKT signal pathway plays critical role in regulation cancer cells survival and proliferation. It is interesting that QRFP seems to activation both kinases involved in survival pathways and apoptosis pathways; however, I am planning to perform downstream

signalling mediators and genes involved in both cell survival and apoptosis process to confirm the actual role of QRFP in prostate cells. It noteworthy to mention for example leptin that have been previously demonstrated to promote various cancer growths does activate AKT, p38 and JNK phosphorylation (Hoda et al., 2012b). To confirm some results from western blot, we evaluate inhibit MAPK pathway and p-Akt expressions.

CHAPTER 6

THE ROLE OF QRFP ON AGR-2, MMPs, CASPASE-3 AND AMPK IN PCa

6 INTRODUCTION

ANTERIOR GRADIENT PROTEIN-2 HOMOLOG (AGR-2)

Human AGR2, is a secreted protein that is exclusively expressed in several organs such as stomach, lung, colon, and prostate (Hu et al., 2012). Besides, AGR2 is implicated in an array of biological processes in different cancer models such as regulation of p53, cell proliferation, migration, invasion, metastasis, cellular transformation, and cell adhesion (Wang et al., 2008). Recently, researchers have focused to find specific roles of AGR2 in cancers since it is involved in tumor development, metastasis and drug resistance (Salmans et al., 2013). They have detected elevated AGR2 levels in multiple human carcinomas including lung, colon, ovarian, breast and prostate cancers compared to the normal tissues. In prostate cancer cells, AGR2 is one of many such genes recognized as overexpressed (Dumartin et al., 2011). Additionally, AGR2 is elevated in PCa cells compared to non-malignant prostatic epithelial cells at the transcriptional and protein levels. The signal level for AGR2 in the PCa cell is ~50-fold higher than that in normal counterpart luminal epithelial cells (Maresh et al., 2010). AGR-2 is stated to be an androgen inducible and just over-expressed through early stages of carcinogenesis in prostate cancer (Chanda et al., 2014). Contrariwise, modern studies have observed lower level of AGR-2 expression in high-grade tumors (al, 2013) & (Chanda et al., 2014).

MATRIX METALLOPROTEINASES (MMPs)

A family of endopeptidases identified, as MMPs are a possible objective for cancer therapy. The Matrix metalloproteinases (MMP) family contains zinc and calcium -dependent proteinases that response to remodelling and degrades basement membrane and extracellular matrix components such as elastin, proteoglycans, glycoproteins, and denatured collagen (Gong et al., 2014). Thus, MMPs influence several significant processes, such as cell differentiation, proliferation, migration, apoptosis, and cell–cell interactions. Wherever degradation of the basement membrane and extracellular matrix is the first stage in invasion and metastases (Bonnans et al., 2014). The proteinases 72 kDa MMP-2 and 92 kDa MMP-9 have been identified as markers for cancer invasion and metastases in a variety of different cancers include colon, breast and specifically associated with prostate cancer (Roomi et al., 2009). Moreover, the expression of MMP-2 and MMP-9 in benign and malignant prostate is detected in tissue and serum samples (Zhang et al., 2004). Activated MMP-2 can activate other MMPs such as MMP-9 through enzymatic cleavage. It has been shown increase in expression of MMP-2 extensively reported in prostate cancer and correlated with larger tumor size, higher Gleason score (Oguic et al., 2014). Recent publications indicated that MMP-2 expression has been found in metastatic cancer, however has not expressed in micro-metastasis and surrounding stromal cells of low grade disease, strongly proposing that increased MMP-2 expression is related with prostate cancer

development and metastasis (Trudel et al., 2003). frequently used Prostate cancer cell lines LNCaP, DU-145, and PC-3 have been determined as low, moderate, and high metastatic possible in Matrigel invasion assays, respectively. Thus, this study aimed to identification of the role QRFP and molecular markers that can predict PCa readiness and progression through activation MMP2 pathway.

CASPASES (cystein-dependent aspartate-specific protease expression)

It is unclear whether caspases are fundamental for apoptosis or other proteases may control their functions. activation of caspases cascades in PCa plays a crucial role in the execution-phase of cell apoptosis (Wong, 2011). Cytochrome –C release from the mitochondria functions as a critical stage in the activation of downstream caspase-3, an apoptosis executioner (Ow et al., 2008). Also that is fundamental for the nuclear changes related with apoptosis, counting chromatin condensation. Caspase-3 is commonly expressed in human tissue include prostate and human prostate cell lines (Jager and Zwacka, 2010). Overexpression or loss of expression of caspase-3 has been stated in malignancies and many of human cell lines. Caspease-3 mediated cleavage of P21 has been revealed to transform prostate cancer cells from the arrest to apoptosis. This leads to acceleration of chemotherapy induced apoptotic process in the PCa cells (Eastham) & (Persad et al., 2004). In several examples caspase-3

generates a constituent kinase activities responsible for transduction of the apoptotic pathway. Investigation of these kinases especially their deregulation, will allow us finding new targets for gene therapy in PCa. In this study, to demonstrated the effect of QRFP on the activation of caspase-3 by immunoblot in PCa cell lines PC3 and DU145 cell.

ADENOSIN 5′-MONOPHOSPHATE-ACTIVATED PROTEIN KINASE (AMPK)

The main role of activation AMPK is to restore cellular energy balance through controlling ATP process by enhancing processes that increase ATP meantime inhibiting consuming ATP processes (Woodard and Platanias, 2010) & (Hardie, 2011). In active proliferating cells, it has been stated that activation of AMPK lead cell cycle prevents via up-regulation of the p53-p21 alliance (Motoshima et al., 2006). Since PC3 cells do not contain wild-type p53 alleles, the induction of p21 by AICAR observed in the present study was probably p53-independent (Motoshima et al., 2006b) & (Aimola et al., 2012). Collectively, these findings suggest that AMPK activation can inhibit the growth of prostate cancer cells by at least three distinct mechanisms (Towler and Hardie, 2007). Conversely, other reports indicate that AMPK might play tumour-promoting roles in the prostate and AMPK activation is higher in PCa than in normal prostate tissue. Certainly, it has been suggested that AMPK can really contribute to resistance to anti-cancer therapy in some situations (Viollet et al., 2010) &

(Jurmeister et al., 2014). AMPK activation is produced after phosphorylation at threonine 172 in the AMPK structure via upstream kinases. Furthermore, AMPK phosphorylation at Thr172 is detectable in some of PCa cell lines, including the androgen-independent DU-145 and PC-3 cells and the androgen-sensitive LNCaP cells (Park et al., 2009). Some of molecules and signalling pathways have been described to be control by AMPK through direct phosphorylation like AMPK substrate and as well as indirectly by gene expression regulation. Signalling pathway of the apoptotic Fas death receptor is reduced in DU-145 cells, in spite of high cell-surface expression of wt Fas, and due to the enhanced AMPK activity. Other pharmacological regulator of AMPK such as AICAR is shown to suppress the proliferation and viability of a PCa cells, including the androgen-dependent LNCaP cell and androgen-insensitive (PC3, DU145 and C4-2) cells (Zhou et al., 2009) & (Zadra et al., 2014). In view of the paradoxical findings on the role of AMPK in cancers, I seek to improve our grasp of AMPK signalling pathway in PCa cells by characterizing the transcriptional production of AMPK activation. Also to emphasis on the effect of QRFP through AMPK activation on prostate cancer cell lines (PC3 & DU145) and discuss the possibility that AMPK might be a therapeutic target for PCa.

MAPK PATHWAY FUNCTION STUDIED BY INHIBITION

Biochemical reagents such as inhibitors of components of the MAPK pathway are overwhelmingly a viable alternative or an integral tool in understanding the functional requisite in a given pathway. Such inhibitors have enabled researchers to distinguish between the major MAP kinase cascades and to recognize the function of various MAP kinases in cellular activation by extracellular stimuli (Burkhard and Shapiro, 2010). Several studies suggested that some neuropeptides which regulate energy homeostasis, can as well either directly or indirectly be implicated in the progress of benign prostatic hyperplasia and prostate malignancy (Malendowicz et al., 2011). In this concern, special attention is being founded on obesity and prostate cancer incidence and accumulating data suggest the higher risk of aggressive cancer development in obese patients.

6.1 HYPOTHESIS

It is important to comprehend the mode of regulation of different cellular processes by signalling modules, which is one of the essential functions of signal transduction study. Therefore, it is significant to understand the downstream effects of various signaling pathways and the cellular reactions that they regulate. Understanding cell signalling in cancer can provide possible targets for anticancer drugs to enhance apoptosis and/or inhibit tumour progression (Tang et al., 2014). The common way to

determine the involvement of signaling pathway in the researched cellular regulate is through blocking its activity followed by measuring effects of this inhibited on the cellular study (2011). Thus, this study targeted to identification of the role QRFP and molecular markers that can predict PCa readiness and progression through activation various downstream signaling pathways.

6.2 AIMS

- To demonstrate the effect that QRFP has on anterior gradient 2 (AGR2) expression in PCa cell lines.
- To demonstrate the effects of QRFP on matrix metalloproteinases (MMP) in PCa cell lines.
- To demonstrate if QRFP activates Caspase-3 in PCa cell lines.
- To demonstrate if QRFP activates AMPK in PCa cell lines.
- To demonstrate if QRFP has a role by knockdown it's receptor GPR103 PCa.
- To demonstrate the effect that QRFP has on MAPK, PI3K/AKT and AMPK inhibitors in PCa cell lines.

6.3 RESULTS

6.3.1 METHODS FOR ASSESSING THE EFFECTS OF QRFP ON AGR2 EXPRESSION

PC3 and DU145 cell lines were cultured as described above until confluent in 6 well plates. Cells were incubated in serum starvation media for overnight prior to treatments. AGR2 protein evaluates were varying doses 0=(basal),10 nM and 100 nM QRFP supplemented to the media and the cells were incubated for 24 hours and the resultant protein lysates were quantified as before and then analysed using western blot as described above using the AGR2 primary antibody and GAPDH 1:1000. In order to evaluate the pathways by which QRFP eventually applies its effects on AGR2 activation and to demonstrate if QRFP induced migration and invasion in prostate cancer cells by activated AGR2 protein expression at 18 KDa. Result showed QRFP induced significant activation of AGR2 on PC3 cells on both doses 10 and 100 nM *$p < 0.05$ and **$P<0.01$ respectively at 24 hours compared to basal levels (Figure 6.1).

