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**SOMATOSTATIN EFFECTS ON
THE PROTEOME OF HUMAN
PROSTATE CANCER CELLS**

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**Karolinska
Institutet**

Stockholm 2009

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Published by Karolinska Institutet. Printed by Tidtryck, Uppsala

Doctoral Thesis

Somatostatin effects on the proteome of human prostate cancer cells

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ISBN 978-91-7409-288-2

ABSTRACT

The transition of prostate cancer from androgen dependent to the therapy resistant androgen independent state, is a large and persistent problem in the clinical management of prostate cancer.

The aim of this thesis was to study somatostatin effects on the proteome of prostate cancer cells, to identify differences in protein expression in androgen dependent and androgen independent prostate cancer cells, and to explore possibilities to enhance the somatostatin receptor expression through pre-treatment with sms, 5-aza and TSA (DNA methylation/HDAC inhibitors). The results may give insight into the feasibility of using somatostatin as a part of the therapy of advanced prostate cancer. Somatostatin (sms) is important in the regulation and physiological control of many organs including the prostate. Sms can inhibit tumor growth and angiogenesis through binding to its specific receptors (SSTRs) transducing growth inhibitory, anti secretory and apoptotic signals. Because of these properties sms is of interest for the management of prostate cancer and also of other malignancies. Sms is an unstable peptide and therefore more stable sms derivatives have been developed that are suitable for clinical use.

Sms and its derivative smsdx (smsdx is a stable long acting glycosylated derivative of natural sms) were incubated with LNCaP and DU145 cells. Proteomic analysis using two-dimensional electrophoresis (2-DE) was performed to determine and compare the effect of the sms and smsdx treatment. Protein expression patterns were analysed with 2-DE and mass spectrometry (MS). Using cDNA obtained from these cell lines, the difference in expression level of SSTR mRNA transcripts before and after sms/smsdx, 5-aza and TSA treatments was analyzed by RT-PCR.

Marked quantitative differences were observed in the protein expression profiles in sms/smsdx treated LNCaP and DU145 cells compared to the control cells. One third of the detected proteins were differentially expressed (PRDXs, hnRNPs). Concordance in protein expression patterns were observed between smsdx and sms treated cells with strong agreement between the up/down regulation of proteins. Catalytic mitochondrial and mitochondrial-associated proteins were significantly affected (fold change ~2 or higher). The treatment triggered up-regulation of the catalytic mitochondrial proteins. Apoptotic related proteins were also affected.

An increased induction of mRNA expression of all five SSTR subtypes was observed in the LNCaP cells when incubated with sms/smsdx (dose dependent). The results indicate a positive feedback loop between sms and its receptors. Inhibition of DNA methylation and histone acetylation resulted in up regulation of SSTR5 mRNA expression.

The results show that smsdx affects important proteins of the proteome in prostate cancer cells both in androgen dependent and in androgen independent prostate cancer cells. Different treatment protocols in terms of derivatives, doses, dose frequency and treatment duration may trigger/enhance effects on proteins involved in the survival/death of the tumor cells. Epigenetic manipulation in combination with sms, might be a possibility to enhance treatment sensibility for somatostatin therapy. Further clinical research on sms is needed to reveal its full potential in the management of prostate cancer.

LIST OF PUBLICATIONS

- I. **Liu Z**, Márquez M, Nilsson S, Holmberg AR and Alaiya A. Proteomic analysis of a human prostate cancer cell line after incubation with a novel somatostatin14 derivative. *Cancer Genomics & Proteomics*. 2005; 2: 347-352.
- II. **Liu Z**, Bengtsson S, Krogh M, Márquez M, Nilsson S, James P, Alaiya A and Holmberg AR. Somatostatin effects on the proteome of the LNCaP cell-line. *Int J Oncol*. 2007; 30(5):1173-9.
- III. **Liu Z**, Márquez M, Nilsson S and Holmberg AR. Comparison of protein expression in two prostate cancer cell-lines, LNCaP and DU145, after treatment with somatostatin.
Manuscript
- IV. **Liu Z**, Márquez M, Nilsson S and Holmberg AR. Incubation with somatostatin, 5-aza decitabine and trichostatin up-regulates somatostatin receptor expression in prostate cancer cells.
Oncol Rep. 2008; 20(1): 151-4.

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LIST OF ABBREVIATIONS

2-DE	2-Dimensional Gel Electrophoresis
AD	Androgen Dependence
ADT	Androgen Deprivation Therapy
AIPC	Androgen Independent Prostate Cancer
AR	Androgen Receptor
CBB	Coomassie Brilliant Blue
DIGE	Differential Gel Electrophoresis
DTT	Dithiothreitol
GCPRs	G-Protein-Coupled Receptors
hnRNP	Heterogeneous Nuclear Ribonucleoproteins
HPLC	High Performance Liquid Chromatograph
HRPC	Hormone Refractory Prostate Cancer
ICAT	Isotope Coded Affinity Tags
IPG	Immobilized pH Gradient
LCM	Laser Capture Microdissection
MALDI	Matrix Assisted Laser Desorption/ionization
MMP	Mitochondrial Membrane Permeabilization
MS	Mass Spectrometry
NE	Neuroendocrine
NED	NE Differentiation
PSA	Prostatic Specific Antigen
PTMs	Post-translational Modifications
PTPC	Permeability Transition Pore Complex

SDS	Sodium Dodecyl Sulphate
SELDI	Surface-enhanced Laser Desorption/ionization
Sms	Somatostatin14
Smsdx	Somatostatin-dextran conjugate
SSTR	Somatostatin Receptor
TCTP	Translational Controlled Tumor Protein
TOF	Time of Flight
VADC	Voltage Dependent Anion channel

1. INTRODUCTION

1.1 PROSTATE CANCER PROGRESSION

Prostate cancer continues to be the most common malignancy diagnosed in men in western countries. In Europe 2004, there is about 190,000 new cases (1). As the population ages, these numbers are expected to increase. Incidence of prostate cancer is increasing steadily in almost all countries, yet we know little about its causes. However, in the past 15 years interest in and funding for prostate cancer research have increased and several promising risk modifiers have been identified - e.g., genetic predisposition, insulin growth factor (IGF) concentrations, and lycopene consumption. Initially, almost all metastatic prostate cancers require testosterone for growth, and the role of androgen deprivation as a first-line therapy for metastatic prostate cancer has been recognized for more than 60 years (2, 3).

Androgen-deprivation therapy (ADT) is the mainstay of therapy for metastatic disease and is principally palliative in nature. ADT removes androgen stimulation, initially inducing apoptotic involution of prostatic tissue. The disease eventually progresses to an androgen-independent (AI) state with poor prognosis and a survival of approximately 15–20 months (4). Despite research efforts and recent advances using chemotherapeutic agents, minimal progress has been made in the treatment of advanced prostate cancer in the last 50 years, and the life expectancy of patients with metastatic disease has changed very little. ADT, while extending length and quality of life for many patients, induces biological changes in prostate cancer cells that promote an AI state.

Hormone-refractory prostate cancer (HRPC) is a clinically heterogeneous disease that is uniformly defined by persistent cancer-cell growth and progression in spite of low testosterone concentrations. Although HRPC can proliferate in an environment depleted of androgens, androgen-receptor (AR) activity remains a vital component of survival and proliferation. Activation of downstream targets varies widely between the androgen-sensitive clinical state and the androgen-independent clinical state (5, 6).

Androgens are primary regulators of normal prostate as well as prostate cancer cell growth and proliferation. During androgen-dependent progression, prostate cancer cells depend on the AR as the primary mediator of growth and survival (7-9). When testosterone enters the cell, it is converted by the enzyme 5 α -reductase to its active metabolite, dihydrotestosterone (DHT), a more active hormone with a 5- to 10-fold higher affinity for the AR. DHT binds ARs in the cytoplasm, causing phosphorylation, dimerization, and subsequent translocation into the nucleus, thereby binding to the androgen-response elements (ARE) within the DNA, with consequent activation of genes involved in cell growth and survival (7). During AI progression, prostate cancer cells develop a variety of cellular pathways to survive and flourish in an androgen-depleted environment.

The mechanisms by which prostate cancer cells become hormone refractory are not fully understood and are probably diverse. Despite this diversity, an increasing body of evidence suggests that the AR has a central role in prostate cancer progression, representing a final common pathway for many of these activating mechanisms. Postulated and documented mechanisms include AR gene amplification, AR gene mutations, involvement of coregulators, ligand-independent activation of the AR, and the involvement of ^{tumor} stem cells (Figure 1) (10-12).

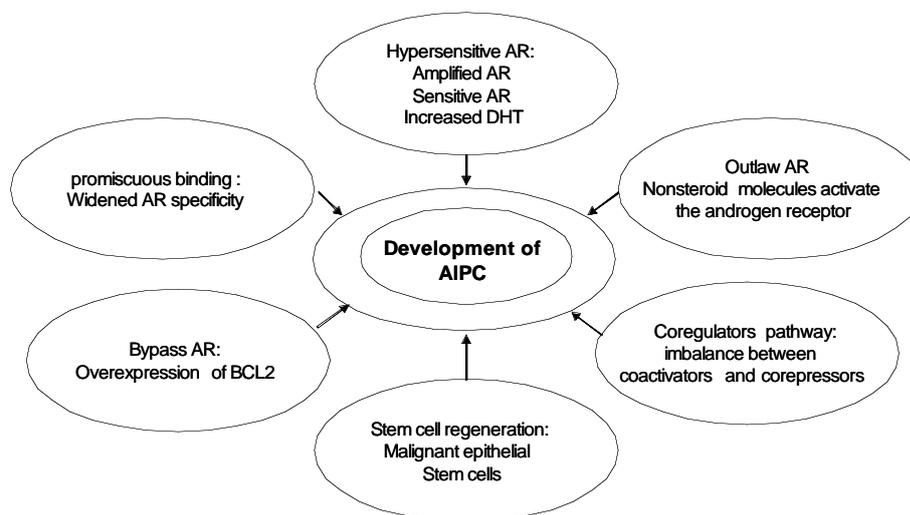


Figure1. Mechanisms of androgen independence of prostate cancer. AR: androgen receptor. AIPC: androgen independent prostate cancer. DHT: Dihydrotestosterone.

Physiological concentrations of testosterone in prostate samples of recurrent disease after androgen deprivation and expression of downstream targets of the androgen

receptor support the role of AR as a final common pathway in the phenotype of HRPC (13, 14).

The acquisition of an AI phenotype is the most serious event during prostate cancer treatment. To understand the biological characteristics of the disease, experimental model systems reflecting the different disease conditions are needed. At the present, there are only a few models that may resemble only a subgroup of human prostate cancer progression. Human cancer cell lines derived from the clinical tumor tissues often fail to exert the tumorigenicity even in immunodeficient mice. Androgen-dependent human prostate cancer LNCaP cells rarely form tumors without prostate stromal cells in SCID mice and even if the tumor is formed, the tumor growth is very slow (15). LNCaP has been established from supraclavicular lymph node metastases (16). These cells are well-differentiated and express ARs and exhibit androgen-sensitive cell growth. Although there is a physiologically equivalent level of androgenic activity in the regular culture condition, LNCaP cells gradually lose their androgen requirement for growth upon passage, and clearly exhibited more aggressive growth and lower androgen responsiveness, mimicking tumor progression in patients (17-20).

1.2 NEUROENDOCRINE DIFFERENTIATION IN PROSTATE CANCER

The cellular signaling pathways of the prostate play a central role in the induction, maintenance, and progression of prostate cancer. Neuroendocrine (NE) cells of the prostate were originally described by Pretl in 1944 (21). Neuroendocrine differentiation (NED) is a common feature of prostatic adenocarcinomas and is usually determined by immunoreactivity for neuroendocrine markers, e.g. CgA, NSE, or bioactive eutopic hormones such as somatostatin (22). NE cells with the dual properties of endocrine cells and neurons, i.e. acting in secretory and autocrine/paracrine fashions, are widely distributed in normal prostatic acini and ducts. In a recent study, human prostate NE cells were found to represent a cell lineage of their own, being of neurogenic origin and therefore distinct from the urogenital sinus-derived prostate secretory and basal cells (23). NE cells demonstrate attributes that suggest they are an integral part of these signaling cascades. The role of prostatic NE cells in this biologic process has recently become the focus of much attention. Known changes in the number, histology, and functions of NE cells during prostate cancer progression indicate that they may play a regulatory role. The fact that the majority of NE cells may not exhibit ARs is of special

interest in the androgen-deprived patient (24). In these patients, NE cells may allow continued prostate cancer growth through paracrine stimulation of neoplastic epithelial cells. Indeed, mitogenic and oncogenic activity has been demonstrated for many of the factors NE cells are known to produce. Data suggest that ADT may facilitate NED and thereby accelerate cellular mechanisms that contribute to the AI state.

NED is very common in prostate cancer specimens. For example, Bostwick (25) reported a prevalence of 92%. The wide ranges that have been reported (30–100%), is probably due to different sampling techniques and testing methods (26). It has been suggested that NED is a part of the oncogenic process. The aggressive malignant potential of NE cancer cells associated with hormonal independence is partly due to the ability of the NE tumor cells to escape apoptosis. Fixemer showed that apoptosis is an extremely rare event in the NE phenotype of prostate cancer cells. This finding further substantiates why NED is associated with tumor progression and hormonal escape in adenocarcinomas of the prostate (27). Studies have shown that regulation and function of normal and malignant NE cells is under the influence of several NE hormones. For instance, somatostatin, 5-HT, bombesin, calcitonin and PTHrP are known to manifest tumor growth-promoting activity *in vitro* and *in vivo* and appear to be a potent mitogen associated with prostate cancer. It was shown that sms is likely to counteract NE and other growth regulatory systems through somatostatin receptors present on secretory, NE, stromal and endothelial prostatic cells. The receptors were found to be up-regulated in carcinoma specimens while the potential mechanisms of somatostatin receptor antitumour effects include inhibition of angiogenesis, proliferation and promotion of apoptosis (28).

NE cells are the third cell type in the epithelial compartment of normal prostate glands in addition to basal and secretory epithelial cells (29), though prostatic NE cells were described decades ago, their functional roles in prostate, especially in prostate cancer progression, have received attention only recently. In normal prostate, NE cells are apparently involved in regulating epithelial cell growth and differentiation in an AI manner. In prostate cancer, the NE cell population that is increased in tumor foci, evidently correlates with tumor progression, poor prognosis, and the hormone-refractory stage. The role of NE cells in prostate cancer progression is suggested by the fact that those cells express and secrete a variety of neuropeptides which have mitogenic effects on adjacent cancer cells and thus can contribute to the AI

proliferation of prostate cancer cells (30-32). Further studies also show that NE differentiation is found in the metastatic lesion and significantly associated with the mortality of prostate cancer patients (33, 34). Although the origin and mechanism of NE cell enrichment during prostate cancer progression are not fully understood, accumulated evidences indicate that prostate cancer cells can undergo a transdifferentiation process to become NE-like cells and these cells acquire the NE phenotype and express NE markers.

NE cells are also present in prostate cancer, and many studies have shown that their number increases in high-grade and high-stage tumors, particularly in hormonally treated and hormone-refractory (androgen independent) prostate cancer. Unlike the non-neuroendocrine secretory-type prostate cancer cells, NE cells lack AR and are likely androgen independent. Therefore it is conceivable that hormonal therapy for advanced or metastatic prostate cancer, which consists of inhibiting androgen production or blocking androgen function, will not eliminate NE cancer cells. Instead, these cells may be enriched after the therapy and they may establish paracrine networks to stimulate AI proliferation of prostate cancer, leading to tumor recurrence.

Androgen ablation therapy is the main option for treating metastatic prostate cancer; however, androgen withdrawal also contributes to increased NE differentiation in prostate cancer (35). It has been shown that patients who had received a longer course of androgen ablation therapy would have higher levels of NED (36). A similar result (37) was also shown in a study, which supports the notion that androgen ablation therapy increases NED of prostate cancer cells.

The population of NE-like cells in prostate cancer is increased with tumor progression, poor prognosis, and the androgen-independent state. In a recent review (38), the author argued that those NE-like cells in prostate cancer lesions were originated from cancerous epithelial cells, but not from normal NE cells, and should be defined as 'NE-like prostate cancer cells'. Although several studies have shown interesting results regarding the NE transdifferentiation of prostate cancer cells in cultures as well as in animal models, it remains for further investigations to determine whether those transdifferentiated NE-like cells exhibit the similar biochemical properties as that of clinical NE-like prostate cancer cells. For example, the biochemical information of those transdifferentiated NE-like cells, such as the expression profiles of NE markers,

the paracrine effect and the tumorigenic activity, is limited. To elucidate the molecular basis of NED and their roles in prostate cancer progression, stable NE-like prostate cancer cell lines of clinical relevance are imperatively needed.

1.3 SOMATOSTATIN AND ITS RECEPTORS

Natural somatostatin (sms14) is characterized as an inhibitory peptide with exocrine, endocrine, and autocrine activity. The general inhibitory function of sms is wide ranging and it affects a number of organ systems. Sms prevents cell proliferation by inducing cell cycle arrest and apoptosis. These effects are mediated by somatostatin receptor (SSTR) on tumour cells. Sms can also inhibit the secretion of hormones and growth factors involved in promoting tumor cell growth, inhibiting angiogenesis, promoting vasoconstriction and modulating immune cell function (Figure 2).

Sms's effect on tumor growth may be also the result of indirect effects i.e. through suppression of the secretion of growth-promoting hormones and growth factors that are involved in tumor cell growth. For example, insulin-like growth factor-1 (IGF-1) is produced by hepatocytes through GH-dependent and -independent mechanisms. It is an important modulator of many neoplasms. The clinically established sms analogue Octreotide, controls serum IGF-1 levels through inhibition of GH secretion via SSTR2 and SSTR5 resulting in a direct effect on IGF gene expression (39). Clinical studies have demonstrated a reduction of IGF-1 gene expression and serum levels of IGF-1 after treatment of breast cancers with Octreotide and these effects are increased when combined with the antioestrogen tamoxifen (40).

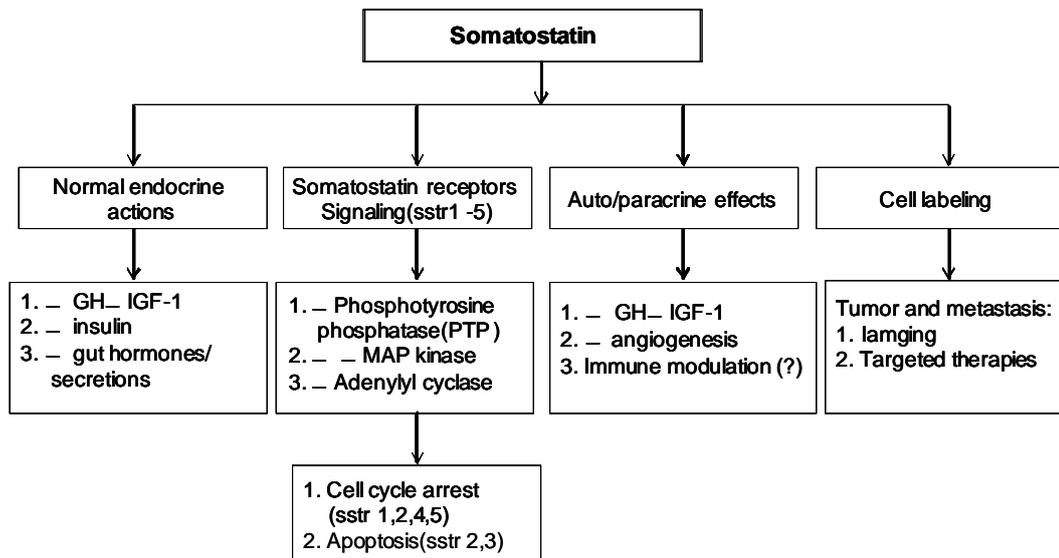


Figure2. Mechanisms of sms and its analogues can potentially contribute to cancer diagnosis and treatment. SSTR: somatostatin receptor. GH: growth hormone. IGF: insulin growth factor.

To date five subtype receptors (SSTR1–SSTR5) have been identified and cloned in human tissue. SSTRs are encoded by five genes localised on separate chromosomes. Four of these genes are intronless, the exception being SSTR2 which is alternatively spliced in rodents to generate two isoforms named SSTR2A and SSTR2B which diverge in their C-terminal sequence. SSTR subtypes belong to the family of G-protein-coupled receptors (GPCRs) with seven transmembrane spanning domains and present a high degree of sequence identity (39–57%). They all bind sms14 and sms28 natural peptides with similar affinity (nanomolar range) although with a slightly higher affinity for sms 14. Only SSTR5 displays a 10-fold higher affinity for sms 28 (41).

The occurrence and localization of SSTRs in the prostate has been a matter of debate with conflicting results. By the immunohistochemical analysis of radical prostatectomy specimens, Dizeyi et al identified SSTR1 in cancerous and neuroendocrine prostate cells, where SSTR2 was found in the stroma (42). They also found that there are lower levels of SSTR2 mRNA expression in prostate cancer cell lines PC-3, DU-145 and LNCaP. Hansson et al confirmed that mRNA expression for SSTR2 is preferentially located in the stromal compartment, while SSTR4 is confined to the epithelial prostate cells (28). In benign and malignant human prostate tissue, Reubi et al noted that Octreotide only binds to the stromal structures of prostate tissue and not to prostate cancer cells, suggesting high SSTR2 expression only in fibromuscular cells (43). The

same group further evaluated SSTR subtypes in about 200 various tumors and found that prostate carcinomas predominantly express SSTR1, while SSTR2 is expressed in non-neoplastic human prostate stroma (44). Halmos et al investigated mRNA expression in 22 human prostate cancer samples by RT-PCR technique (45). They found that detection rate of mRNA was 86% for SSTR1, 14% for SSTR2 and 64% for SSTR5. In conclusion, SSTRs are present in human prostate cancer and cell lines, but SSTR2 is not prominent, if present in cancerous tissue and cells, at very lower level.

Sms and its analogues can also indirectly control tumor development and metastasis by inhibition of angiogenesis. Tumor angiogenesis is essential for tumor growth, invasion and metastasis. Over-expression of peritumoural vascular SSTRs with high-affinity for sms and Octreotide has been reported in human primary colorectal carcinomas, small cell lung carcinoma, breast cancer, renal cell carcinoma and malignant lymphoma. This expression appears to be independent of receptor expression in the tumor and may be related, at least in part, to SSTR2 (46). Furthermore, SSTR2 receptors have recently been detected by immunohistochemical staining and in vivo scintigraphy in proliferating angiogenic vessels of human vascular endothelium (47). Thus, sms may inhibit angiogenesis by acting directly on SSTRs-positive cells such as endothelial cells and monocyte cells and indirectly inhibiting the production of angiogenic factors.

The antiproliferative effect of sms can also result from apoptosis. In breast cancer MCF-7 cells, the cytotoxic effect of sms is dependent on SHP-1 and results from caspase 8 activation, cell acidification and mitochondrial dysfunction (48). Apoptosis is induced by SSTR3 as a result of the induction of p53 and Bax (49). Apoptosis is also induced by SSTR2 in HL-60 cells that express endogenous SSTR2 (50) and in human pancreatic cancer cells expressing mutated p53 and devoid of endogenous SSTR2, after correction of the deficit by expression of SSTR2 (51). Thus, sms can induce apoptosis by p53-dependent and -independent mechanisms. SSTR2 induces apoptosis in a tyrosine phosphatase SHP-1-dependent manner.

Sms may also inhibit the secretion of gastrointestinal and pancreatic hormones involved in the regulation of tumor growth such as gastrin and glucagon via SSTR2, and insulin via SSTR5 (39, 52). NE cells not only produce sms, they also have receptors indicating autocrine as well as paracrine function (53). In prostate cancer cells, sms induces cell-cycle arrest and apoptosis (54), perhaps through SSTR3, which induces Bax (55).

Newly developed sms analogues may also be useful agents in the treatment of prostate cancer (56-58). While all five subtypes display an affinity similar to natural sms, there are major differences in the binding of currently available sms analogues to various SSTR subtypes (59, 60). Although many studies indicated that sms-14 was efficacious in certain conditions, its short plasma half-life of < 3 min. limited its clinical usefulness (61). Therefore, much effort was devoted to the development of a more stable sms analogue suitable for clinical use. Octreotide was the first developed analogue and is the most widely used for symptomatic treatment of hormone secreting neuroendocrine tumors. It has higher affinity for the SSTR2 and shows significant anti-neoplastic actions in tumor expressing SSTR2 (62). It remains the drug of choice for application in a majority of pure NE tumours because such tumours most often predominantly express SSTR2 (46). However, other sms analogues such as Lanreotide, which have good affinity for SSTR5 in addition to that for SSTR2, may advantageously identify SSTR5 expressing tumours.

Smsdx is a novel derivative of native somatostatin 14 that has been developed in our laboratory (63, 64). It is a glycosylated sms where up to 4 sms have been conjugated to a dextran backbone. The resulting conjugate retains the biological properties of sms and its affinity to the SSTR subtypes and has high *in vivo* stability (half-life in blood is ~27 hours). The universal affinity of smsdx may be of importance when treating HRPC that predominantly expresses this SSTR1 subtype. Clinically available sms analogues (Octreotide, Lanreotide) have shorter half-lives and affinity only to SSTR2 and 5 lack affinity to SSTR1 subtype. Smsdx has completed a clinical phase 1 study revealing low toxicity and high tolerability (65).

The primary effect of sms analogues is not a direct cytotoxic effect of NE cells but rather inhibition of the release of various peptide hormones secreted by NE cells (66). The observation that sms analogues inhibit the release of various NE products has stimulated interest in their use as antiproliferative and pro-apoptotic agents.