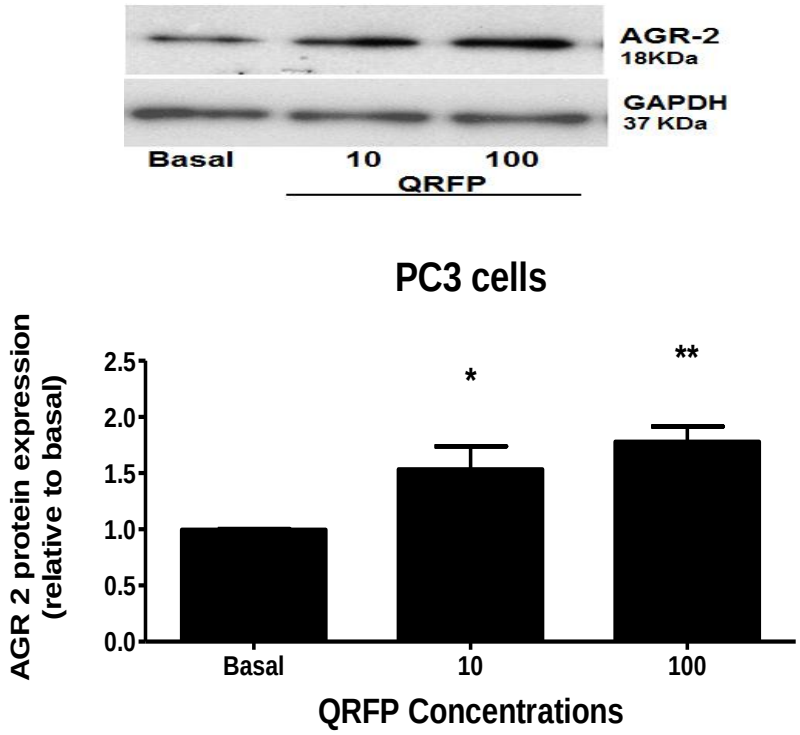

Figure 6.1The effect of QRFP on AGR-2 protein expression in PC3 cells.

Representative western blot analysis showing changes in AGR-2 protein activity and corresponding GAPDH protein expression following treatment with 10 and 100 nM QRFP for 24 hours of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in AGR-2 activation in PC3 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

6.3.2 THE EFFECT OF QRFP ON MMP-2 PROTEIN EXPRESSION IN PC3 and DU145 CELLS

PC3 and DU145 prostate cancer cell lines were cultured as described above until confluent in 6 well plates. Cells were incubated in serum starvation media for overnight prior to treatments. Exposer PC3 and DU145 cells at time dependent (2, 4, 6, 8 and 12 hours) with 100 nM QRFP which corresponded to the greatest dose migration and invasion functional effect. The resultant protein lysates were quantified and then analysed using western blot as described above using the MMP-2 primary antibody and GAPDH at 1:1000 dilution in order to evaluate the pathways by which QRFP eventually applies its effects on MMPS activation and to demonstrate if QRFP induced migration and invasion in prostate cancer cells by activated MMP-2 protein expression at 65 KDa Result showed QRFP induced activation MMP-2 on PC3 cells which reached significant activation *$p < 0.05$ at time dependent 2 hr and the level remained significantly higher even at 8 hr later turned back to basal levels (Figure 6.2). An increase in activation of MMP-2 showed on time pointe in DU145 cells higher than in PC3 cells, the maximum increase significant activations were seen at 2, 8, and 12 hours **$P<0.01$ and slightly drop to significant activation at 4 and 8 hours *$p < 0.05$ all data compared to cell basal (Figure 6.3).

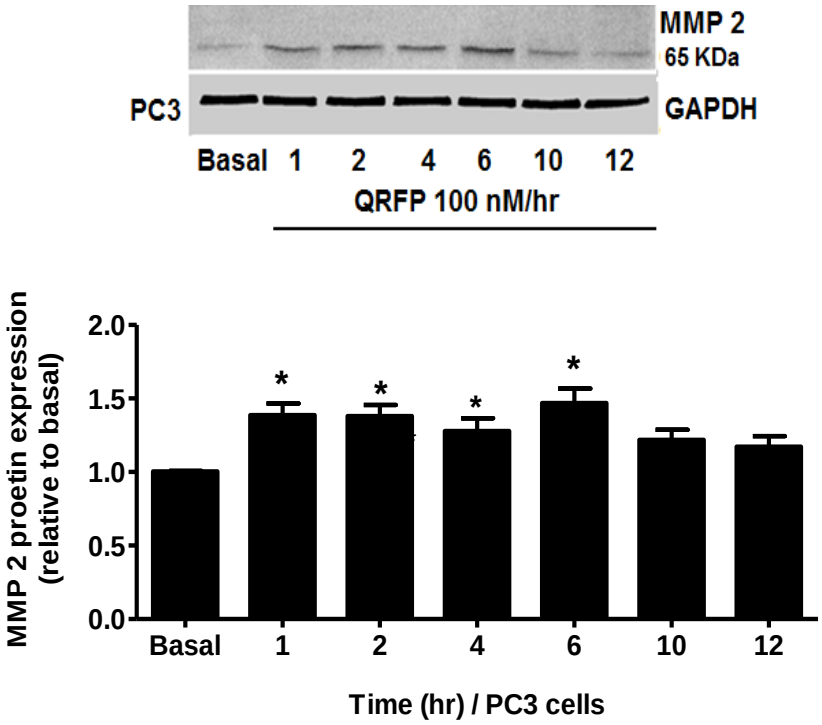

Figure 6.2 The effect of QRFP on MMP-2 protein expression in PC3 cells

Representative western blot analysis showing changes in MMP-2 protein activity and corresponding GAPDH protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 1, 2, 4, 6, 10 and 12 hours) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in MMP-2 activation in PC3 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 compared to basal expression.

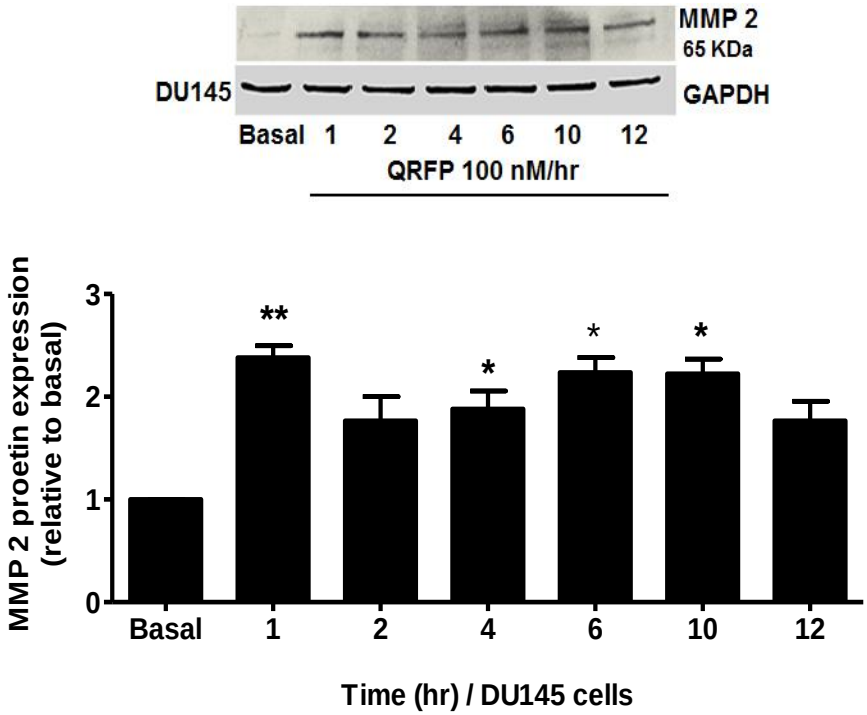

Figure 6.3 The effect of QRFP on MMP-2 protein expression in DU145 cells

Representative western blot analysis showing changes in MMP-2 protein activity and corresponding GAPDH protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 1, 2, 4, 6, 10 and 12 hours) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in MMP-2 activation in DU145 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

6.3.3 THE EFFECT OF QRFP ON TOTAL CASPASE-3 ACTIVITY IN PC3 and DU145 CELLS

PC3 and DU145 prostate cancer cell lines were cultured as described above until confluent in 6 well plates. Cells were incubated in serum starvation media for overnight prior to treatments. Incubation PC3 and DU145 cells for time dependent (1, 2, 4,6,10 and 12 hours) with 100 nM QRFP which corresponded to the greatest apoptosis functional effect. The resultant protein lysates were quantified and then analysed using western blot as described above using the Caspase -3 primary antibody and GAPDH at 1:1000 dilution to determine whether QRFP promoted apoptosis of prostate cell lines by measured protein expression of Caspase-3. A significant activation of Caspase-3 in PC3 cells were indicated at time point 1, 2, 6 and 12 hours $*p < 0.05$ and appeared to come towards basal levels at time 4 hours and 10 hours (Figure 6.4). Similar activation shown in DU145 cells were statically significant activation at time pointe 1, 4, 6 and 12 hours $*p < 0.05$ and rapid decreased to basal levels at time point 2 hours and 10 hours (Figure 6.5).

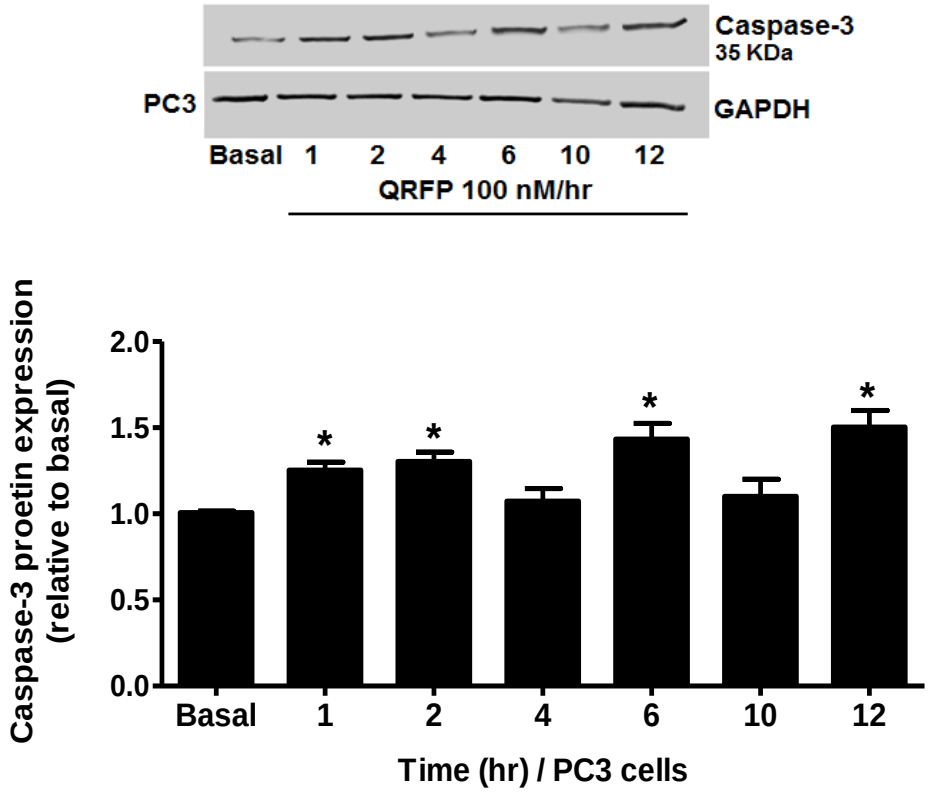

Figure 6.4 The effect of QRFP on total Caspase-3 protein expression in PC3 cells

Representative western blot analysis showing changes in Caspase-3 protein activity and corresponding GAPDH protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 1, 2, 4, 6, 10 and 12 hours) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in Caspase-3 activation in PC3 cells. Data represent mean ± S.D from three independent experiments; *$p < 0.05$ compared to basal expression.