NE cells have been found in all stages of prostate cancer. A number of researchers have found that NE cells lack or do not express the AR (24, 26, 67-69). NED of prostate

adenocarcinoma is a possible target for therapeutic strategies, such as administration of GH analogues (e.g., sms), especially in patients with HRPC.

There are several treatments attempting to inhibit NED or at least block pathways that “drive” prostate cancer progression. Three known pathways that have raised interest are bombesin/GRP, serotonin and sms. Sms is successfully used for symptomatic treatment of neuroendocrine tumors (29). The actions of sms in prostate cancer are more complex and the treatment effect may be through secondary mediators such as decreasing certain growth factors from NE cells (66). In addition, several SSTR types exist and established sms analogues have limited affinities to the different SSTR subtypes. A recent review of the literature including seven studies using sms analogues as monotherapy showed ‘negative’ results (70).

Sciarra et al proposed combination therapy with sms analogues and estrogen (70). Rationale for this combination is that some studies have shown that the number of neuroendocrine tumor cells increase during hormone therapy in prostate adenocarcinoma (71, 72). Sciarra et al (73) suggest that sms may influence the microenvironment in which prostate cancer cells reside, allowing other treatments to more effectively kill the tumor cells. Recognizing the direct cytotoxic effects of estrogen on prostate cancer (74), Robertson et al (1996) used the sms analogue Lanreotide in combination with ethinyl estradiol, theorizing a synergistic effect. Fourteen of 20 stage D3 patients demonstrated extended response time and symptomatic improvement. The results were encouraging and supportive of the rationale for this combination therapy in both progression-free survival and biochemical response. In addition, serum CgA decreased significantly, suggesting that a decrease in NE cell number or activity may be partially responsible for the results.

1.4 EPIGENETICS OF PROSTATE CANCER

With prostate specific antigen (PSA) test used clinically in 1998, there has been dramatic increase in the diagnosis of prostate cancer (75). The American Cancer Society recommends annual screening of men above the age of 50 for prostate cancer with PSA and rectal examination (76). However, it is not clear whether the PSA is effective in the diagnosis of prostate cancer as it lacks both specificity and sensitivity (77, 78). About 25% of men with normal PSA may harbor prostate cancer (78) and a

PSA value of less than 20 ng/ml may not differentiate between prostate cancer and benign conditions (78). This leads to unnecessary prostate biopsies and the risk of failure to diagnose prostate cancer in patients with low PSA values. Similarly, there is lack of effective prognostic markers to predict the behaviour of prostate cancer and outcome following definitive treatment. Novel biomarkers based on epigenetic profiling are being explored to aid in the diagnosis and management of prostate cancer (79-81).

The standard treatment for newly diagnosed metastatic prostate cancer is androgen ablation. However the disease eventually progress to HRPC. Recently a taxan derivative (TAX327) has shown treatment efficacy with survival advantage in HRPC (82). Additionally, recent results with sms derivatives combined with dexamethasone or in combination with estrogens seem promising (73). Intensive investigations have shown that aberrant epigenetic features including aberrant DNA methylation, make an important contribution to carcinogenesis as well as genetic alterations. Hypermethylation of CpG islands in promoter regions can lead to silencing of tumor-suppressor genes, while hypomethylation of the genome leads to instability. Epigenetics is one of the rapidly expanding fields in cancer related research. Recent studies have shown that epigenetics plays an important role in cancer biology, somatic gene therapy, viral infections, genomic imprinting. Epigenetic changes, particularly DNA methylation, were found to be involved in a variety of cancers including colon, lung, breast, ovarian and prostate cancer (80). Unlike passively transferred genetic mutations, the epigenetic changes must be actively maintained and its “reversibility” makes them a potential therapeutic target (83).

Epigenetic changes are defined as heritable changes in gene expression that occur without changing the order of bases in the DNA sequence (84) and it contributes to the malignant transformation and progression of prostate cancer. One of the first identified hallmarks of epigenetic alterations is DNA methylation i.e. the addition of a methyl group to the 5'-carbon of cytosine in CpG sequences catalyzed by three active DNA methyltransferases (DNMTs), DNMT1, DNMT3a and DNMT3b. DNA methylation (Hypo- or hypermethylation) and histone modification (acetylation or deacetylation) have been shown to be involved in the malignant transformation and progression of prostate cancer (81).

Epigenetic changes are heritable and potentially reversible. Hence, it is reasonable to expect that these can be used as potential therapeutic targets. Currently there are several drugs which are at different stages of development. They can be broadly classified in two groups: (i) DNMT inhibitors and (ii) Histone Deacetylase (HDAC) inhibitor.

5-aza-2' - Deoxycytidine (5-aza-dC) is one of the early drugs identified as a DNMT inhibitor after being used as cytotoxic drug in the -90ies. This drug forms irreversible covalent bonds with DNMT1 after its incorporation in to DNA, thereby inducing degradation of DNMT1 (85, 86). Myelosuppression is a known side effect of this drug which is otherwise well tolerated. 5-aza-dC has been recently approved by FDA for clinical use in certain haematological conditions. Another drug in the same group, Zebularine can be administered orally or intraperitoneally. It has to be given in high doses, however, is chemically stable and has low toxicity (87). Other drugs in this category which are being studied include Epigallocatechin-3 - Gallate (EGCG), Procainamide, Procaine and MG 98 (88).

A variety of natural products exhibit HDAC inhibitory activity. Commonly used HDAC inhibitors which are being tested include trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA) and valproic acid (81). Many of these drugs have exhibited antitumor activity. SAHA and sodium butyrate have shown prostate cancer inhibition in animal models (89, 90). Overall, low toxicity rates of these drugs are encouraging for conducting further studies. The combination of HDAC and DNMT inhibitors has synergistic effect in the reactivation of silenced gene (81). Another interesting possibility is the combination of epigenetic drugs and conventional anti androgens and chemotherapeutic agents. It should be cautioned that the epigenetic drugs currently lack gene specificity and some of them are associated with significant toxicity. Hence, efforts are being made to develop gene specific epigenetic drugs (89).

1.5 CANCER PROTEOMICS

The term 'proteome' was first coined in 1994, and refers to all the proteins in a cell, tissue, or organism. Proteomics refers to the study of the proteome. Because proteins are involved in almost all biological activities, the proteome is a rich source of biological information. The total number of proteins in human cells is estimated to be between 250-500.000, and only a small percentage have been sequenced or identified.

The complete proteome has not been characterized for any organism. In contrast, the genome or the entire set of genes for several organisms has been sequenced, including humans. The human genome is estimated to contain about 35,000 protein-encoding genes. Besides the difference in quantity, another important difference between the genome and proteome is that the genome is static and relatively unchanged from day to day. Cellular proteins, on the other hand, are continually moving and undergoing changes such as binding to a cell membrane, partnering with other proteins, gaining or losing a chemical group such as a sugar, fat, or phosphate, or breaking into two or more pieces. Proteins play a central role in the complex communication network within and between cells and are constantly responding to the needs of the organism.

Proteins and/or modified proteins may vary among individuals, between cell types, and even within the same cell under different stimuli or different disease-states. One gene can produce more than one protein and one protein can be modified in multiple ways, which may change its behaviour. This can happen when the cell uses a single gene DNA template to produce several different messenger RNAs, which are then used as templates to make different proteins, or it may happen when a protein is modified by cellular processes after it is created. The result is that instead of one gene producing one protein, one gene can produce as many as 1,000 different proteins. On average, however, a gene produces five to ten different proteins from its messenger RNAs (91). The quantity of different proteins can vary greatly. For example, in human blood, the concentration of the protein albumin is more than a billion times greater than another protein, interleukin-6. There is no laboratory amplification technique for proteins like there is for amplifying genes. This means that it is not possible to make copies of proteins that are present in very small amounts.

The goal of clinical proteomics is to develop proteomics technology for the benefit of patient care. This new research technology is now being used in clinical research studies ranging from cancer to cardiovascular disease and organ transplants (92). Proteins that can be used as early biomarkers of disease, or that may predict response to therapy or the likelihood of relapse after treatment in blood, urine, or diseased tissue (93,94) are needed.

1.5.1 Proteomics technologies for cancer research

Advances in proteomics are contributing to the understanding of the pathophysiology of neoplasia, cancer diagnosis and anticancer drug discovery. The recent progress of proteomics has opened new possibilities for tumor-associated biomarker discovery. With the advent of new and improved proteomics technologies, such as the development of quantitative proteomic methods, high-resolution, -speed and -sensitivity MS and protein arrays, as well as advanced bioinformatics for data handling and interpretation, it is now possible to discover biomarkers that can reliably and accurately predict outcomes during cancer management and treatment. Proteomics technologies will bring forward novel strategies to accelerate the translation of basic discoveries into clinical practice.

Cancer proteomics encompasses the identification and quantitative analysis of the entire complement of proteins in a biological sample. The commonly used technologies available today include 2D-polyacrylamide gel electrophoresis (2-DE), isotope-coded affinity tags (ICAT), matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS), liquid chromatography–MS/MS (LC–MS/MS), imaging MS, protein arrays, and autoantibody expression techniques.

Proteomics in its classical definition involves protein separation by 2-DE and identification by MALDI-TOF-MS. 2-DE has the advantage that it enables the simultaneous visualization of thousands of protein spots, the quantification of their levels and the detection of post-translational modifications. It is a well-established, robust and the most widely used approach in neuroproteomics and other research fields. In the first-dimensional separation, usually immobilized pH-gradient (IPG) strips (95, 96) are used. The increased application of 2-DE today is to a large extent due to the introduction of the IPG strips.

Clinical proteomics can have important applications that may directly change clinical practice by affecting critical elements of care and management. Prostate cancer treatment decisions are complicated by the biologic heterogeneity of the disease and potential proteomic biomarkers of prostate cancer can benefit not only the early detection of disease, but can also be used for determining cancer risk, stratifying disease stage and grade, monitoring response to therapy, and in general assisting in therapeutic decision making. Proteomic platforms are fast becoming powerful tools for deriving protein “signatures” in a wide range of cancers, including prostate cancer (97).

1.5.2 Prostate Cancer Proteomics

The first study describing an alteration in global protein expression in prostate cancer was published in the early 1980ies. In this study, Edwards et al described a protein series common to the urine and prostatic tissue of patients with prostatic adenocarcinoma (98). With the use of the 2-DE approach, several differences in protein expression were observed. Unfortunately, none of these proteins were identified. Since this time, several studies of global protein expression in prostate cancer by proteomics technology have been conducted on surgical specimens or in cell culture models (99-101). A large number of molecular markers have been identified, but clinical applications of most of these markers are lacking.

Proteomic analyses have revealed a significant lack of correlation between expression patterns in cultured cell lines and microdissected cells from the same patient (99). Therefore, tissue-based proteomic analyses are more attractive for relating protein biomarkers directly to disease. In a current study (102), prostate needle biopsy specimens from patients with prostate cancer or BPH were analyzed using a proteomic approach to identify proteins specifically regulated in prostate cancer. These findings may shed light on the molecular events underlying prostate cancer progression and may aid in the identification of biomarkers and therapeutic targets for the treatment of prostate cancer.

In one study, the proteome patterns correlating with the three anatomical zones of the prostate: the peripheral (PZ), the transition (TZ) and the central (CZ) zone were investigated (100). Ten proteins with significant zonal differential expression were identified, eight of them with underexpression in the CZ versus the PZ and the TZ (arginase II, ATP synthase, cytokeratin 8, lamin A/C, peroxiredoxin 4, protein disulfide isomerase A3, tropomyosin, and vimentin), and two with overexpression in the CZ (peroxiredoxin 2 and creatine kinase B). The PZ and TZ, have epithelium with highly similar major protein expression profiles, whereas the protein profile of the CZ differed suggesting functional differences.

Proteins responsive to androgen may be involved in the development and progression of prostate cancer and the ultimate failure of androgen-ablation therapy. These proteins

represent potential diagnostic and therapeutic targets for improved management of prostate cancer. Several studies using prostate cancer cell lines have investigated different aspects of androgen proteins using proteomic platforms. Proteins differently expressed in androgen-sensitive prostate cancer cell-line LNCaP-FGC and androgen-resistant line LNCaP-r (a model for development of androgen resistance in prostate cancer) was identified by 2-DE and mass spectrometry (103). HSP60 was upregulated in LNCaP-r, nm23 in LNCaP-FGC, and titin (two isoforms) in either LNCaP-r or LNCaP-FGC. In non-malignant prostate, HSP60-staining was in the glandular compartment, particularly basal epithelial cells, whilst in prostate cancer, most epithelial cells showed moderate–strong staining without apparent correlation between staining intensity and Gleason grade. This study highlighted that the identification of HSP60 correlated with clinical results, indicating that this model can be used to explain mechanisms involved in transformation to androgen resistance. Rowland (104), on the other hand, investigated the effect of androgen (R1881) and anti-androgen (bicalutamide) on the androgen-responsive prostate cancer LNCaP cell line using a quantitative gel-based proteomic approach analysed by 2-DE DIGE. Following androgen supplementation, 108 spots (68 proteins) were increased and 57 spots (39 proteins) were decreased. Essentially, no difference was observed between control and anti-androgen-treated samples, confirming the absence of “off-target” effects of bicalutamide. Identified proteins were shown to be involved in diverse processes, including the stress response and intracellular signaling. This study of androgen responses has provided a number of potential candidates for development as diagnostic/prognostic markers and drug targets.

1.6 ADVANCES IN TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

The 2-DE technique was developed 30 years ago and has traditionally been the standard exploratory tool for proteomics (105, 106). 2-DE is currently the most powerful high resolution technique for the separation and quantitative analysis of complex mixtures of proteins. The technology of high-resolution 2-DE has been considerably improved, making the method more reliable and reproducible. 2-DE separates proteins according to isoelectric point (pI) in the first dimension and molecular mass (M_r) in the second dimension (105). Proteins separated on 2D gels can be visualised by either staining with Coomassie blue dye, silver stains, fluorescent dyes (Figure 3). Theoretically, 2-DE is capable of resolving up to 10,000 proteins

simultaneously, with approximately 2000 proteins being routine, and detecting and quantifying protein amounts of less than 1 ng per spot.

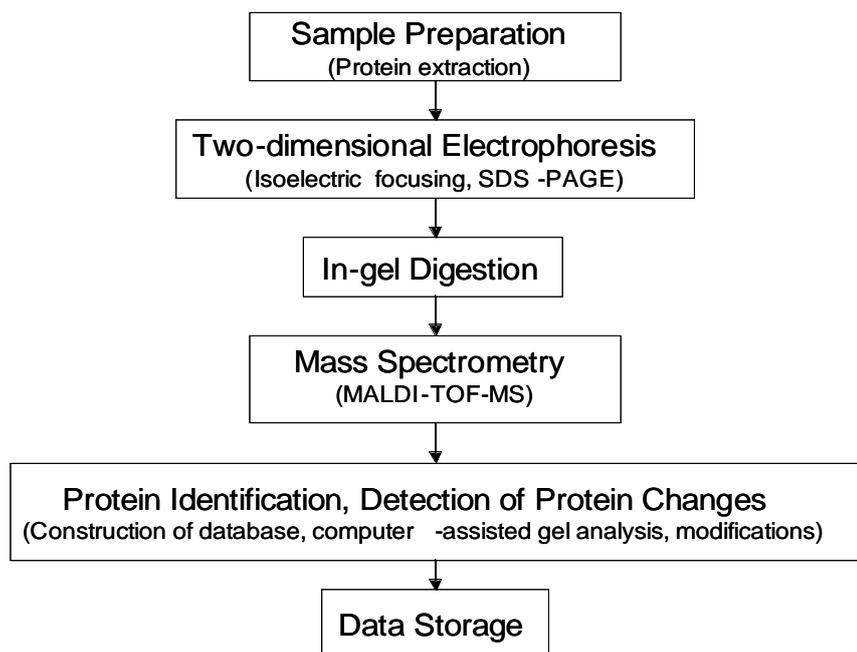


Figure3. *Workflow schemes in proteomics. Proteome analysis using two-dimensional gel electrophoresis and mass spectrometry (2-DE/MS). Protein from cell lysates is separated in the first dimension by isoelectric focusing (pI) and then in the second dimension by SDS-PAGE (molecular mass). Separated proteins are visualized by staining. Protein spots of interest are then excised and subjected to in-gel proteolytic digestion (e.g. trypsin) prior to sequence analysis by MS. In the case of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, peptide mass mapping techniques are used to create a unique mass fingerprint of the protein. For protein identification by MS/MS, a single peptide ion is isolated and fragmented to form smaller product peptide ions that contain the amino acid sequence information. The protein is then identified based on these unique identifiers (mass mapping or tandem mass spectrum) by computer sequence database matching.*

1.6.1 Sample preparation

Sample preparation for a proteomic analysis is a delicate and critical work. Multiple factors, such as protein molecular sizes, charge, hydrophobicity and hydrophilicity indices, protein conformational states, post-translational modifications, complexation with other macromolecular biological entities and enzymatic co-factors and cellular distribution, can influence the sample preparation. It's not possible to use a single sample preparation protocol that sufficiently captures the entire proteome for a given biological system. An ideal sample preparation protocol would reproducibly and non-intrusively isolate the full complement of proteins in a given biological sample, concurrently eliminating post-extraction modifications and non-specific contaminants.

Proteins are not expressed in equal amounts and there are large differences in the protein levels in all proteomes. Low abundance proteins are present at about 100 copies per cell, while the high abundant proteins may be present at 100,000,000 copies per cell (107). A proteomic analysis has to employ the proper technologies for the detection of all proteins. There exist a lot of approaches which can be used for the enrichment and visualization of the low-abundance proteins and also for the depletion of the high-abundance proteins and the latter may hinder the detection of the less abundant counterparts. For low-abundance proteins, in order to be present in sufficient quantities and be detected, the low-abundance proteins should be enriched from crude extracts. Enrichment is performed by biochemical protein-enriching approaches. The original protein mixture is separated into less complex fractions and each contains a lower number of total proteins in comparison with the starting material. This fractionation increases the likelihood of detecting low-abundance proteins. Two approaches are usually employed: (i) separation of the mixture into subcellular fractions and organelles, and (ii) enrichment of proteins from larger volumes by selective fractionation, immunoprecipitation, chromatographic, or electrophoretic methods (108-110).

Samples having proteins with high abundance make the detection of other less abundant proteins difficult. Examples of such samples are plasma and cerebrospinal fluid. The latter is widely used in neuroproteomics studies. Body fluids, like plasma, are interesting from the medical point of view in that many cells communicate in the plasma and release other components there. Although plasma and cerebrospinal fluid are easily obtained and can provide important biomedical information, the proteomic analysis is difficult (111). The reason is that plasma contains about ten high-abundance proteins which together represent about 98% of the total protein content. Probably, less than 1% of all proteins are prime targets for the identification of novel markers (112). The most abundant proteins are albumin and the immunoglobulin heavy and light chains, together representing about 80% of the plasma protein content.

Detection of hydrophobic and membrane proteins in 2-DE gels is related to certain limitations, and a relatively low number of real hydrophobic and membrane proteins have been detected. Using current 2-DE products (i.e., non-ionic or zwitterionic detergents like CHAPS, and non-ionic, mild chaotropes like urea or thiourea), hydrophobic and membrane proteins can be resolved and visualized (108, 113, 114).

Analysis of membrane proteins is of particular interest because they exert important functions in signal-transduction pathways, ion transport, cell–cell interactions, and other processes. Many of those proteins are drug targets, and are consequently of medical interest. Hydrophobic membrane proteins, which are one of the most interesting targets for new drugs, they are difficult to dissolve. However new zwitterionic detergents such as sulfobetaines (e.g. SB 3–10 or ASB 14) can increase the solubilisation of hydrophobic proteins (115, 116).

2-DE still experiences several shortcomings due to, in part, its capacity to resolve thousands of proteins simultaneously. Today, there is no standard 2-DE system that separates all kinds of proteins equally well, i.e. hydrophilic and hydrophobic proteins, low and high Mr proteins and highly basic proteins. It is impossible to display all proteins in a single gel. So, several gels are most probably required for one sample. Unfortunately, 2-DE cannot fully meet all these challenges.

In general, the great complexity of higher eukaryotic proteomes, their constantly changing nature, and most importantly, the wide diversity of protein properties and behaviour suggest that no single proteome analysis method will be able to effectively address all proteome analysis problems within the near future. Hence, it seems most likely that all the major current analytical technologies including 1D and 2D gels, multi-dimensional chromatography coupled with MS instruments, and antibody arrays will continue to play important and complementary roles in proteome analysis studies in the near future.

Despite some limitations, 2-DE gels remain as the gold standard to which any competing method should be compared. At the present, it is not apparent that any non-2-DE gel protein separation method provides major advantages over 2-DE gels in terms of the number of proteins detected, analysis time or sensitivity.

1.6.2 First dimension: isoelectric focusing (IEF)

Proteins are amphoteric molecules containing acidic and basic groups. These become protonated or deprotonated, depending on the pH environment. In basic environment, the acidic groups become negatively charged; in acidic environment the basic groups become positively charged. The net charge of a protein is the sum of all negative or

positive charges of the amino acid side chains. When an electric field is applied, it will start to migrate towards the electrode of the opposite sign of its net charge. At the pI, the protein has no net charge and stops migrating. The introduction of commercial IPG strips offer greater reproducibility and allows to establish comparisons among laboratories (117, 118). The pH gradients in these gels are prepared by co-polymerising acrylamide monomers with acrylamide derivatives containing carboxylic groups. In this case, the gradient cannot drift and it is not influenced by the sample composition. The use of immobilised pH gradients in the first dimension has allowed many methodical innovations for 2-DE (96). Immobilised pH gradients are stable and capable of simultaneously focusing both acidic and basic proteins on a single gel prepared with a broad pH gradient. With IPGs, several wide gradients have been used (3–10, 4–7). New types of gel strips with narrow and basic gradients are being developed.

1.6.3 Second dimension: SDS-PAGE

The second dimension of 2-DE separates proteins on the basis of their apparent molecular weights in polyacrylamide gels in the presence of SDS. The methodology for the second dimension did not change as much as for IEF. However, for SDS-PAGE some developments in the chemistry and instrumentation also contributed to improved handling and reproducibility of the spot positions. In this step, large amounts of SDS are incorporated into the SDS-protein complex in a ratio of approximately 1.4 g SDS/g protein. So, SDS masks the charge of the proteins themselves and the anionic complexes formed have a more or less constant net negative charge per unit mass. Thus, the electrophoretic mobility of proteins treated with SDS depends on the molecular weight of the protein. At a certain polyacrylamide percentage, there is an approximately linear relationship between the logarithm of the molecular weight and the relative migration distance of the SDS-polypeptide complexes of a certain molecular range. The molecular weights of the sample proteins can be estimated with the help of co-migrated standards of known molecular weights.

1.6.4 Detection of protein spots

In practice, the techniques most applied in proteomics laboratories are Coomassie Brilliant Blue (CBB), silver and fluorescence staining. Colloidal Coomassie Blue staining contains ammonium sulphate, which increases the strength of hydrophobic

interactions between the proteins and the dye. The procedure is time consuming and requires several steps, but gives sensitivity similar to that of silver staining.

Silver staining often produces a pattern different from the pattern achieved with Coomassie Blue. CBB detects approximately 0.1 µg of protein, and silver staining improves protein detection up to five-fold. The CBB procedure shows linear responses over a range of protein concentrations from 0.5–20 µg, whereas silver staining is linear at protein concentrations from 0.02–0.8 ng/mm² (119). Silver staining is useful when searching for qualitative variations (presence/absence of spots). Therefore, the preference should be decided before hand i.e. number of spots, good definition or relative isolation of spots. However, when the detection of quantitative variations is the aim, Coomassie staining with the assistance of computer programs is more reliable (120).

Coomassie Blue stained gels are usually compatible with mass spectrometry analysis because the dye can be completely removed from the proteins. Moreover, CBB stained gels contain enough protein for the identification and characterisation using mass spectrometry. Nevertheless, silver staining can be modified for mass spectrometry compatibility by omitting the glutaraldehyde from the sensitising solution and formaldehyde from the silver solution (121).

An important recent advance was the development of high-sensitivity non-covalent fluorescent stains for general proteins, glycoproteins and phosphoproteins (122). A major advantage of fluorescent stains with conventional chromogenic stains is a wider linear detection range, and they are therefore very well suited for quantification of proteins. Most of them show sensitivity similar or below that of the Coomassie Blue dyes. Fluorescence staining is mass spectrometry compatible. Sypro Ruby from Molecular Probes, Flamingo from Bio-Rad and Deep Purple from GE Healthcare are very sensitive, and as sensitive as mass spectrometry compatible silver staining. A fluorescence scanner is required for visualisation and detection. A recent study showed that the Deep Purple stain can result in increased peptide recovery compared to the Sypro Ruby stain and can improve MS-based identification of lower intensity proteins (123).

1.6.5 Image analysis

Once the gels are stained, the images are then analyzed. Specialized computer programs have been developed for the analysis of 2-DE protein profiles. However, for the detection of qualitative changes visual analysis is essential. Therefore, the gel images have to be converted into digital data using a scanner or camera and analyzed with a computer. Most 2-DE programs follow these steps for the evaluation of 2-DE gels: spot detection, spot filtering, spot editing, background correction, gel matching, normalization, comparison, quantification and reporting and exporting of data. One of the crucial steps is normalization. Before the gels are compared for differences in the spot pattern, the spot volumes of the different gels have to be adjusted by normalization. This step corrects for different protein loads and staining effectiveness. Gels are normalized according to the total spot volume or the volume of a single prominent reference protein.