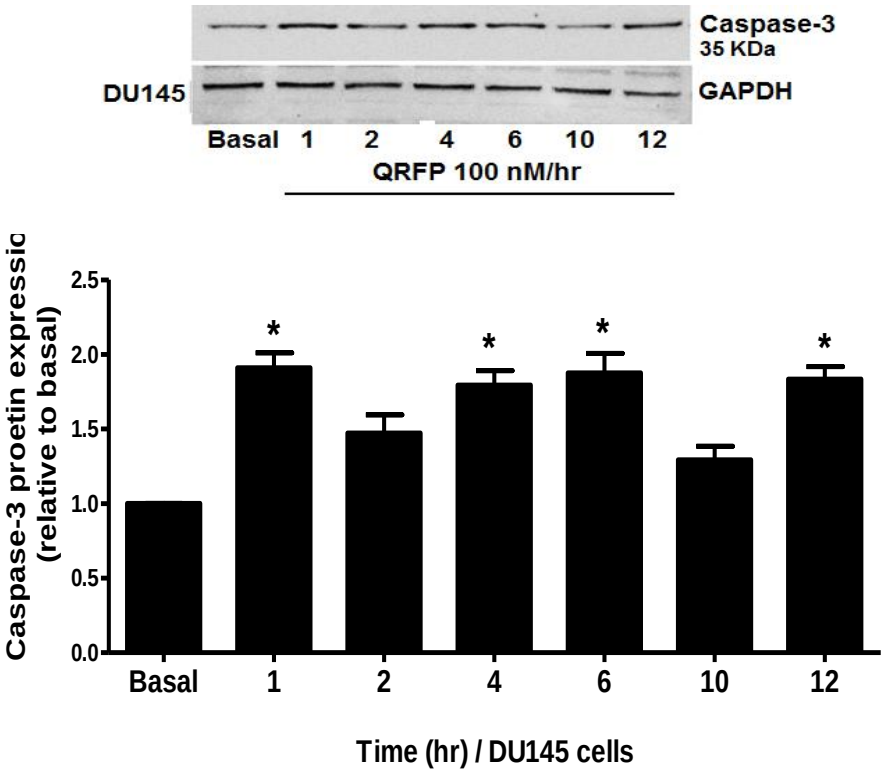

Figure 6.5 The effect of QRFP on total Caspase-3 protein expression in DU145 cells

Representative western blot analysis showing changes in Caspase-3 protein activity and corresponding GAPDH protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 1, 2, 4, 6, 10 and 12 hours) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in Caspase-3 activation in DU145 cells. Data represent mean ± S.D from three independent experiments; *$p < 0.05$ compared to basal expression.

6.3.4 THE EFFECT OF QRFP ON AMPK ACTIVITY IN PC3 and DU145 CELLS

PC3 and DU145 prostate cancer cell lines were cultured as described above until confluent in 6 well plates. Cells were incubated in serum starvation media for overnight prior to treatments. Stimulate PC3 and DU145 cells for time dependent (2, 4, 6, 8 and 12 hours) with 100 nM QRFP which corresponded to the greatest apoptosis functional effect. The resultant protein lysates were quantified and then analysed using western blot as described above using the AMPK primary antibody and GAPDH at 1:1000 dilution to demonstrated the effect of QRFP mediated the AMPK expression on prostate cancer cell lies QRFP induced strong activation of all at time points AMPK activity in PC3 cells reached significant activation at 2 hr *p < 0.05, high significant at 4 hr and 8 hr **P<0.01 and highest increase activation at 6-12 hours ***P<0.001 **(**Figure 6.6). DU145 cells presented also QRFP increase significant effect on AMPK activity at time pointe 2, 8 and 12 hours **P<0.01 and significant effect at 4 and 6 hours *p < 0.05 all data related to basal cells **(**Figure 6.7).

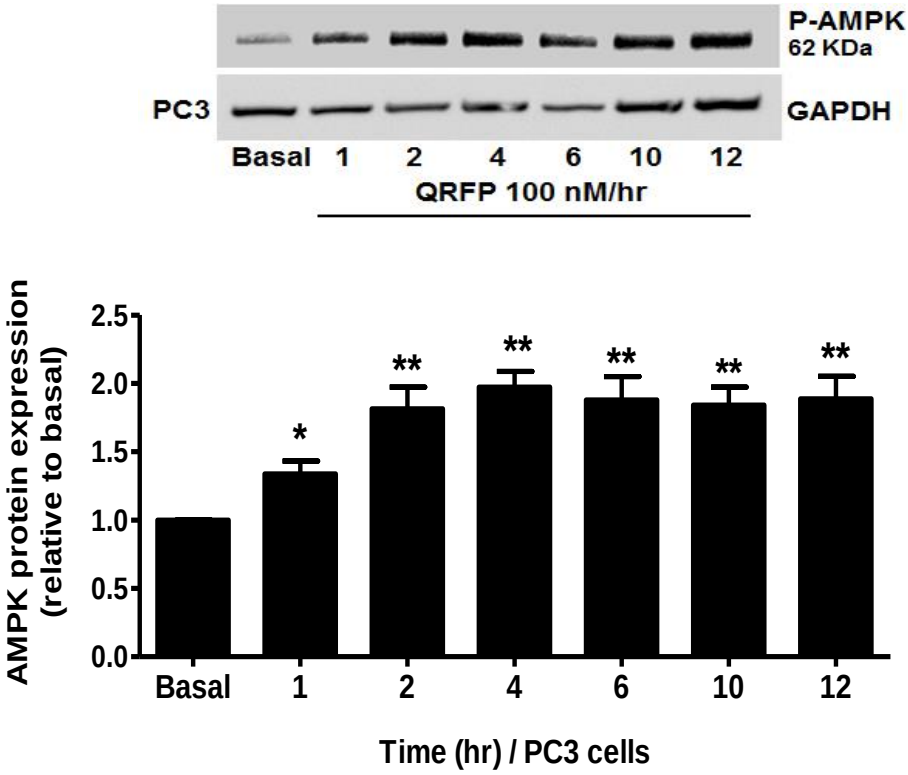

Figure 6.6 The effect of QRFP on AMPK protein expression in PC3 cells

Representative western blot analysis showing changes in AMPK protein activity and corresponding GAPDH protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 1, 2, 4, 6, 10 and 12 hours) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in AMPK activation in PC3 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

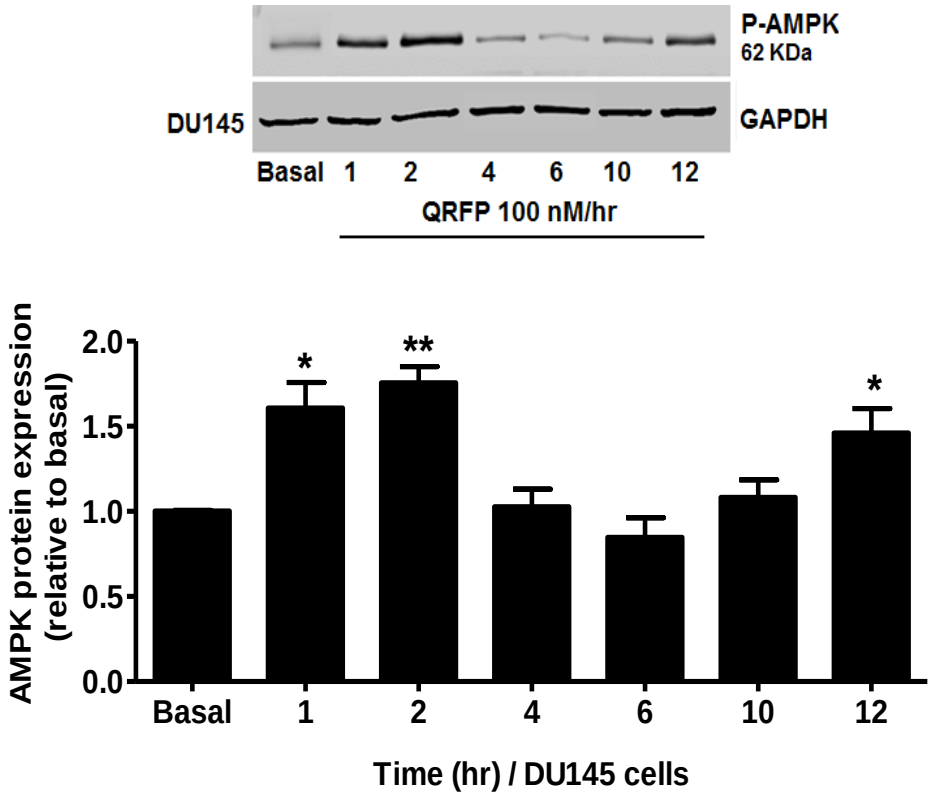

Figure 6.7 The effect of QRFP on AMPK protein expression in DU145 cells

Representative western blot analysis showing changes in AMPK protein activity and corresponding GAPDH protein expression following treatment with 100 nM QRFP at varying time points (0 = Basal, 1, 2, 4, 6, 10 and 12 hours) of incubation; lanes correspond to bar chart.

The graph showing quantification of changes in AMPK activation in DU145 cells. Data represent mean ± S.D from three independent experiments; *p < 0.05 and **P<0.01 compared to basal expression.

6.3.5 GPR103 GENES SILENCING INHIBITTS p-AKT PATHWAY IN PC3 cells

To investigate the potential role of GPR103 in QRFP induced AKT signaling, siRNA specific for the silence GPR103 receptor was employed in according to manufacturer's instructions in method chapter. The mRNA level of GPR103 significantly decreased in PC3 following siRNA transfection at increasing concentration of siRNA. Figure 6.8. A, has shown silence the expression of GPR103 mRNA decreased significantly with increasing doses of siRNA *P<0.05, **P<0.01 and ***P<0.001 respectively. I observed and dose dependent reduction in GRP103 expression and maximum reduction was observed at 10nM siRNA concentration. Future studies were conducted using this 10nM siRNA concentration for AKT phosphorylation experiments.

The maximum inhibition of GPR103 was achieved at siRNA (10 μM) concentration. AKT phosphorylation studies were conducted in cells transfected with GPR103 siRNA. The QRFP induced p-AKT protein expression was significantly decreased in cells transfected with GPR103 siRNA compared to control in PC3 cells as showed in (Figure 6.8. B).

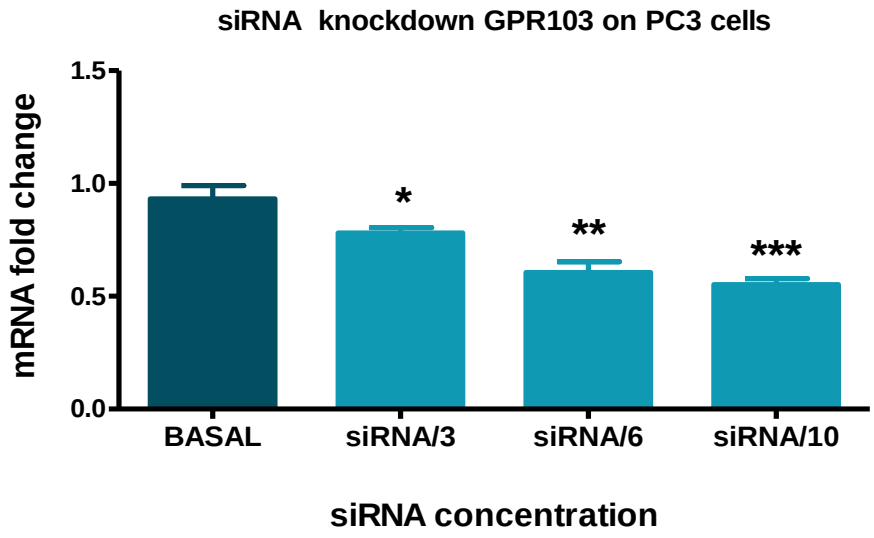

Figure 6.8. siRNA mediated knockdown of GPR103 receptor PC3 cells. Bar chart representing the effects of siRNA mediated knockdown of genes targeted using real-time PCR. By using siRNA concentration from 3, 6 and10 nM range and complexed with 2 µg/ml Lipofectamine™ 2000 and transfected for 24 hours. The following day mRNA expression for GPR103 expression were measured using real-time PCR to quantify GPR103 expression. Data showed dose dependent significantly decreased in GRP103 expression and maximum reduction was observed at 10nM siRNA concentration. Data are presented as mean ± S.D, the experiment was performed in 4 wells *P<0.05, **P<0.01 and ***P < 0.001 as compared with control (basal) cells.

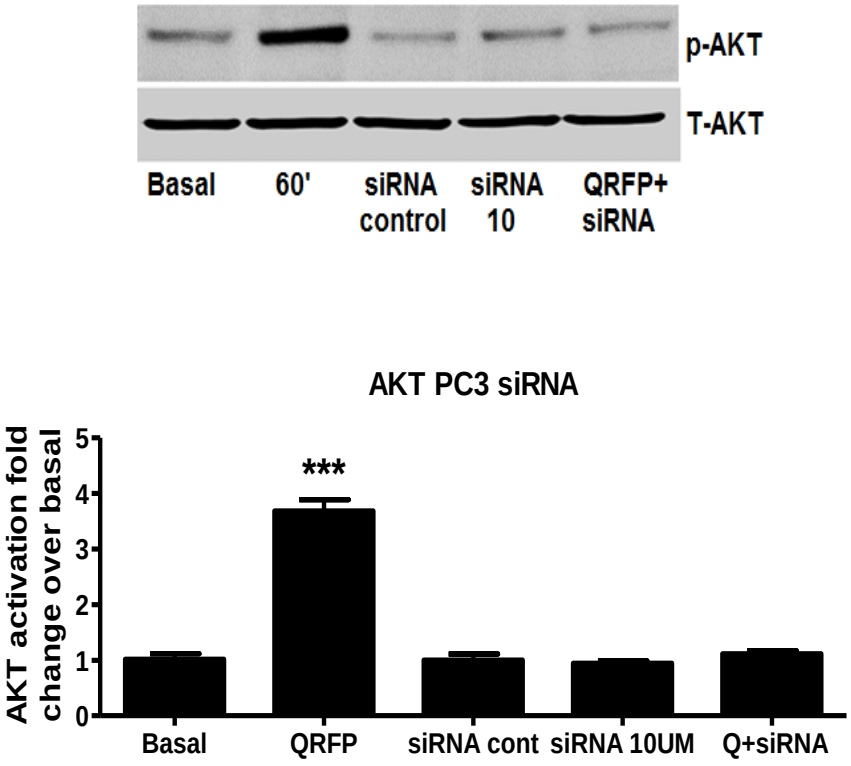

Figure 6.9 Effect of GPR103 receptor knockdown on QRFP induced AKT phosphorylation in PC3 cells.

By used 10 nM siRNA complexed with 2 µg/ml Lipofectamine™ 2000 and transfected for 24 hr. The following day cell culture media was changed and cells were allowed to settle for 3 hours prior to stimulation with or without QRFP. Representative western blot and graph presented PC3 cells transfected with GRP103 siRNA QRFP induced AKT phosphorylation was completely abolished compared to non-transfected cells. Data represent mean ± S.D from three independent experiments; ***P<0.001 compared to basal expression.

6.3.6 THE EFFECT OF QRFP WITH MAPK, PI3K/AKT AND AMPK IINHIBITORS IN PC3 AND DU145 CELLS.

To further determine whether mitogen-activator protein kinase (MAPK), AKT, and AMPK signaling pathways are involved in QRFP mediated prostate cells immigration. Approximately 70% confluent PC3 and DU145 cells were seeded in CIM-Plate and incubated with designated signaling inhibitors for 1 h prior to subsequent treated with QRFP peptide (100 nM) alone or combined p38 (SB202190; 10 µM), PI3K inhibitor (LY294002; 10 mM), AMPK inhibitor (compound C;) and MAPK inhibitor (U0126; 10 mM) for 8 hours. Following stimulation xCELLigence system applied to determine cell migration.

As showed in Figure 6.10/6.11 incubation PC3 and DU145 cells with AMPK and P38 inhibitors combined with the peptide significantly increased the migration rate compare to the cell incubated with the peptide alone *P<0.05, **P<0.01 respectively. In contrast, the effect of QRFP with PI3K inhibitor in PC3 and DU145 cells showed significantly reduced cell migration compared to basal cells or basal related with QRFP **P<0.01. PC3 and DU145 cells were treated with a selective MEK/ERK1/2 inhibitor U0126 combined with the peptide or alone shows no QRFP change effects on this pathway.

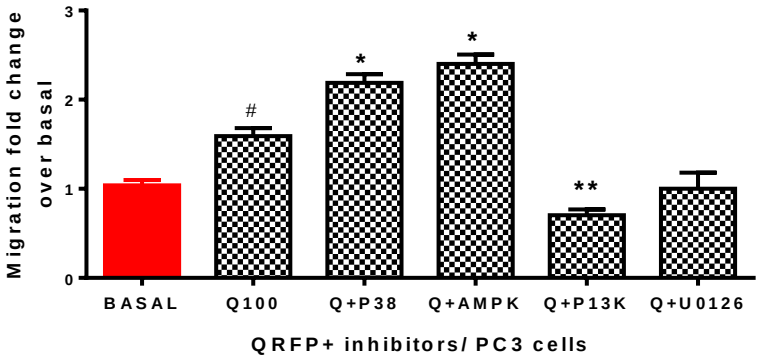

Figure 6.10 The effects of QRFP and combination inhibitors on migration signalling pathways in PC3 cell. Representative graph rate of QRFP 100 nM, p38, AMPK P13K and U0126 inhibitors were incubated for 8 hours compared to QRFP alone and to basal cells. Data were evaluated using the xCELLigence RTCA software mean ± SD, (n = 5) $^{\#}P < 0.05$, *P<0.05 and **P<0.01.

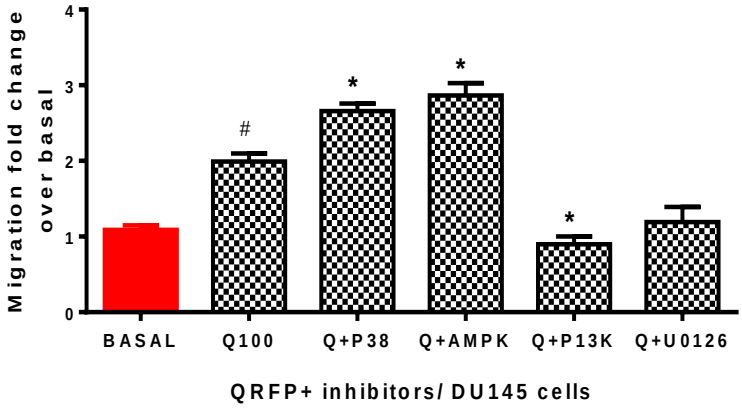

Figure 6.11 The effects of QRFP and combination inhibitors on migration signalling pathways in DU145 cell. Representative graph rate of QRFP 100 nM, p38, AMPK, P13K and U0126 inhibitors were incubated for 8 hours compared to QRFP alone and to basal cells. Data were evaluated using the xCELLigence RTCA software mean ± SD, (n = 5) $^{\#}P < 0.05$ and *P<0.05.

6.4 DISCUSSION

Anterior gradient 2 (AGR2) appears high-expressed in prostate cancer and involved to promote cell migration, invasion, cellular transformation and drug resistance (Hu et al., 2012). Also controls the cell adhesion rates of detached cells, which may correlate with the ability of metastatic cells (Hrstka et al., 2010). The interesting data obtained from western blot presented QRFP induces increase in anterior gradient (AGR2) expression in PC3 cells compared to DU145 cells on dose dependency. However, this referred to the low expression of AGR2 whereas overexpressed in PC3 cells. Again QRFP at 100 nM seems to show strong effect on prostate cancer cells. Interestingly Bu, *et al*. reported that while high-expressed AGR2 in prostate cancer cells increased migration and invasion and apparently metastatic spread, however, AGR2 supressed cell growing and proliferation (Maresh et al., 2010) . This finding might explain our previous data which QRFP induce prostate cancer cell migration and invasion and suppression cell proliferation. It has been confirmed that AGR-2 expression was strong associated with cyclin D1 and negative association with P53 and p21 in prostate cell lines. Also it has been revealed that downregulation of AGR2 was related to cellular senescence, by active p16, p21, p27 and suppression PI3K/Akt/ ERK in prostate cancer cells (Hu et al., 2012).

The mechanism of expression AGR2 could be involved in molecular crosstalk among ER and other pro-oncogenic signalling pathways including insulin-like growth factor receptor (IGFR), epiderma-l growth factor receptor (EGFR), ERK, and PI3K /AKT pathway (Hrstka et al., 2013). However, this leads to a potential role of QRFP in the manipulation of AGR2 activation and consequently a role for AGR2 in prostate cancer progression.

Several reports have showed that increased expression levels of MMP-2 in the serum or tissue samples of prostate tumor patients are associated with late stage. As well indicated the expression of MMPs associate with migration, invasive and metastatic possible of prostate cancer cell lines (Xiao et al., 2012). Expression of MMP-2 is linked to prostate cancer advance, whereas the Inhibition of MMP-2 expression suppresses the prostate cancer metastatic. To understanding the mechanisms by which QRFP effect activation of MMP-2 mediate activation MAPK is sought after for the possible therapeutic applications of cancer migration and invasion. Further examined has been early performed of the upregulate MAPK signaling pathways of MMP-2 associated with cellular migration and invasion. It is acknowledged that the MAPK pathway regulates cancer cell metastasis. Several recent suggestions have been connected with the activation of ERK1/2 signaling pathway and MMPs (Shuvojit Moulik, 2014).