1.7 MASS SPECTROMETRY PLATFORMS

MS has revolutionized proteomics. It is a technology used to measure the masses of individual molecules that have been converted into ions. Analysis of peptides in biological fluids by MS can provide diagnostic and prognostic information for cancer (124). In general, a mass spectrometer has three components: a chamber that holds the ion source; a detector that registers the number of ions at each mass-to-charge (m/z) value; a mass analyzer that measures the m/z value of the ionized analytes. Time-of-flight (TOF) refers to the length of time required for proteins/peptides ionized from the surface of a sample plate travelling through the MS chamber to the detector plate. The fundamental principle that permits MS to separate analytes is the fact that small ions fly faster than larger ones. The m/z values of the ions are calculated by the time of each ion reaching the detector plate. A mass spectrometer has the ability to analyze samples processed on a variety of platforms. MS platforms provide a sensitive system for differential protein/peptide expression, given its low sample requirement and high-throughput format. Modern MS instrumentation, such as the Fourier transform ion cyclotron resonance (FT-ICR) MS has achieved excellent performance. Higher abundance proteins can be detected from subpicogram-sized (total) proteome samples (125).

The basis of MALDI-TOF-MS technology is the absorption of short pulses of laser light by a chromophoric matrix resulting in volatilization of the sample molecules

dispersed in the matrix. The matrix isolates sample molecules in a chemical environment that enhances the probability of ionization without fragmentation. The ions formed are accelerated by a high voltage supply and then allowed to drift down a flight tube where they separate according to mass. Arrival at the end of the flight tube is detected and recorded by a high-speed recording device. MALDI-TOF-MS has developed into a valuable tool in oncoproteomics. It is envisioned to be most useful in conjunction with conventional biochemical techniques. They can be applicable to identify blocked amino termini, PTMs and mutation sites in known proteins. A significant amount of preliminary structure determination is possible on very small amounts of analyte (<10 pmol). Detailed protein characterization using the latest MALDI-TOF/TOF-MS/MS technology enables ultrasensitive protein identification. However, for ladder sequencing and in-source fragmentation studies, it is important to minimize potential peptide impurities. Careful attention must also be given to synthetic peptide samples so as not to confuse the fragment ion signals with protonated molecular ions originating from low levels of incomplete synthesis impurities.

MALDI mass spectrometry has also been used for generating patterns of proteins from clinical samples, such as serum and plasma, and does not rely on protein identity so can be used to generate a diagnostic fingerprint. MALDI has also been used for molecular imaging of tissue sections (126) for generating disease-related protein mass profiles of normal and malignant breast cells (127).

Protein expression profiling using either MALDI-TOF or SELDI-TOF approaches has seen a wide application to many disease sites including prostate cancer (97, 128-134). The SELDI-TOF approach utilizes a chip-based affinity capture procedure to reduce sample complexity and then “profiles” the bound intact proteins by mass. The technique is sensitive, needs minimal amount of protein, and has relatively high throughput (135-137). Other investigators have employed a combination of chromatographic paramagnetic beads and MALDI TOF/TOF MS presenting a powerful and sensitive analysis of pre-fractionated samples (138). The paramagnetic beads allow for reasonable high throughput processing and reproducible fractionation of proteins/peptides followed by MALDI-TOF MS analysis. Since the introduction of this technology, the technique has been widely used for single or multidimensional separation of proteins/peptides on the beads. The fractions are then spotted on target plates for MALDI-TOF analysis (139). Although not yet fully realized, this approach

via sophisticated TOF/TOF capabilities offers direct protein identification with little or no additional work-up. A new technique immuno-MS (140) was first reported in early 2000. The incorporation of immuno-MS provides for early validation of biomarkers discovered on the same platform and offers distinct advantage over ELISA in that isoforms, modifications and cleavage products can be evaluated with the same antibody.

MS technologies will play an increasing role in biomarker identification and validation in cancer, especially in prostate cancer. After a selected group of potential protein biomarkers are identified, and their expression levels can be monitored and correlated in normal and different disease states, a prostate biomarker database can be developed useful for monitoring the expression levels during prostate cancer progression and treatment.

2. AIMS OF THE STUDY

- I. To determine how smsdx affect the protein expression of the prostate cancer cells in comparison to sms.
- II. To identify proteins affected by sms and smsdx in prostate cancer cells.
- III. To compare the protein expression in androgen dependent and androgen independent prostate cancer cells after sms and smsdx treatment
- IV. To investigate SSTRs expression in prostate cancer cell lines and attempt to enhance the SSTRs expression

3. MATERIALS AND METHODS

3.1 SAMPLE PREPARATION FOR 2-D ELECTROPHORESIS

Sms and smsdx were incubated with androgen-dependent cell LNCaP and androgen-independent cell DU145 cultures. The cell cultures were treated with smsdx or with sms for three days, 1 nM per day, as described by Brevini (141). Controls were untreated cells (negative control) and cells treated with sms (positive control). The cellular extraction from LNCaP and DU145, the preparation of the total cell lysate were performed as described by Franzén (142). Protein determination was made using Pierce BCA protein assay reagent (Rockford, IL, USA.).

The samples were diluted to a total volume of 250 μ l, 0.2% Pharmalyte, 8 M urea, 0.3% DTT, 2 M CHAPS and a trace of bromphenol blue (Sigma). An amount of 100 μ g of protein was loaded on each strip via rehydration using non-linear pH 3-10 Ready Strip IPG, strips (Bio-Rad, Hercules, Ca, USA). Focusing was carried out for a total of 45,500 Vh in a PROTEAN IEF cell (Bio-Rad). Precast gels (12.5% homogenous Tris-HCl Criterion) SDS-PAGE (Bio-Rad) were run using a Criterion Dodeca cell gel apparatus (Bio-Rad). A total of 4 gels were run per sample group. The electrode running buffer was 25 mM Tris, 192 mM Glycine, 0.1% w/v SDS. Gels were run at 250 V for approximately 1 h until the bromophenol blue marker had reached the bottom of the gel at a temperature of approximately 15°C. Proteins were visualized by silver staining as described by Rabilloud (143).

3.2 GEL SCANNING AND IMAGE ANALYSIS

2-DE gels were scanned at 100 μ m resolution (12-bits/pixel) using a GS-710 laser densitometer (Bio-Rad). Data was analyzed using PDQuestTM software Version 7 (Bio-Rad). After auto-detection of all protein spots, gel-images were carefully edited. The individual proteins quantities were expressed as ppm of the total integrated OD. All 2-DE maps were matched and evaluated independently. Quantitative data sets were generated and subjected to hierarchical cluster analysis using the J-Express software. The clustering pattern is represented diagrammatically as a dendrogram (144). The variables used in the cluster analysis were selected using both the Mann-Whitney

signed –Ranked test and Student’s t-test, ($P < 0.05$) for statistical analyses between control and sms-treated cells.

The methodological reproducibility of the 2-DE analysis was determined using group correlation analysis (145). Briefly, the total optical density is directly correlated to the total protein concentration. Minor differences in gel loading, running conditions, and silver staining may affect sample comparisons and affecting the 2-DE gel reproducibility. Four gels were run from each treatment group and comparisons of the intensity of matched spots between 2-DE gels were performed using the correlation coefficient analysis. A correlation coefficient was measured between two gels based on the optical densities of the same spots in the two gels being compared. A correlation coefficient of 1 will imply that the two samples being compared are identical. In a group consisting of 4 samples, a maximum of six pair wise comparisons are possible. The average correlation coefficient among the smsdx samples was 0.85 ($n = 6$ gel pairs, range 0.80 – 0.92).

3.3 PREPARATIONS FOR MASS SPECTROMETRY

500 μ g of protein was mixed with rehydration buffer (8 M urea, 2% CHAPS, 0,002% bromophenol blue, 18,2 mM DTT, 0,5% Pharmalyte (pH 3-10 non-linear), left at room temperature for 15 min and centrifuged for 10 min before being applied to a 24 cm immobilized pH gradient strip (pH 3-10 non-linear) for overnight rehydration. First-dimension isoelectric focusing was carried out on GE Healthcare IPG-phor according to manufactures instructions, with a total focusing time of around 70 kVhrs. The strips were equilibrated in 15 ml equilibration solution (6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (w/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue) reduced with 65 mM DTT for 15 min, followed by 15 min equilibration in equilibration solution with 135 mM iodoacetamide added. The IPG-strips were then loaded and run on a 12,5% SDS-PAGE gel (25°C) with 10 W/gel until the bromophenol blue dye front had run off the base of the gel. The gels were stained in colloidal coomassie (LabDesign Boule Nordic AB) over night and then distained in water according to manufactures instructions. The gels were scanned using GE Healthcare ImageScanner. Spot detection and matching was done in ImageMaster 2D Platinum (GE Healthcare) with three manual landmarks set.

Statistical analysis: Three gels were run for each of the three groups; control, smsdx, and sms. The master gel belonged to the smsdx group. The 1083 spots on the master gel were matched with spots on the other 8 gels, when possible. If no matching spot was found on a gel, it was designated as a missing value. A spot can be missing either due to an experimental error or due to low abundance. The two treated groups, sms and smsdx, were compared separately with the control group using a Mann-Whitney test. Since the control group and a treated group contains three measurements each, the lowest possible two-sided Mann-Whitney p-value is 0.1, which is achieved exactly when all 3 values in one group are lower or higher than all 3 values in the other group. This can only happen when there are no missing values. The number of spots without any missing values in the smsdx and control group was 468. Out of these 137 had a p-value of 0.1, leading to a false discovery rate of 0.29. To include the rest of the spots, missing values were set to zero in the Mann-Whitney test. So, the spots with a p-value of 0.1 are exactly those spots with the three highest measurements in one group, and all measurements in the other group either lower or missing. The number of spots in the control versus smsdx comparison with at least one missing value was 615. Out of these 100 had a p-value of 0.1, leading to a false discovery rate of 0.62. The total number of spots with a p-value of 0.1 for the control versus smsdx comparison is hence 237 out of 1083, leading to a false discovery rate of 0.46. For the control versus the sms group, the corresponding numbers were: 140 out of 436 (FDR=0.31) without missing values, 83 out of 647 (FDR=0.78) with missing values, and 223 out of 1083 (FDR=0.49) in total.

3.4 MASS SPECTROMETRY ANALYSIS

Proteins were identified with a vMALDI-LTQ instrument (Thermo Electron, San José, CA, USA). The spot picking, destaining, digestion, extraction, sample preparation and spotting on MALDI target plates were carried out using a spothandling workstation (ETTAN “Spothandling” workstation, GE Healthcare) and a standard protocol provided by GE Healthcare. The plate containing the combined extracts was evaporated to dryness. Each sample was prepared by constituting the dried peptides in 2,5 µl of matrix solution (2,5 mg/ml of α -cyano-4-hydroxy-cinnamic acid (Sigma) in 50% acetonitrile containing 0.05% TFA). 2,0 µl sample was spotted on a clean MALDI target slide surface and allowed to dry. The samples were analysed with a vMALDI-LTQ (Thermo Electron, San José, CA, USA). The analysis was done using Xcalibur 1.4 software in data dependent mode. A survey scan (MS) was followed by MS/MS

scans on the 5 most abundant ions. This string of 6 scan events was repeated six times for each sample spot. Dynamic ExclusionTM ensured that in total 30 different peptides were selected and fragmented for each sample. The MS spectra were collected in the 900-2000 Da mass range while the mass range for the MS/MS spectra were automatically selected by the system based on a Q value of 0.25. The standard collision energy of 38 was set for all the analysis. A time limit of 5 minutes/sample was selected, whether or not the 30 MS/MS spectra could be acquired. Database searches were done using both the MASCOT and Sequest search algorithm against the human session of the IPI protein database (version 2.38). The two searches were compared in the in house developed software Promiscuous MS/MS. A minimum of two peptides and A Mascot score of 45 were required for a protein to be accepted as identified.

3.5 RNA EXTRACTION AND RT-PCR

Total LNCaP cellular RNA, after treatment with sms and smsdx in different concentration (0, 0.1, 1.0, 10, 50, 100 nM for 24 h) and treatment time (0, 1, 4, 8, 16, 24 h for 10 nM), was extracted using the Trizol® (Invitrogen, Carlsbad, CA), according to the manufacturers' instruction. cDNA was synthesized using random primers (N6) and MMLV reverse transcriptase. The PCR for PRDX2, TCTP and HSP27 mRNA were performed by using the following primer pairs (table 1):

Table 1. The RT-PCR primers sequences and amplified genes

Amplified gene	Primers	Sequence	Product
PRDX2	sense	5 ^f - GCCCACGCAGCTTTCAGTCA -3 ^f	623bp
	antisense	5 ^f - AGCCAGCCTAATTGTGTTTG -3 ^f	
TCTP	sense	5 ^f - GAGGGGAAGATGGTCAGTAGG -3 ^f	278bp
	antisense	5 ^f - TGCTTGATTTGTTCTGCAGC -3 ^f	
HSP27	sense	5 ^f - CCAGAGCAGAGTCAGCCAGCAT -3 ^f	576bp
	antisense	5 ^f - CGAAGGTGACTGGGATGGTGA -3	

A total of 32 PCR cycles at 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 60 seconds for PRDX2; at 94°C for 60 seconds, 60°C for 60 seconds for TCTP; at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 40 seconds for HSP27, and 72°C for 60 seconds for was carried out. Beta-actin expression was used as a control for RNA

loading and reverse transcription efficiency and amplified with its specific primers using 25 cycles. PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and visualized in UV light.