Association of MMPs in EGFR transactivation by GPCRs has been described formerly (Amorino et al., 2007). For the first time to our knowledge that QRFP is found to effect activation MMP-2 expression on prostate cancer cell lines, QRFP is found to affect activation MMP-2 expression in prostate cancer cell lines, which potential supports the role of QRFP on cell migration and invasion. The expression and the activities of MMP-2 upon exposure to 100 nM QRFP at time dependent showed increase in activation in PC3 cells than DU145 cells. In order to fully clarify the mechanisms implicated in QRFP induced PC3 and DU145 cell migration and invasion. Attributed to the overexpression of MMP 2 observed in a progression metastasis bone which related to PC3 cells. MMP-2 expression has been shown regulate by many transcriptional factors g AP-1/2, ETS, Sp-1, and NF-κB (Gong et al., 2014). MAPK signalling pathways (ERK, p38 JNK) and AKT have been indicated to upregulate MMPs expression (Yang et al., 2013). Though, the previous studies indicated the association between activation of EGFR and downstream signal pathway Raf/MEK/ ERK1/2 signalling pathway and MMP expression (Chen et al., 2015). Moreover, there is a potential direct association between MMP2 and ERK1/2 activity which could involve with cFos and STAT3 (Shuvojit Moulik, 2014). Enhance MMP-2 expression by p38 MAPK pathway mediated TGF-β- has been involved in migration and invasion in prostate cancer cell lies (Huang et al., 2005). Chen et al, presented shikonin (Chinese medicine) could supressed metastasis PC3

and DU145 cells through inhibited MMP-2 expression mediated inactive AKT/mTOR and activation ROS/ERK1/2/ and p38/JNK pathway (Chen et al., 2014b). This data clearly demonstrated that QRFP involved in increase activation of MAPK signalling pathways and AKT signlaing and activities downregulates MMP-2 expression in PC3 cells and DU145 cells. QRFP may has potential roles in developing prostate cancer metastasis on dose and time dependent manner. It is of interest, therefore, to define whether apoptosis is induced by the QRFP through the MAPK and AKT signaling pathway mediate downregulate caspase-3 and induce apoptosis in prostate cancer cell lines. The data obtained from immunoblot showed the first time unprecedented that QRFP increase the activation of caspase-3 in both of prostate androgen insensitive in time dependent manner. Therefore, the unusual observed from the results is unstable of increase activation in all time points started treatment from 1 hr pending 12 hrs which has been shown in PC3 cells and DU145 cells. Activation and or inactivation of some protein kinases such as Ras/Raf/MEK and the stress-activated kinase JNK and an essential for the cysteine protease caspase pathway have also been stated (Drew et al., 2002). The MAPK p38 activity could regulate downstream caspases cascade and is significant for the activation of Caspase-3 in prostate cancer cell lines. It has been shown that Gyromagnetic fields (GMFs) inhibit cell growth and induce cell death mediated by activation of the p38 MAPK pathway mediate Caspases (Lei et al., 2015). PC3 cells treated with

a growth factor TGFβ1, has shown suppress cell proliferation and induction cell apoptosis through activation MAPK p38/ JNK/SAPK pathway which turn to enhance activation Caspease-3 (Al-Azayzih et al., 2012). Moreover, aurothiomalate (ATM) causes activation of ERK/ p38 and JNK MAPK phosphorylation mediate activation Caspase-3 and induced apoptosis in PC3U cells (Trani et al., 2009). It is complex to explain the variable of Caspease-3 activation in both cell lines investigated might be as explained early QRFP has dual function in the same time in prostate cancer cells. Otherwise these finding indicate that potential additional kinase is involved in this pathway and that this molecule might be particularly activated by the QRFP. This result demonstrated that QRFP has potential role effect to induce apoptosis in prostate cancer cells.

In further to confirming apoptosis induction, the present study clearly demonstrated that QRFP increase activation of AMPK expression in PC3 cell and DU145 cells. Our data shows higher expression level of AMPK activity in PC3 cell at all-time point treatment peaking at 6 hours. However, the effects of QRFP on activation AMPK was less in DU145 cells just at two-time point significance activation 2 hr and 4 hr later on the activation decreased to level basal cells. Recent studies have demonstrated that Increased expression AMPK levels in androgen dependent cells LNCaP and androgen independent cells DU145 while PC3 cell higher expression (Choudhury et al., 2014) & (Grossi et al., 2015). Pharmacologically activation of AMPK has been shown to suppression cell proliferation and

viability of many prostate cancer cell lines (Jurmeister et al., 2014). Besides the roles in controlling metabolism and energy balance, AMPK is found for its association to a wide range of cellular survival pathways, such as the phosphatidylinositol 3-kinase (PI3-K) pathway. AMPK activation can inhibit the activation of the protein target of mTOR, a key downstream component of the PI3-K pathway. Thus, AMPK's site in both the metabolic pathway in addition to its position upstream of the mTOR signaling pathway make it a possible therapeutic target for the treatment of PCa (Shackelford and Shaw, 2009) & (Kim and He, 2013). Many mechanisms can be involved in activation of AMPK results pleiotropic effects in the suppression of prostate cancer and tumor development, such inhibition of PI3k/Akt/mTOR pathway, cell cycle progression and up-regulation of p53 and p21 axis leading to induction apoptosis (Zadra et al., 2014). Furthermore, the AMPK pathway associated with a number of tumor suppressor genes, including upstream activator LKB1 and direct activates p53 and TCS2 (Kim et al., 2014). However, Induction of apoptosis is a challenge, since it could enhance the effects of classical anticancer agents. The data are in line with literature observations highlighting the role of AMPK activated by QRFP induced suppression proliferation cells and induced apoptosis in prostate cancer cell lines depend on dose and time manner.

To prove whether the QRFP mediated effects were through GPR103 receptor. The receptor was knockdown with GPR103 siRNA. The findings from study showed that QRFP induced AKT activation was abolished when

GPR103 receptor was knockdown in PC3 cells. These findings suggest that QRFP effects were mediated through GPR103 receptor in PC3 cells. Mechanistic analysis of QRFP mediated migration studies revealed that multiple signalling pathways are involved in QRFP induced migration of prostate cells. Pharmacological reagents that inhibit MAPK pathways were used to determine whether MAPK signaling is involved in the cells immigration. Previous studies showed that PCa cell migration and invasion are mediated through the p38 MAPK pathway; this leads to the activation of heat shock protein 27 that involved in regulates of MMP-2 activation and cell invasion. Other studies suggested that the overexpression of p38 MAPK can induce the high sensitivity to apoptosis induction leading to cell death, p38 is not an essential for cellular apoptosis. Furthermore, it has been shown that inhibition of p38 MAP kinase pathway caused inhibition of the DNA synthesis, survival, and proliferation of PC3 cells. p38 MAPK has been found plays a dual role as a regulator of cell death, and it can mediated cell proliferate or cell death depending on a cell type specific manner also in type of stimulus (Koul et al., 2013). Stimulates AMPK activation can influence many effectors proteins implicated in many regulatory processes which control or contribute to the pathogenesis of PCa (Choudhury et al., 2014). The role of AMPK PCa pathogenesis remains in debate. Studies have presented that AMPK is expressed in human PCa cell and inhibition of its activity, using the inhibitor compound C, has suppression effects on cell growth (Frigo et al., 2011). Conversely,

some medicines which are used in the cure of diabetes and other metabolic diseases, such as metformin, have been shown to decrease cancer risk and to supress PCa cell proliferation and cell growth in animal models mediate AMPK activation (Zadra et al., 2013). At present, though, it is unclear how AMPK regulates in PCa cell migration and invasion. Given together in this study the effect of QRFP mediate inhibit p38 and AMPK pathway observed increased significantly cell migration in both cell lines PC3 and Du145. In contrary, the results from this study exhibited that inhibition of PI3K-pathway reduced significantly the effect of QRFP migration in PC3 and DU145 cells. While, inhibited the MAPK/ ERK with U0126 showed no change effect of QRFP migration on this pathway in both cell lines PC3 and Du145. The MAPK ERK and PI3K-Akt signal pathway have been revealed to control the progression pathway leading to metastasis of PCa in addition to the development and progress of many other tumors. This means, over-expression of AKT/PI3K is highly related with the development metastasis of PCa. Many other studies have also presented that targeting PI3K-Akt signaling pathway with antisense, siRNA or small molecule inhibitors outcomes in the down-regulation of migration and invasion and tumorigenesis in cancer cells. It have been presented that the activation of MMP-2 and the invasiveness to be not mediated via ERK, signaling pathway in human melanoma cells (Denkert et al., 2002). In addition, Inhibition of the MAPK ERk and PI3K/Akt signaling pathway can inhibit cancer cell proliferation, migration and invasion, and metastasis

(Hennessy et al., 2005) & (Chen et al., 2011). Taken together, our data suggest that QRFP promotes migration and invasion of prostate cells which consistence with result obtained from functional assay and also with litters reviews.

CHAPTER 7

THE REGULATION OF QRFP & GPR103 EXPRESSION BY ADIPOKINES IN PROSTATE CANCER

7 INTRODUCTION

A variety of hormones, adipokines and bioactive peptides regulate the growth prostate gland. Obesity results in an abnormal release of these hormones , which can form part of the motive that stimulates prostate carcinogenesis (Mistry et al., 2007). World widely, the WHO estimates presented that by 2015 more than 2.3 billion people are overweight and around 700 million people are obese (Suzuki et al., 2010). The relationship between prostate cancer and obesity is complicated. Many reviews suggested that the obesity is linked with increased risk of prostate cancer. However, various molecular mechanisms have been suggested to clarify these explanations (Lughezzani, 2012). However, the details of these mechanisms remain uncertain. Thus it is important to continue to investigate the pathways that are associated with obesity and prostate cancer (Alshaker et al., 2015). As elucidated in the introduction chapter white adipose tissue is core site of energy storage in human, while, brown adipose tissue mostly utilizes energy and produces heat. Adipocytes produces and secretes bioactive molecules which are termed as adipokines. The adipose tissue energy metabolism and the immune system is well determined by two main adipokines which has opposite in function namely leptin and adiponectin. In latest years, researchers have extensively focused particularly on both adipokines for their ability to regulate cancer cell phenotype directly. Many properties of adipokines

might have impact on progression of prostate carcinogenesis (Al-Hamodi et al., 2014) & (Burton et al., 2010). Both circulating leptin levels and adipose tissue leptin mRNA increases in obesity. Clinical studies showed that leptin levels in blood are correlated with prostate cancer progression (Ribeiro et al., 2006) & (Mitsuyama et al., 2015). Leptin has been shown to stimulate intracellular signalling pathways correlated with cell proliferation, migration, invasion and inhibition of apoptosis mainly in androgen independent PCa cell lines (PC3 and DU145). Conversely, leptin has not been found as a growth promoter of androgen dependent LNCaP cells (Hoda et al., 2012b). Leptin receptor mRNAs for both long and short forms were determined in the androgen-independent PC3 and DU145 human prostate cancer cell lines. In vitro studies showed that exogenous leptin administration induced the growth of these two cell lines. However no mitogenic response to the ligand was observed in the androgen dependent LNCaP prostate cancer cell line (Baillargeon and Rose, 2006). Adiponectin receptors mRNA has been found to be expressed in both beneigh and malignant human prostate tissues and also in PCa cell lines androgen-independent PC3 and androgen-dependent LNCaP. Adiponectin levels are inversely correlated with the risk of prostate cancer. Lower adiponectin concentrations are independently correlated with high-aggressive prostate cancer (Mistry et al., 2006). Recently, a new member of adipokine family was discovered named chemerin, a chemoattractant protein. In adipose tissue expression of chemerin and its receptor, (CMKLR1, or ChemR23)

are significantly higher in obese adipose tissue. Chemerin and CMKLR1 are involved in regulation of adipogenesis (Sell et al., 2009). Many of neuropeptides have distinguished expression patterns in the hypothalamus and main effect on the central regulation of energy balance. QRFP is a potent agonist of GPR103, involved in enhanced food intake, increased body weight and fat mass, adipogenesis and decreases thermogenesis. QRFP mRNA level has been found expressed in peripheral tissues specific in white adipose tissue in mouse model. However, the expression of QRFP in adipose tissue is reduced in diet-induced obesity (Jossart et al., 2014). While, the role of GPR103 in regulating peripheral metabolic pathways is unclear (Mulumba et al., 2010). In rats, QRFP management changes macronutrient selection and particularly increases the intake of a calorically heavy high fat diet (Primeaux et al., 2013). Several studies found that effects of QRFP on food intake are mediated by the hypothalamic neuropeptides and adiposity signal, including leptin (Primeaux et al., 2013). Bernard Beck et al, 2009, resulted the role of leptin in the ventromedial hypothalamus by presenting an influence on the orexigenic drive, as proposed by the reverse relationship between plasma leptin and QRFP in this part. Plasma leptin concentration increased in animals chronically injected with QRFP 43. Moreover, expression of QRFP is up regulated with leptin deficiency in obese animal models (Beck and Richy, 2009). This leads that QRFP could has a function downstream of leptin in the regulation of feeding behavior.