4. RESULTS

4.1 CHANGED PROTEIN EXPRESSION AFTER SMS/SMSDX TREATMENT (PAPER I)

High resolution and reproducibility of protein spots from all samples were obtained with the criterion mini 2-DE gels. Analysis of the total protein expression profiles from the three sample groups (untreated, sms treated, smsdx treated) in prostate cancer cell line LNCaP revealed both similarities and differences. Significant quantitative differences were observed in the protein expression patterns between negative control cells and smsdx treated cells. A set of 63 protein spots was ≥ 2 -fold up regulated in smsdx treated cells compared to negative control samples ($P < 0.05$) (Fig 4). All the samples were distinctively separated into two main branches on the dendrogram in hierarchical cluster analysis. The first branch consists of all 4-control samples, while the second main branch is subdivided into 2 minor branches that separate sms from smsdx.

When similar analysis was done between control and sms, the expressions of a set of 44 protein spots were ≥ 2 -fold up regulated in sms treated cells compared to negative control samples ($P < 0.05$ Mann Whitney analysis).

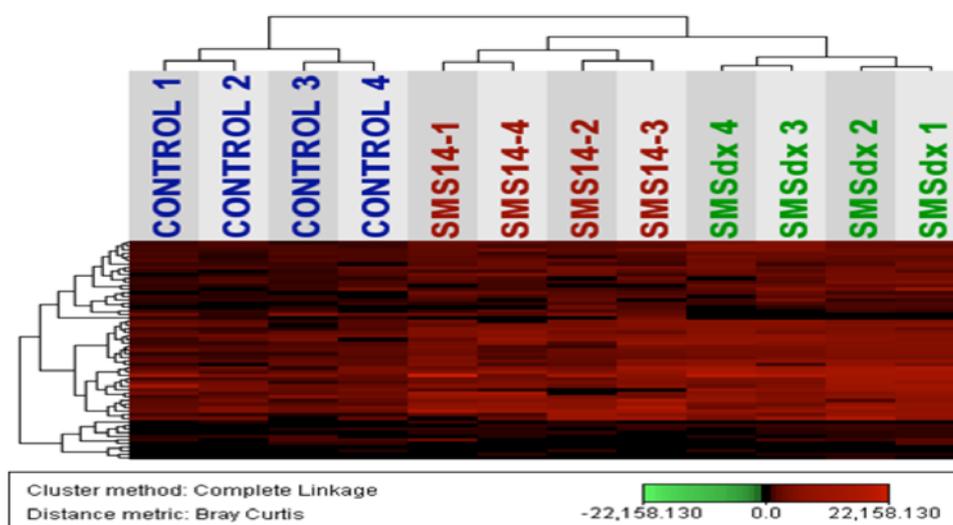


Figure 4. Hierarchical cluster analysis of 12 LNCaP control and treated samples. The dendrogram represents expression patterns of 63 protein spots in 4 control (Blues), 4 sms treated (Red) and 4 smsdx treated (Green) cells. The columns represent individual samples, while the rows represent the proteins. The colours in each cell unit correspond to the

expression levels of a given protein in a particular sample (Red= up-regulation, Black= down-regulation).

Quantitative differences were also observed in the protein expression patterns between sms and smsdx treated cells. A set of 44 protein spots was ≥ 2 -fold up regulated in smsdx treated cells compared to sms samples. The difference is statistically significant using Mann Whitney signed –ranked test ($p < 0.05$) (Fig 5). The 44 protein spots were subjected to hierarchical cluster analysis and classified (Fig 2). In this analysis, both the negative control and smsdx samples were closely classified on the same main branch while the sms samples were on the second main branch. On the contrary, 49 protein spots were ≥ 2 -fold up regulated in sms treated cell compared to smsdx treated cells. Approximately 50% of the total number of the resolved spots of smsdx treated and sms treated cells had similar expression patterns compared to negative control.

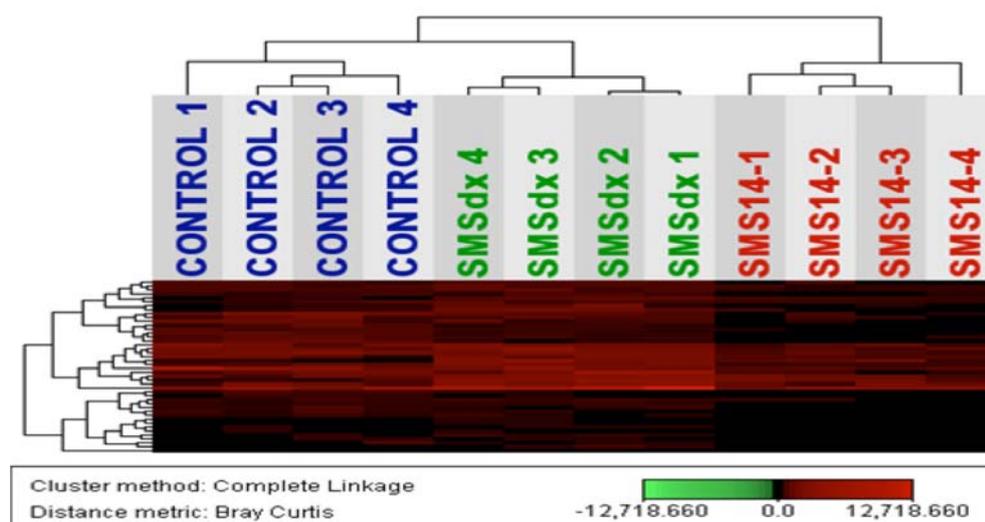


Figure 5. Comparison of sms and smsdx samples. Cluster analysis of a set of 44 spots were > 2 -fold up regulated in smsdx treated cells than sms samples.

4.2 IDENTIFICATION OF PROTEINS AFFECTED BY SMS/SMSDX TREATMENT (PAPER II)

Paper II identifies some of the affected proteins in an effort to elucidate pathways and proteins that might be of importance for the potential usefulness of sms treatment in prostate cancer. After incubating the LNCaP cell-line with sms/smsdx, comparative proteomics was used for analysing and identifying affected proteins. Protein expression patterns were analysed with 2-DE and mass spectrometry. Catalytic mitochondrial and

mitochondrial-associated proteins were significantly affected (fold change ~2 or higher) and they were in general up-regulated. Apoptosis-related proteins were both up-regulated (VDAC1, VDAC2) and down-regulated (PRDX2, TCTP). The fold change was >2 for PRDX2 and <2 for the others. There was a strong agreement between sms and smsdx on the up- and down- regulation of proteins. Sms/smsdx triggered up-regulation of catalytic mitochondrial proteins and seemed to affect apoptotic related proteins. Several of the proteins, e.g. VDAC, IDH3A and NADP involved in the MMP event which is critical in the process leading to chemotherapy induced apoptosis. The VDAC-Bax complex promotes pore formation, outer MMP, and the release of AMPs to activate caspases and induce apoptosis. Bcl-2 family proteins also regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. VDAC1 has been shown to co-immunoprecipitate with the anti-apoptotic protein Bcl and suggested to be involved in forming the mitochondrial pore which release cytochrome c during apoptosis.

Table 2 Proteins with strong differential expression (>1.6 in fold change) between smsdx vs. control and sms vs. control.

Protein name	Short Name	UniProt ID	Fold change smsdx vs. ctrl	Fold change sms vs. ctrl	Trends sms/smsdx vs. ctrl
Apoptosis					
Voltage-dependent anion channel 1	VDAC1	P21796	1.65	1.72	sms/smsdx > ctrl
DNA binding					
TATA-binding protein associated factor 2N	RBP56	Q92804	3.71	2.62	sms/smsdx > ctrl
Heterogeneous nuclear ribonucleoprotein D0	HNRPD	Q14103	2.24	1.79	sms/smsdx > ctrl
Electron transport					
Protein disulfide-isomerase	PDIA1	P07237	Inf	Inf	unique to sms/smsdx
Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial	UCRI	P47985	Inf	Inf	unique to sms/smsdx
Metabolism					
Short chain 3-hydroxyacyl-CoA dehydrogenase, mitochondrial	HCDH	Q16836	1.66	1.68	sms/smsdx > ctrl
Glycerol-3 phosphate dehydrogenase, mitochondrial	GPDM	P43304	1.62	1.75	sms/smsdx > ctrl
Acetyl-CoA acetyltransferase cytosolic	THIC	Q9BWD1	Inf	Inf	unique to sms/smsdx

Isocitrate dehydrogenase (NAD) subunit, mitochondrial	IDH3A	P50213	-1.78	-1.72	ctrl > sms/smsdx
Aconitate hydratase, mitochondrial	ACON	Q99798	2.50	2.17	sms/smsdx > ctrl
Proliferation					
Peroxiredoxin 2	PRDX2	P32119	-2.06	-2.14	ctrl > sms/smsdx
Heterogeneous nuclear ribonucleoproteins C1/C2	HNRPC	P07910	2.56	2.65	sms/smsdx > ctrl
Protein binding					
Early endosome antigen 1	EEA1	Q15075	Inf	Inf	unique to sms/smsdx
Lamin A/C	LMNA	P02545	Inf	Inf	unique to sms/smsdx
Transcription					
Chromobox protein homolog 3	CBX3	Q13185	Inf	Inf	unique to sms/smsdx
Transcription intermediary factor 1- β	TIF1B	Q13263	Inf	Inf	unique to sms/smsdx
Transport					
Voltage-dependent anion-selective channel protein 2, mitochondrial	VDAC2	P45880	1.84	1.79	sms/smsdx > ctrl

No missing values and the lowest possible two-sided Mann-Whitney p-value (= 0.1). Protein accession numbers from SwissProt/TrEMBL are given and “trend” indicates the direction of the change in expression level. The proteins are grouped according to molecular function. Smsdx and sms appear to have similar effect demonstrated by the concordance in protein expression levels .Inf: infinite.

4.3 DIFFERENTIAL PROTEIN EXPRESSION BETWEEN LNCAP AND DU145 (PAPER III)

A set of 222 proteins (isoforms and variants) for 2-DE map in LNCaP cell line were identified. One hundred and twenty five protein spots in DU145 cells were matched with the reference protein map of LNCaP cells. Fifty-eight (isoforms of 49 proteins) protein spots were identified and found differentially expressed at 2-fold change between LNCaP and DU145 cells. One third of the detected proteins were differentially expressed (PRDXs, hnRNPs). Concordance in protein expression patterns were observed between smsdx and sms treated cells with strong agreement between the up/down regulation of proteins. There was an agreement between sms and smsdx regarding the protein expression regulation in DU145 cells. Thirty-one and twenty-seven proteins have lowerer and higher expression (2-fold) in DU145 than in LNCaP cells, respectively. These proteins are involved in several cellular processes, including

proliferation, apoptosis, energy production and metabolism, vesicle trafficking, cytokinesis. Of the identified differentially expressed proteins (more than +/-2-fold change) between LNCaP and DU145 cells, PRDX2, TCTP, HSP27 were tested on the mRNA expression after treatment with sms and smsdx in different concentration and treatment time. PRDX2 was found to be up regulated in a dose dependent manner. TCTP mRNA was down regulated in this current study. HSP27 mRNA was also down regulated. Figure 6 and 7 showed the different expressions of PRDX2, TCTP regulated by sms and smsdx at the level of protein and mRNA.

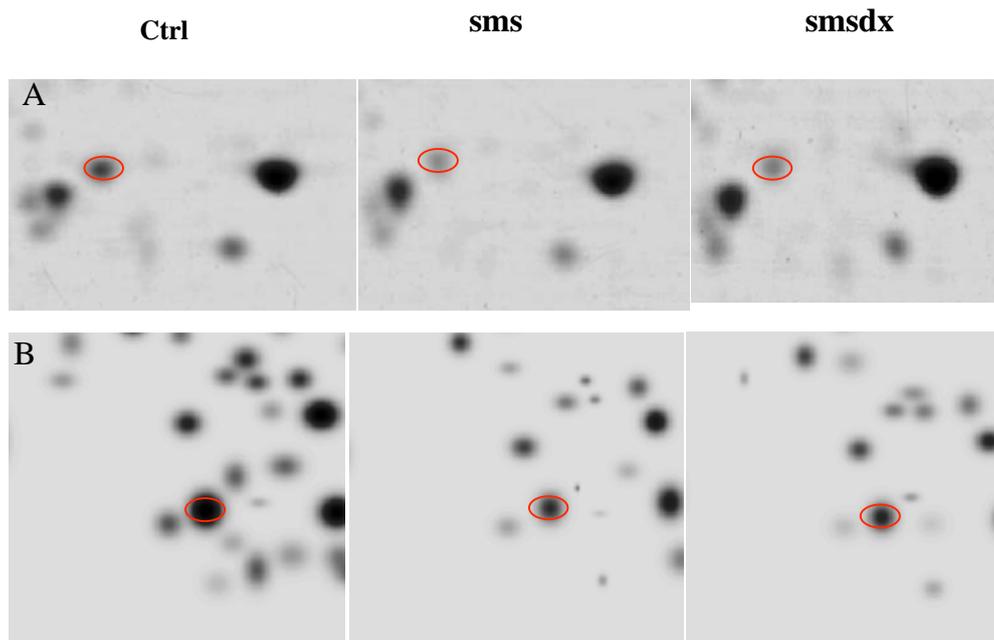


Figure 6. The identified protein expression of PRDX2 (A) and TCTP (B) (2 fold down-regulation) after treated with 1nM sms/smsdx for three days in the prostate cancer cell line LNCaP.

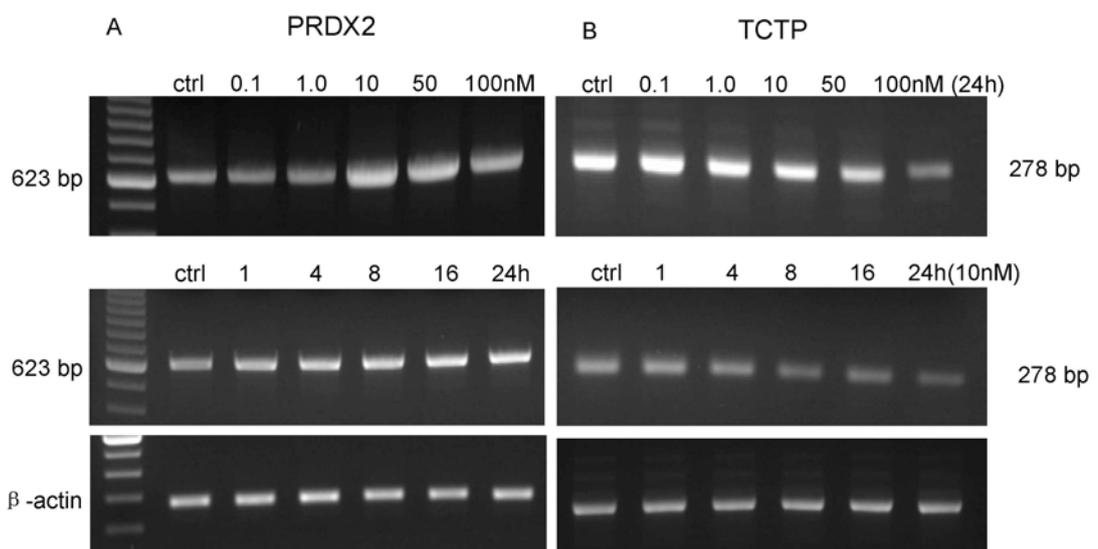


Figure 7. *PRDX2* and *TCTP* mRNA expression in LNCaP cell line after treatment with *smsdx*. Up-regulation of *PRDX2* (A) and down-regulation of *TCTP* (B) mRNA in *smsdx* treated LNCaP cell line with different concentration (0, 0.1, 1.0, 10, 50,100 nM for 24h)(upper lane) and treatment time (0, 1, 4, 8, 16, 24h for 10 nM)(middle lane), Control: lower lane.

4.4 ALTERATION OF SSTRs MRNA EXPRESSION BY SMS/SMSDX AND DNA METHYLATION INHIBITOR/HDAC INHIBITOR IN PROSTATE CANCER CELL LINES (PAPER IV)

The results indicate a positive feedback loop between *sms/smsdx* and SSTRs. The exposure of LNCaP cells to *sms* or *smsdx* for 3 days significantly up-regulated SSTR1 – 4 mRNA expression. The effect was clearly dose-dependent within 0.1 to 10 nM, and the strongest induction was seen at 10 nM for both *sms* and *smsdx* (Fig. 8).

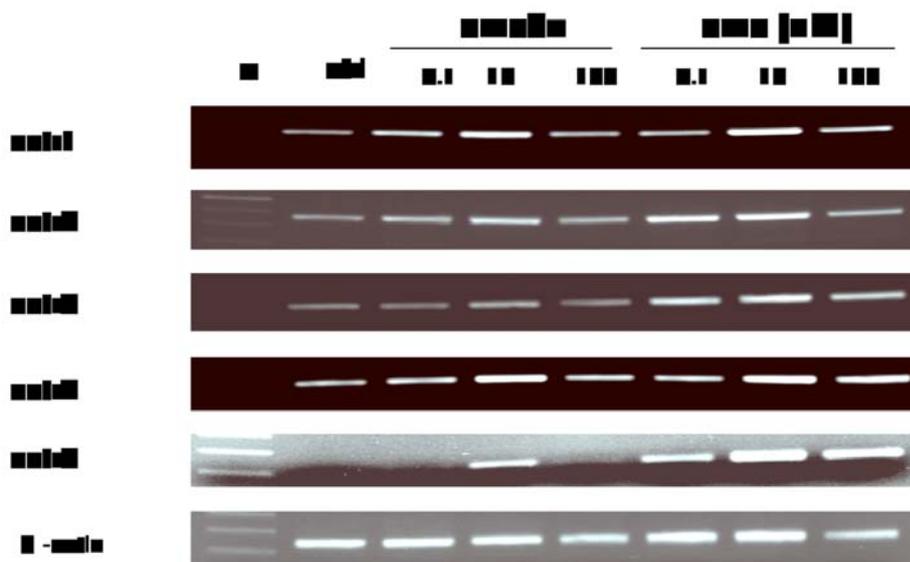


Figure 8. *Sms* and *Smsdx* induce up-regulation of SSTR mRNA expression in LNCaP cells. The cells were treated with either *sms* or *smsdx* for 72 hours and then harvested for analyses of SSTR mRNA by using RT-PCR. M: Molecular Marker and C: Control cells without *sms* or *smsdx* treatment.

Interestingly, SSTR5 mRNA is absent in untreated cells, but appeared after 3 days treatment with *sms* or *smsdx*. Both *sms* and *smsdx* were most efficient at 10 nM. SSTR5 mRNA was undetectable in LNCaP cells, although 5-aza and TSA failed to stimulates SSTR5 expression in LNCaP cells, treatment either with 5-aza or TSA led to a significant induction of SSTR5 mRNA in LNCaP-r cells, a hormone-resistant sub-

line. The combination of two compounds had additive effect on the transcriptional activation of the gene (Fig. 9).

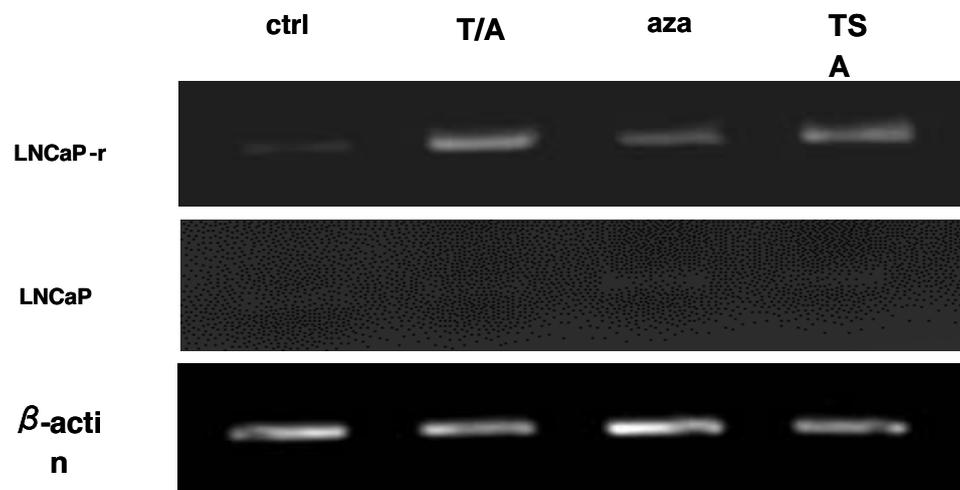


Figure 9. DNA methylation and histone deacetylase inhibitors stimulate SSTR5 mRNA expression in LNCaP-r cells. The cells were treated with 5'-aza (A), TSA (T), and A plus T, as indicated and then harvested for analyses of SSTR5 mRNA expression by using RT-PCR.

5. DISCUSSION

During androgen-independent progression, prostate cancer cells develop a variety of cellular pathways to survive and flourish in an androgen-depleted environment (e.g., AR gene amplification and enhanced sensitivity) (7). The transition of prostate cancer from androgen dependent (ADPC) to androgen independent (AIPC) and hormone refractory (HRPC) making anti-androgen therapy ineffective remains a significant clinical problem. Chemotherapy has only limited efficacy in HRPC (146, 147).

Treatment with sms analogues represents an alternative approach in prostate cancer management. The insulin-like growth factors (IGFs) are integral components of multiple systems controlling both growth and metabolism. For example, the IGFs play a critical role in both cell cycle control and apoptosis. Apparently, GH dependent and independent production of the IGF family is involved in the pathological growth of prostate cancer cells (148, 149). IGF-1 can stimulate prostate cancer cell growth. This mitogenic effect is modulated by IGF-binding proteins. Androgen withdrawal induces neuroendocrine differentiation of prostate cells which secrete neuropeptides and IGF-1, two epigenetic mechanisms of tumor resistance (148). One of the mechanisms of sms actions is related to a reduction of available IGF-1. The use of sms analogues as monotherapy in patients with HRPC has produced only modest clinical responses (150). However, Koutsilieris et al (2001, 2004) recently developed a therapeutic “combination concept” i.e. combining sms with other drugs, aiming at suppressing the bioavailability of IGF-1 and downstream biological effectors. A potential mechanism for the efficacy of this combination regimen involves the abrogation of the protective effect of IGF-I on prostate cancer cells. This specific approach was applied recently in the setting of androgen independent prostate cancer and involved administration of a sms analogue and oral dexamethasone in combination with luteinizing hormone-releasing hormone analogues (LHRH-A), providing encouraging preliminary results in HRPC (151-153).

Reubi et al (1996) first detected, via *in situ* hybridization studies, that prostate cancer preferentially expressed the SSTR1 subtype compared with the SSTR2 or SSTR3 subtypes (154). Kosari F (2008) found that SSTR1 is associated with systemic progression in prostate cancer after comparisons of gene expression profiles obtained

from the epithelial cells of non-neoplastic tissue, primary tumor, and metastatic tumor samples (155). SSTR1 was found to be the most prominent candidate prognostic biomarker in aggressive prostate cancer. Sinisi (1997) reported that SSTR1 was expressed only in epithelial cells from prostate cancer, SSTR2 was found only in epithelial cells from normal prostate. SSTR3 mRNA was undetectable in normal and cancer EC, whereas SSTR4 and SSTR5 were present in epithelial and stromal cells (156). Activation of phosphotyrosine phosphatases (PTPs) by SSTRs represents one of the main intracellular mechanisms involved in the antiproliferative effect of sms analogues (157). So far, three family members (SHP-1, SHP-2 and DEP-1/PTP η) have been identified as selective SSTR intracellular effectors to transduce antiproliferative signals. The mitogen activated protein (MAP) kinase cascade represents one of the major regulators of cell growth by hormones and growth factors. Effects of sms on the MAP kinase cascade are regulated through SSTR1 (158, 159). The activation of SHP-2 by SSTR1 may mediate the antiproliferative activity of sms. The antiproliferative activity mediated by SHP-1, following SSTR2 stimulation, is dependent on the inhibition of the entry in the S phase of the cell cycle and accumulation of the cells in G1, through the over-expression of p27kip1 and increase of hypophosphorylated the retinoblastoma gene product (Rb) (160). RKIP (down regulated by smsdx in the current study) inhibits the MAPK signaling pathway, which is also inhibited by activation of SSTR2, and SSTR subtypes 3 and 5 (161, 162). Interaction between human SSTR1 and SSTR5 also affects some functional properties (163). For example, activation by ligand induces SSTR dimerization, can alter the functional properties of the receptor such as ligand binding affinity and agonist-induced receptor internalization and up-regulation. Probably, crosslinking between SSTR1 and SSTR5 can be of importance for the efficacy of smsdx.

To date the SSTR subtypes involved in the induction of apoptosis are considered to be SSTR2 and SSTR3 (164), acting through the modulation of the intrinsic intracellular apoptotic pathways. This pathway leads to apoptosis *via* the activation of pro-apoptotic genes (i.e. p53, members of the Bcl2 superfamily) in responses to alteration of mitochondrial activity (164). Susini et al found that TCTP antagonizes apoptosis by inserting into the mitochondrial membrane and inhibiting Bax dimerization (165). So deregulation of TCTP by sms/smsdx in the current study suggested its involvement in the sms/smsdx cytostatic effect via mitochondrial-mediated pathway. Induced expression of SSTRs and regulation of certain proteins

(e.g., RKIP, VDACS) by sms/smsdx in the present study suggest that sms/smsdx exerts its effects on prostate cancer cells via MAPK pathway and by regulating the activities of phosphotyrosine phosphatases (PTPs).