The study suggested that the QRFP could be good target for the pharmacological treatment of diet-produced obesity (Beck and Richy, 2009).

7.1 HYPOTHESIS

In this chapter, I have investigated whether QRFP & GPR103 expression is regulated by adipokines leptin, adiponectin and chemerin.

7.2 AIM

- To demonstrate the role of adipokines in regulation of mRNA QRFP and GPR103 expression in prostate cancer cell lines.

7.3 RESULTS

7.3.1 REGULATION OF QRFP & GPR103 EXPRESSION BY ADIPOKINES AND HORMONES IN PROSTATE CANCER

Cytokines produced by adipocytes are associated with prostate cancer progression. Real time PCR was used to investigate the effect of the adipokines on QRFP and GPR103 expression in prostate cancer cell lines. PC3 cells were treated by Leptin (100 nM), adiponectin (10 nM), and Chemerin (1 nM) for 24 hours then assessed by real time PCR assay. The RNA was converted into cDNA and primers for the following genes (QRFP and GPR103) were used as previously described. The using primers for GAPDH as an internal control are showed below. The data obtained showed that both QRFP and GPR103 mRNA were significantly increased with Leptin stimulation**P<0.01 compared to basal. However other drugs used in the current study did not show any statistical significant changes in the expression of QRFP and GPR103 mRNA expression (Figure 7.1/7.2).

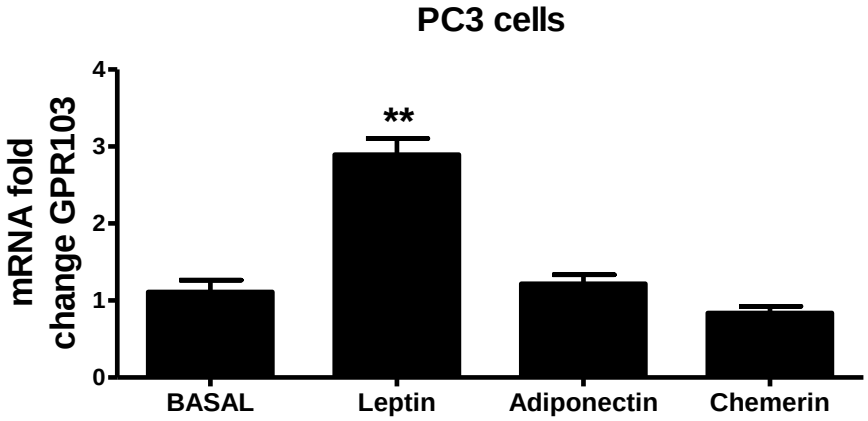

Figure 7.1 Effects of adipokines on GPR103 expression in PC3 prostate cell lines. PC3 cells were incubated for 24 hours with different drugs. GPR103 levels following stimulation with adipokines are shown. Data shown are mean ± S.D. Leptin significantly increases GPR103 production at 24 hours. The experiment was performed in triplicate wells **P<0.01 as compared with basal.

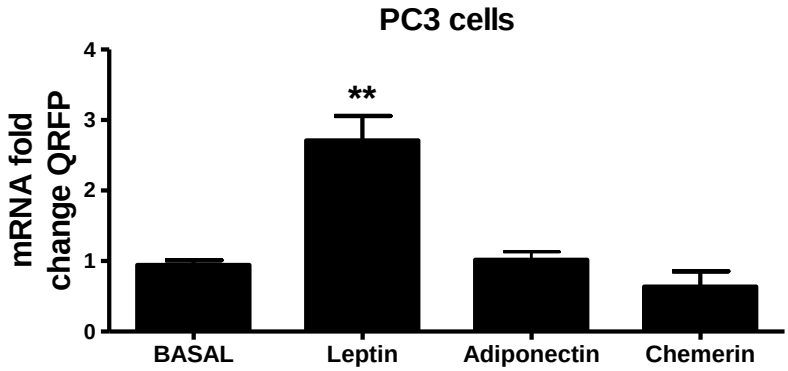

Figure 7.2 Effects of adipokines on mRNA QRFP expression in prostate cell lines. PC3 cells were incubated for 24 hours with different drugs. QRFP levels following stimulation with adipokines are shown. Data shown are mean ± S.D. Leptin significantly increases mRNA QRFP production at 24 hours. The experiment was performed in triplicate wells **P<0.01 as compared with basal.

7.3.2 THE EFFECT OF LEPTIN ON REGULATION OF QRFP & GPR103 mRNA EXPRESSION IN PROSTATE CANCER

In order to examine what is the optimal dose of leptin that induces significantly increase in QRFP and GPR103 expression. PC3 cells were stimulated with concentration of leptin ranging from 1 to 100 nM. Total RNA was extracted from PC3 cells after treatment with leptin 1, 10, 50 and 100 nM for 24 hours as previously described. RT-PCR demonstrate mRNA QRFP and GPR103 expression a significant up-regulation from 50 nM Leptin *p < 0.05 and peaks at a treatment dose-dependent effect of 100 nM **P<0.01 (Figure 7.3/7.4). Similarly, to examine what is the optimal time required for leptin to significantly increase in QRFP and GPR103 expression. PC3 cells were treated with 100 nM of leptin time dependently from 4-24 hours. RNA was extracted from PC3 cells after treatment with 100 nM leptin for 4, 8, 12 and 24 hours as previously described. mRNA QRFP and GPR103 expression demonstrate a significant increase of from 12 and 24 hours *p < 0.05 and**P<0.01 respectively (Figure 7.5/7.6). To assess the relative changes of Leptin time dependency effect on mRNA QRFP and GPR103 in DU145 cells. RNA was extracted from DU145 cells after treatment with leptin 100 nM for 4, 8, 12 and 24 hours as previously described. QRFP and GPR103 gene expression showed a significant increase from 4 and 8 hours *p < 0.05 and**P<0.01 respectively. The levels then begin to return to basal at 12 hours and significant decreased at 24 hours (Figure 7.7/7.8).

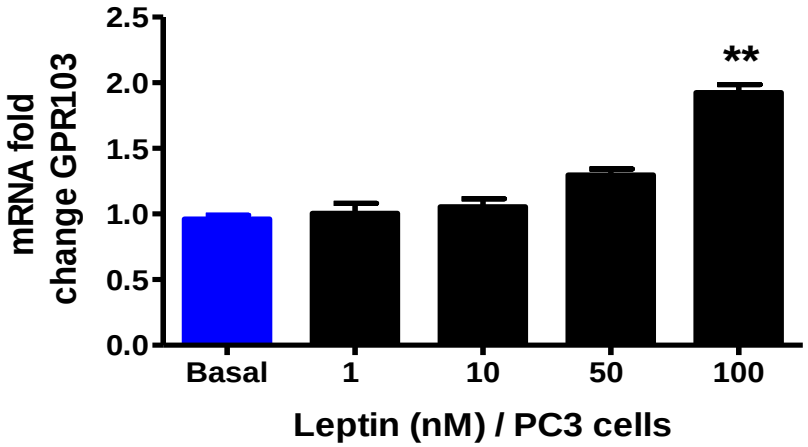

Figure 7.3 Effects of Leptin (100 nM) stimulation for 24 hours on GPR103 genes expression in PC3 cells was evaluated by using RT-PCR. mRNA expression levels were normalized against GAPDH mRNA expression. Data are expressed as mean ± S.D, from three independent experiments *p < 0.05 and **P<0.01.

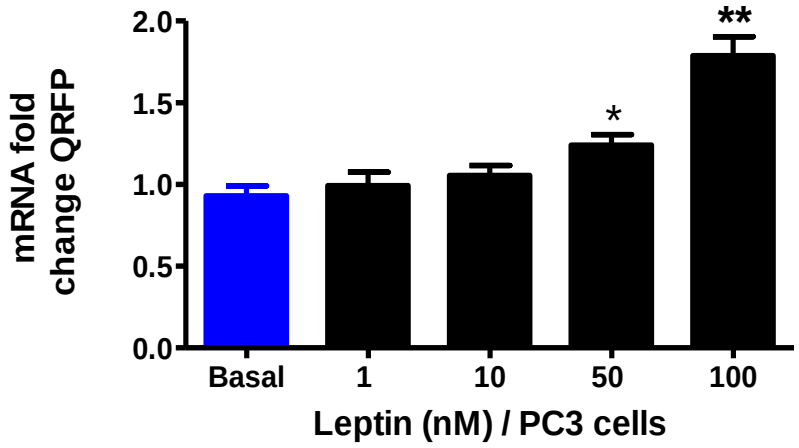

Figure 7.4 Effects of Leptin (100 nM) stimulation for 24 hours on QRFP genes expression in PC3 cells was evaluated by using RT-PCR. mRNA expression levels were normalized against GAPDH mRNA expression. Data are expressed as mean ± S.D., from three independent experiments *p < 0.05 and **P<0.01.

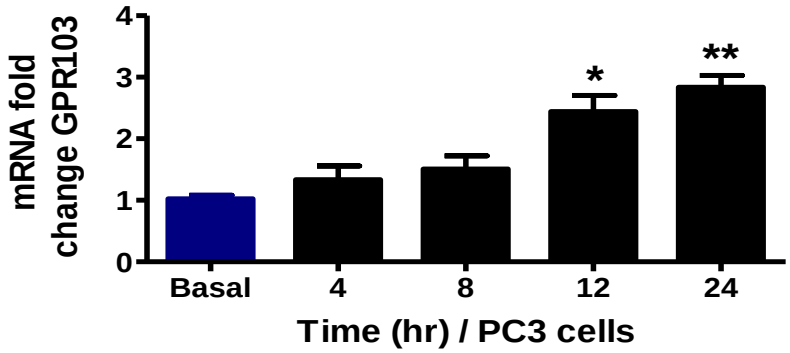

Figure 7.5 Effects of Leptin on GPR103 expression in prostate cell lines. PC3 cells were incubated for time dependence 4,8,12 and 24 hours and evaluated by using RT-PCR. Leptin significantly increases GPR103 gene expression at 24 hours. Data shown are mean ± S.D, the experiment was performed in triplicate wells *P<0.05 and **P<0.01, as compared with basal.