Marked quantitative differences were observed in the protein expression profiles in sms/smsdx treated LNCaP and DU145 cells compared to the control cells. One third of the detected proteins were differentially expressed (PRDXs, hnRNPs). Concordance in protein expression patterns were observed between smsdx and sms treated cells with strong agreement between the up/down regulation of proteins indicating preservation of sms effects of smsdx. Based on these identified proteins affected by sms/smsdx, we classified the proteins into different functional groups. The proteins were categorized on the basis of their known biochemical functions including metabolism, signaling transduction, maintenance of cell structure, cell-cell interactions, transport/trafficking, transcriptional regulation and cell cycle regulation. The findings implicate broad effects of smsdx on the prostate cancer cell proteome. Catalytic mitochondrial and mitochondrial-associated proteins were significantly affected. The treatment triggered up-regulation of the catalytic mitochondrial proteins. Apoptotic related proteins were also affected. Apoptotic cell death can be induced by a number of triggers released from the mitochondrial outer membrane (e.g. cytochrome c, Smac/Diablo). The release can be blocked by over-expression of Bcl-2 (166). Several of the proteins involved in the MMP event which is a critical event in the process leading to chemotherapy induced apoptosis. The VDAC-Bax complex promotes pore formation, outer MMP, and the release of AMPs to activate caspases and induce apoptosis. VDAC1 has been shown to co-immunoprecipitate with the anti-apoptotic protein Bcl and suggested to be involved in the forming of the mitochondrial pore which release cytochrome c during apoptosis (167). From the present results it seems reasonable to assume that sms/smsdx affects the mitochondria of LNCaP in a way that eventually triggers mitochondrial-mediated apoptosis. The regulation of VDAC1 and VDAC2 was reported to be involved in mitochondria-dependent apoptosis (168). VDAC1 has a higher expression, VDAC2 lower expression in LNCaP than in DU145, both up regulated by sms. Because VDACS were expressed more highly in cancer cell lines than in normal cell lines, it could be appropriate to develop novel anti-cancer drugs that pharmacologically target the VDACS. Lower level expression of VDAC1 and high level expression of VDAC2 in DU145, seems to give be resistance to mitochondria-

dependent apoptosis. Here the regulatory mechanism of sms/smsdx needs further investigation.

Different androgen dependent prostate cancer cells show different biological characteristics. Androgen dependent prostate cancer cells (LNCaP) maintain several characteristics of human prostatic carcinoma, such as the dependence on androgen, the presence of androgen receptors, the production of acid phosphatase (169) and the secretion of prostate-specific antigen (PSA). While androgen independent prostate cancer cells (DU145) are unresponsive to androgens and lacking PSA expression. AR has a central role in prostate cancer progression, representing a final common pathway for “the HRPC mechanisms”. Most probably there are a number of proteins specifically involved in the transition from hormone dependence to hormone independence which are regulated by androgens and its receptor. The characterisation of the androgen response may provide useful biomarkers/drug targets, and increasing the understanding of the molecular events leading to androgen independence. Through differential proteomic analysis, a number of proteins were identified, e.g., RKIP, HSPD1, PRDXs, hnRNPs. RKIP regulates activation of MAPK, NF-kappaB and G protein coupled receptors (GPCRs) (170). RKIP is a major androgen-dependent protein described previously in the rat ventral prostate (171). Its level was found to be reduced or absent in variants of established cell lines derived from metastatic prostate cancer (172). Low RKIP levels in DU145 were suggested to be correlated with enhanced extracellular signal-regulated-kinase (ERK)/MAPK pathway activation. It was found with a higher level expression in LNCaP than in DU145, which was down regulated by sms in LNCaP. This suggests that RKIP has a role in the transition from androgen dependency and could be a potential drug target.

The biological role of HSP60 (HSPD1) and its possible use as a marker in carcinogenesis has been explored previously. It was found that heat-shock protein expression independently can predict the clinical outcome in prostate cancer (173). HSP60 was significantly up-regulated in both early and advanced prostate cancer when compared to normal prostatic epithelium. HSP60 was found more abundant in LNCaP-r (LNCaP derived sub-line, hormone independent) (66), despite the presence of an AR incapable to mediate stimulation of proliferation. HSPD1 was up regulated by both sms and smsdx in DU145 cells in the current study. Since it is known that

HSPs binds to and inactivates ARs, one could assume that increased concentration of HSP60 may block the binding of androgens to the receptor.

In PRDXs family, PRDX2 is known to protect cells from oxidative damage and to confer resistance to oxidative damage to cancer cells (174). PRDX2 was reported higher expression in highly metastatic prostate cancer cells (175). PRDX2 and PRDX3 have a higher expression in DU145 compared to LNCaP. PRDX2 expression was down regulated and PRDX3 up regulated by both sms and smsdx. The different regulating manners by sms/smsdx in PRDXs are thought to be related to phosphorylation, overoxidation and proteolysis of PRDXs. The roles of PRDXs in regulating levels of hydrogen peroxide, an intracellular signaling molecule common to many cytokine-induced signal-transduction pathways, possibly lead to different expressions in LNCaP and DU145 cells.

The hnRNPs family has a central role in DNA repair, telomere biogenesis, cell signaling and in regulating gene expression at both transcriptional and translational levels. The hnRNPD and hnRNPC have lower expression up to 2-fold change, higher expression with hnRNPH1, hnRNPA2B1 in DU145 cells compared to LNCaP cells. The hnRNPs were regulated by sms/smsdx in different manners, sometime up sometime down. Different hnRNPs member functions as regulator of tumourigenesis especially relating to different events of tumour development and progression such as inhibition of apoptosis, proliferation, invasion and metastasis (176). The different expressions of hnRNPs are thought to be related to the different aggressive and metastatic abilities between LNCaP and DU145 cells.

Different protein isoforms due to the posttranslational modifications showed different expressions and modulations by sms/smsdx. These proteins also involved in a number of cellular processes including proliferation and apoptosis. The differentially expressed proteins might help to elucidate the underlying transition mechanism from prostatic androgen dependence to androgen independence.

Smdx has an average of ~ 3 sms14 conjugated to the dextran70 backbone, retains the biological properties of sms and the affinity to the SSTR subtypes together with high *in vivo* stability. Rens-Domiano (1991) found that sialic acid residues in the SSTRs carbohydrate α 2, 6-configuration have a role in maintaining the receptor in a high

affinity state for agonist binding (177). If the lysine sidegroups of sms were substituted with non charged sidegroups, the peptide's binding affinity to SSTRs was greatly reduced. In general, the sialic acid residues are oriented towards the extracellular surface of the cells. It is conceivable that differences in the carbohydrate processing may contribute to the physical heterogeneity of the SSTRs. The cationic charge of the smsdx conjugate could contribute to the conjugate's ability to bind to the SSTR's through electrostatic interaction with the negatively charged sialic acid residues exposed by the SSTR's.

Zapata (2002) found that sms inhibits cell proliferation in LNCaP and PC-3 cells through an autocrine/paracrine somatostatin loop which acts by SSTR2 and/or SSTR5 (178). This antiproliferative effect could be mediated by SHP-1 in prostatic cells and regulated, both short and long term, by endogenously secreted sms. It might be so that smsdx, having pan affinity, could trigger all five SSTRs at the same time, possibly inducing a potent effect on the prostate cancer cells e.g. via SSTR1-SHP-2 and/or SSTR2-SHP1 activated PTPs pathways. This could explain the higher protein fold change seen with smsdx compared to sms in certain proteins. These proteins are believed to be involved in the processes of proliferation, apoptosis and the development of androgen-independent disease in LNCaP and DU145 cells (e.g., heat shock protein family (HSPB1, HSPA4, HSPA9), hnRNP family (hnRNPC1/C2, H, D0), TCTP, GRP78 and several mitochondrial proteins).

An increased induction of mRNA expression of all five SSTR subtypes was observed in the LNCaP cells when treated with sms/smsdx (dose dependent). The result indicates a positive feedback loop between sms and its receptors. This regulation pathway may enhance the antitumor activity of somatostatin. Inhibition of DNA methylation and histone acetylation resulted in up regulation of SSTR5 mRNA expression, indicating the involvement of DNA methylation and histone acetylation in controlling SSTR5 expression. It is reasonable to assume that this may increase the direct tumor cell sensitivity to sms/smsdx treatment, in addition to the effects of the DNA methylation/HDAC inhibitors.

In the clinical setting, enhanced treatment sensitivity as a result of increased SSTR expression, will depend on the dose, dose frequency and affinity profile of the sms, as well as the duration of sms treatment. Epigenetic manipulation combined with sms

treatment, may offer a novel alternative for the treatment of advanced prostate cancer. Different combinations of sms analogues have been suggested in the clinical management of prostate cancer (70, 74). The exact protocol for such combination therapy remains to be determined.

In conclusion, the results show that sms/smsdx regulates important proteins of the proteome in prostate cancer cells in different prostate cancer androgen dependency status indicating a potential for somatostatin treatment. Regulation of SSTRs by different sms analogues treatment protocols and epigenetic manipulation in combination with sms might be a possibility to enhance treatment sensibility for somatostatin therapy. As indicated from recent studies, sms analogues treatment needs probably to be combined with other treatment modalities to maximise the efficacy in the management of HRPC.

6. SUMMARY AND CONCLUSIONS

- I. Smsdx can regulate the protein expression of the prostate cancer cell line LNCaP in a sms like manner. Sms like protein regulating effects are preserved in smsdx.
- II. Sms/smsdx induced up-regulation of catalytic mitochondrial proteins and affected apoptosis-related proteins.
- III. Differential expressions of protein were found in hormone dependence and hormone independence prostate cancer cells after sms and smsdx treatment.
- IV. SSTR expression was up-regulated by sms/smsdx and by DNA methylation and HDAC inhibitors. This might increase the tumor sensitivity to sms treatment in addition to the effects of the DNA methylation/HDAC inhibitors.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Karolinska Institutet and Karolinska Hospital for supporting me to perform my study, and everyone who aided, supported and inspired me during the past years. I would especially like to thank:

My supervisor Professor **Sten Nilsson**, for providing me the opportunity to work in your lab and for sharing your genuine interest in prostate cancer research. Thank you for your generosity, kindness and never ending support.

My co-supervisor Dr. **Anders R Holmberg**, Thank you for the meticulously thorough work, amazing quick e-mail replies and endless support with my daily lab work. I'd like to say I couldn't finish my study without your encouragement and support over the years. You are an excellent teacher, teaching me all I know about scientific writing.

Dr. **Ayodele Alaiya**, for your never-failing encouragement, friendship, fully support and sharing your wealth experience with 2-DE.

Dr. **Marcela Márquez**, for your unending support, scientific instructions, constructive suggestions and friendship.

My collaborators at Lund University, Professor **Peter James**, Dr. **Sofia Bengtsson**, and Dr. **Mårten Krogh** for your indispensable support & expertise in protein identification and special thanks to you **Sofia!**

Professor **Anders Zetterberg**, for your enthusiasm for scientific research.

Professor **Håkan Mellstedt** and Professor **Tina Dalianis**, the head of Department of Oncology-Pathology, for creating excellent and comfortable working environment.

Dr **Dawei Xu**, for your vast knowledge and wealth experience in scientific researches.

Drs **Li Chunde, Liu Tao, Zheng Chengyun, Zhang Anju, Xie Yuntao, Liu Cheng, Luo Juhua** for your help and friendship.

My Chinese friends in KI, **Sheng Jinfeng, Ma Suhua, Zhao Jian, Liu Tong, He Xiaobing, Zhu Chaoyong, Yin Shucheng, Zhang Junhang, Liu Anquan, Niu Xianzhi** for help and friendship.

My CCK colleagues: **Lena Lennartsson, Margareta Rodensjö, Lisa Anfalk, Sören Linden, Juan Castro** for help and friendship. **Eva Gripenholm** for your help and arrangement of my salary and agreement.

My friends in nursing field, **Alice Rinell Hermansson, Barbro Gustafsson, Barbara Parfitt** for friendship and successful collaborations.

Professor **Zhan Tao**, the President of Jilin University, the former President of Shandong University, I still keep the word in mind *a great day for Shandong University*. Professor **Meng Qingyue**, the present head of school of public health, Professor **Cheng Lin**, the famous scientist in the field of Energy and Power Engineering, Professor **Wang Haiyang**, the head of Shandong University Network, the elite of Shandong University, for friendship and encouragement.

Fang Xiaolei, Ming Yu, for introducing me to the scientific world and giving me selfless family support during my study in Sweden. **Fan Yidong, Zou Qianli**, for your optimism and encouragement.

Zhao Shengtian, the head of Second Hospital of Shandong University, for your encouragement and friendship.

My Chinese friends, **Lu Ming, Cui Baoxia, Li Xiaomei, Zhang Xi, Cheng Baoquan, Shangguan Hong, Jia Congqi, Lei Gefei, Ge Nan, Meng Yan, Zhu Qinjun** for nice talks and parties. **Wang Wj** for nice talks, support and encouragement.

All the colleagues of Anders Zetterberg's group, Klas Wiman's group, Olle Larsson's group, for support.

My beloved family, my wife **Xuan** and my daughter **Yuyu**

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