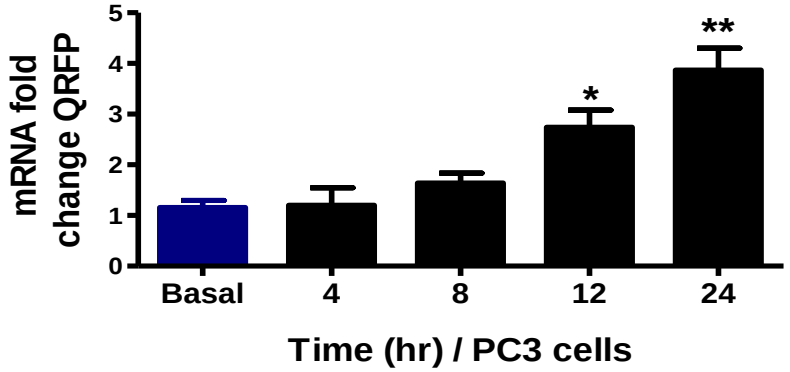

Figure 7.6 Effects of Leptin on QRFP expression in prostate cell lines. PC3 cells were incubated for time dependence 4,8,12 and 24 hours and evaluated using RT-PCR. Leptin significantly increases QRFP gene expression at 12 hr and 24 hr. Data shown are mean ± S.D, the experiment was performed in triplicate wells *P<0.05 and **P<0.01, as compared with basal.

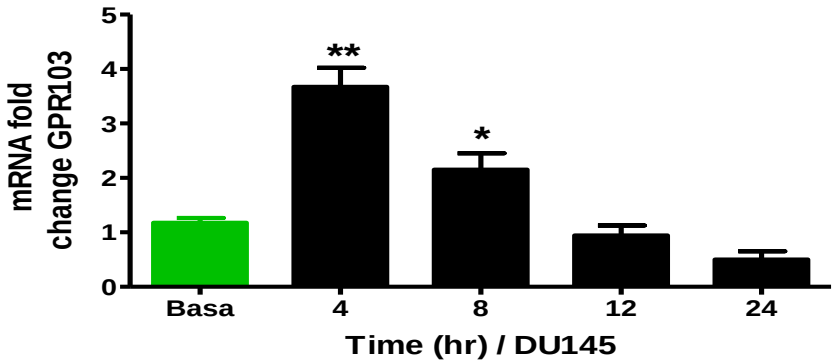

Figure 7.7. Effects of Leptin on GPR103 expression in prostate cell lines. DU145 cells were incubated for time dependence 4,8,12 and 24 hours and evaluated by using RT-PCR. Leptin significantly increases GPR103 gene expression at 4 and 8 hours. Data shown are mean ± S.D, the experiment was performed in triplicate wells *P<0.05 and **P<0.01, as compared with basal.

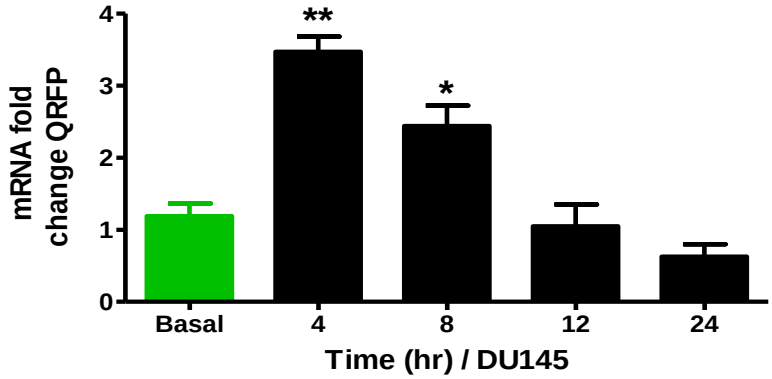

Figure 7.8. Effects of Leptin on QRFP expression in prostate cell lines. DU145 cells were incubated for time dependence 4,8,12 and 24 hours and evaluated by using RT-PCR. Leptin significantly increases QRFP gene expression at 12 and 24 hours. Data shown are mean ± S.D, the experiment was performed in triplicate wells *P<0.05 and **P<0.01, as compared with basal.

7.3.3 LEPTIN REGULATES QRFP & GPR103 GENE EXPRESSION IN PROSTATE CANCER CELLS VIA MAPK AND PI3 KINASE PATHWAY

It is well documented in the literature that leptin induces its functional effects via activation of MAPK and PI3 kinase signalling pathways. In this current study we employed real time PCR to demonstrate the relative change in QRFP and GPR103 gene expression in the presence and absence of MAPK and PI3 kinase inhibitors.

PC3 cells were treated by a combination with or without Leptin (100 nM), MAPK inhibitor (U0126; 10 mM) or PI3K inhibitor (LY294002; 10 mM) for 24 hours. Following stimulation RNA was extracted and converted into cDNA. The primers for the following genes (QRFP and GPR103) were used as previously described. The primers for GAPDH as an internal control are showed below. The data obtained showed that both QRFP and GPR103 mRNA were significantly increased with Leptin stimulation**P<0.0.

However, the effects were inhibited in the presence of PI3K and U0126 inhibitor which showed significant reduction in QRFP and GPR 103 mRNA expression*P<0.05 compared to basal cells stimulated with leptin. *P<0.05 and **P<0.01 on PC3 cells compared to Leptin stimulate levels (Figure 7.9/7.10).

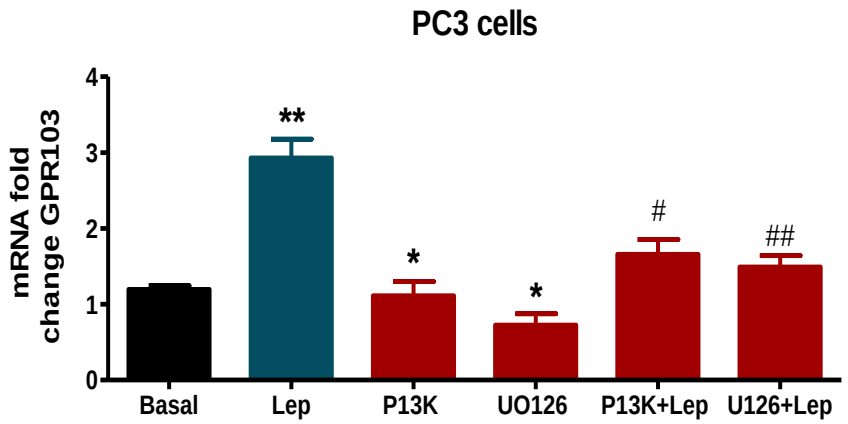

Figure 7.9 Effects of Leptin and MAPK inhibitors on mRNA GPR103 expression in prostate cell lines. PC3 cells were incubated for 24 hours with different Leptin, P13K and U0126 and evaluated using real-time PCR. Data shown are mean ± S.D. The experiment was performed in triplicate wells. *P<0.05 and **P<0.01, as compared with basal while #P<0.05 and ##P<0.01 compared to Leptin.

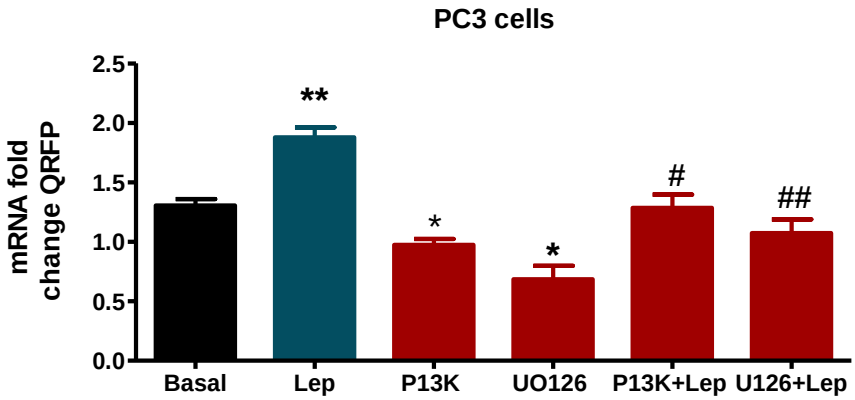

Figure 7.10 Effects of Leptin and inhibitors on mRNA QRFP expression in prostate cell lines. PC3 cells were incubated for 24 hours with different Leptin, P13K and U0126 and evaluated using real-time PCR. Data shown are mean ± S.D. The experiment was performed in triplicate wells. *P<0.05 and **P<0.01 as compared with basal. while #P<0.05 and ##P<0.01 compared to Leptin.

7.4 DISCUSSION

It is essential to understand the associations between obesity and prostate cancer and effects of adipokines on QRFP and GPR103 expression in prostate cancer. In the present study, different adipokines have been used to investigate their effects on mRNA QRFP and GPR103 in prostate cancer cell lines in vitro. The results obtained from these experiments demonstrated for the first time mRNA levels of QRFP and GPR103 are significantly increased with adipokine Leptin. Conversely adiponectin and chemerin stimulation showed no significant effect on QRFP and GPR103 gene expression in PC3 cells. Particular consideration is expecting for the potential role of the leptin with QRFP and GPR103 gene expression, because of its association with the development of prostate cancer. Moreover, my study demonstrated the optimal dose of leptin required to induce significant increase in QRFP and GPR103 mRNA expression in PC3 cells. Time dependent studies demonstrated that effects of leptin on QRFP and GPR103 gene expression in PC3 cells is time dependent. To confirm my results other androgen insensitive cells (DU145) were employed. Inversely, DU145 cells showed different leptin time dependent response compared to PC3 cells. In conclusion, leptin appears to have a time and dose dependent effects on QRFP and GPR103 mRNA expression in different prostate cancer cell lines. Evidence from literature supports that leptin as a growth factor induces prostate cancer cell

migration and invasion (Frankenberry et al., 2004). My previous findings in this thesis showed that QRFP induces prostate cancer cell migration and invasion in prostate cancer cell lines. Given leptin regulates QRFP expression, it is reasonable to preclude that leptin effects might be mediated via involvement of QRFP and QRFP may be downstream target for leptin. This finding could support the hypostasis that increase effects of leptin on QRFP and GPR103 may have association on progression of prostate cancer. Leptin receptors are expressed in androgen independent PC3 and DU145 cells. Leptin binds receptor the long Ob-Rb and activities various pathway including JAK/STAT that activate MAPK and PI3K/AKT pathway leads to induction cellular growth, migration and invasion in prostate cancer. Whereas the short form leptin receptor Ob-Rb mainly activate only MAPK ERK1/2 pathway but not STAT (Miyazaki et al., 2008) & (Dutta et al., 2012). Inhibition of these pathways with specific chemical inhibitors not only prevents the phosphorylation of respective key signal-transduction elements, but also directly blocks the endometrial cancer cell proliferation. Also, prevention of leptin-induced activation of the JAK/STAT pathway by specific chemical inhibition in turn significantly reduces the activation of ERK and AKT pathways (Sharma et al., 2006).

In an attempt to examine the signalling pathways involved in leptin-mediated induction of QRFP and GPR103 expression in prostate cancer cells. I examined the influence of PI3 Kinase, ERK, AMPK and p38 MAPK

kinase pathway inhibitors on leptin induced QRFP and GPR103. Findings from this study demonstrated that PI3K and U126 MAPK inhibitor decreases leptin induced QRFP and GPR103 mRNA expression in PC3 cells, suggesting that leptin induced QRFP and GPR103 expression via involvement of PI3 kinase and ERK MAPK kinase pathway.

CHAPTER 8

GENERAL DISCUSION

GENERAL DISCUSSION

The biology and physiological role of peptide QRFP and receptor systems are described. I demonstrate their presence in tumors, with a focus on prostate cancers. This study has explored a potential novel role for the neuropeptide QRFP in prostate carcinogenesis. Employing most common PCa cell lines in addition to benign and malignant human prostate tissue I managed to demonstrate the expression of QRFP &GPR103 its receptor. It should be noted that, these studies have limitations as to the interactions do not necessarily translate to the circulating in-vivo environment conditions such as absence of blood flow and many factors involve may affect the final outcomes most of these failures are not necessarily published. For this study prostate cancer cell lines PC3, Du145 and LNCaP cell lines were used as surrogates for CaP rather than in-vitro cultures of human prostatic cells. These cell lines are well characterised cellular models most widely used in studies involving prostate cancer research. Three of the most commonly used cell lines of PCa were derived from metastatic lesions: PC3 from bone, DU145 from brain and LNCaP from a lymph node. These cell lines were representing different spectrums of prostate cancer with aggressive form, moderate metastatic potential and low metastatic potential, also hormone refractory was demonstrating both androgen insensitive and androgen sensitive disease respectively. One of the significant biological variances between the three cell lines is the

androgen receptor (AR) express. LNCaP cells with expression of luminal differentiation markers androgen receptor (AR) and prostate-specific antigen (PSA) while the PC3 and DU145 cells presents negative AR or PSA mRNA/protein. Since these cell lines are immortalised cell cultures established from metastases, they therefore useful in studying the biochemical changes in advanced disease cells and in evaluating their response to chemotherapeutic agents. However, they have significantly different characteristics.

In the present study, I reported the presence and function of QRFP and its receptor GPR130 in human prostate tissue and in human prostate cell lines. In mammals, the mRNAs encoding the QRFP and its receptor GPR103, are mainly expressed in the brain, especially in the hypothalamus, and in different peripheral organs such as adipose tissue, testes. Consistent with this report, PCR and immunoassay data reveal that GPR103 and its ligand QFRP is highly expressed in human prostate compared to three prostate cancer cell lines: LNCaP, DU145 and PC3. Moreover, both gene and protein expression studies data showed that QRFP and its receptor are highly expressed in malignant prostate tissue compared to benign tissue both at mRNA and protein level. Interestingly, QFRP is highly expressed in cancerous cells and because this neuropeptide is an α-amidated and that the expression of peptidyl-glycine alpha-amidating monooxygenase (PAM), the enzyme responsible for the

amidation of neuropeptides is overexpressed in high Gleason's score has been reported by other studies (Alonzeau et al., 2013).

The same study also reported that QFRP and GPR103 are present in the same carcinomatous formations, indicating that this peptide may act on cancer cells via an autocrine/paracrine mechanism. Furthermore, QFRP and GPR103 are particularly existing in cancerous structures labelled with EM66, a fragment of SgII that has been earlier recognised as an indicator of neuroendocrine differentiation (Anouar et al., 1998). Supported by modern investigations identifying important roles of peptides in the control of cancer cell proliferation and apoptosis, migration and invasiveness. The present study was to investigate the effect of QFRP on the prostate cancer cells. It was hypothesized that the increase in prostate cancer growth by this peptide might originate not only from mitogenic potential but also from its ability to inhibit cancerous cell proliferate. The impact of QRFP on prostate cancer cells metastasis and inhibit growth was studied by measuring proliferation rate, migration and invasion and apoptosis potential in prostate cancer cells. My findings showed that in prostate cancer cell lines PC3, DU145 and LNCaP, QRFP suppress proliferation and induce apoptosis on doses and time dependency. Also enhanced migration and invasion in the AI cells PC3 and DU145 were found. Most of the PCa deaths are result of the development of an androgen-resistant phenotype of PCa either via some co-activators or by mitogen activated

cell signaling activities that lead to the overexpression of anti-apoptotic genes and survival of the cancer cells. This leads to conduct the further experiments by using androgen insensitive cell lines, furthermore the other reason for halt carrying out the LNCaP cells due to manner of growing these cells over each other on plate.

This study provides the first description of the signaling profile of QRFP actions in the PCa cell lines. Furthermore, the possible involvement of MAPK and PI3K signaling pathways in QFRP-induced effects in these cell lines was also investigated. In an attempt to explore how QRFP and its receptor regulate signal transduction in prostate cells, I studied the effect of QRFP on MAPK and AKT signaling pathways. QRFP upon binding to its receptor in PC3 and DU145 leads to the activation of several signaling molecules including the mitogen-activated protein kinases (ERK/MAPK), p38 MAPK, JNK MAPK and phosphoinositide 3-kinase (PI3K)/Akt pathways. Those pathways are critically involved in either prostate cancer cell survival, migration, invasion and/or cell death pathways, depending on the cell type and cell stimulus. Therefore, the potential involvement of MAPK and AKT signal-transduction pathways in mediating the effects of QRFP in prostate cell lines was studied by measuring the phosphorylation of their downstream components, AKT and ERK1/2, respectively. Inhibition of this pathway in the presence of QRFP neuropeptide on PC3 and DU145 was able to abolish the mitogenic and anti-apoptotic effects. Consistent

with the cell migration and invasion results, incubation of cancer cell lines (PC3 and Du145) with QRFP enhanced MMP2 activation. Evidence from previous studies shows that matrix metalloproteinase-2 (MMP2) is important in the pathogenesis of prostate cancer. Over expression of MMP2 has been associated with neoplasm, prostate, breast, cervical, cutaneous, ovarian and gastric cancer (Jezierska and Motyl, 2009) & (Gong et al., 2014). Further investigation is achieved to confirm QRFP induced migration and invasion by activation of AGR-2 in PCa cells. AGR-2 has been shown a biomarker elevated in metastatic PCa related with the neuroendocrine phenotype (Kani et al., 2013).

This study also approved QRFP has potential apoptosis effect by activation down regulates caspase 3 and AMPK pathways in PC3 and DU145 cells. The direct involvement of GPR103 was evident, since silencing the receptor using siRNA against GPR103 resulted in significant reduction in QRFP induced AKT activation in PC3 cells. Not surprisingly, the QRFP effects were not completely abolished by knockdown of GPR103 receptor. This was possibly due to either not achieving complete knockdown of GPR103 receptor in PC3 cells by siRNA or possibly due to activation of an orphan GPCR. For example, it has been suggested that QRFP may have alternative receptor through which may elicit its functional roles in rodents (GPR103A and GPR103B), however to-date only one subtype of GPR103 receptor has been identified in human.

I attempted to determine the specific QRFP effect on PCa cells by inhibiting MAPK and PI3k pathway. Cryptotanshinone has been shown to prompted caspase-independent cell apoptosis by activation of JNK and p38 and inhibition of ERK1/2 pathways in Rh30 and DU145 cells. This finding is strongly supported by other studies Inhibition of p38 or silencing p38 abolish cryptotanshinone-induced apoptosis. Additionally, expression of substantially active MKK1 enhanced ability of cryptotanshinone inhibition of ERK1/2 expression and induction apoptosis (Chen et al., 2013a). It has shown that activated Akt in DU145 cells and up-regulated glycolysis, AICAR-mediated AMPK expression inhibited proliferation without evidence of increased cell death. Supporting a tumor-suppressing role, silencing AMPK activation or a dominant negative AMPK altered enhanced proliferation, migration PCa cell lines (Choudhury et al., 2014).

These reviews are in line with my outcomes from this study that inhibiting p38 and AMPK increased the QRFP migration effect, however inhibiting PI3k pathway suppress migration cells and also showed no migration respond by inhibit ERK pathway in PC3 and DU145 cells. Based on the data obtained from my study QRFP is involved in activation of different MAPK, AKT and downregulate Caspase-3, AMPK, MMP-9 and AGR-2 pathways, it appears that QRFP activated ERK pathway for short time is not respond to proliferation might be maintenance survival cells. Activation of upstream MAPK JNK and downstream Caspease-3 pathway could be

linked to the suppression proliferation and induction amount of cell death dependent on time and dose dependence. While activation AMPK pathway might be possibly to reduce the migration cells. The potent QRFP effect on enhance cell migration and invasion strongly related mediated activation PI3k/AKT, MMP-9 and AGR-2 pathway.

According to the letters reviews the relation between obesity and PCa remain in debate. Overall, controversy exists over whether obesity enhance the risk of PCa, however, studies strongly indicate that obese individuals is an important risk factor for PCa mortality (Freedland and Aronson, 2004). In this study I highlighted firstly on the information that directly link obesity to prostate cancer. I also briefly explain some of the risk factors linked to obesity and their association with PCa. My study supported that by demonstrated for the first time mRNA levels of QRFP and GPR103 are significantly increased in response to adipokine Leptin in PC3 cells. This finding might support the hypostasis that increase effects of leptin which are higher in obesity on QRFP and GPR103 this might be one of the causes for increased incidence of aggressive prostate cancers in obese individuals. Previous study from Somasundar et al, reported that leptin the obesity gene stimulates human prostate cancer cell lines in vitro (Somasundar et al., 2003). It has been reported that leptin promotes cancer cells proliferation and metastasis potential through activation of MAPK and AKT dependent signal transduction pathways these pathways

are important for cancer cell proliferation oncogenic transformation and tumour progression, and these pathways involve protein kinases at many levels (Hoda et al., 2012a). Furthermore, MAPK inhibitor PI3K and U126 decreases leptin induced QRFP and GPR103 mRNA expression in PC3 cells, suggesting that leptin induced QRFP and GPR103 expression involve PI3 kinase and ERK MAPK kinase pathway. Given that metabolically active neuropeptides are differentially expressed in tissues and in the circulation of normal and cancer patients. For example, circulatory levels of orexin were lower in cancerous patients compared to controls. I sought to investigate the circulatory levels of QRFP using commercially available ELISA kit. The present study suggests that circulating level of QRFP are decreased among cancer patients with benign and advanced disease. This investigation recommends additional studies are required of QRFP in patients with cancer associated anorexia with full detailed assessment including recent oral intake and age and wild range of subjects covered in the study.

In conclusion, induction of apoptosis is a most important challenge, since it could progress the effects of model anticancer agents. The data are in line with literature observations highlighting the role of AMPK and AKT pathway activated by QRFP induced suppression proliferation cells and induced migration and invasion and apoptosis in prostate cancer cell lines depend on dose and time manner.

In the future, the main challenges for successful clinical therapy application will be better understanding, within specific peptide and receptor are over-expressed and at which stage of carcinogenesis and cancer development. Thus, a better knowledge of the QRFP signaling pathways deregulated in PCa might lead to better preventive and therapeutic strategies for this disease.

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