Antibody Microarray Analysis of Signaling Networks Regulated by Cxcl13 and Cxcr5 in Prostate Cancer

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Abstract

Advanced prostate cancer (PCa) often spreads to distant organs, leading to increased morbidity and mortality. It is now well established that chemokines and their cognate receptors play a crucial role in the multi-step process of metastasis. We have previously identified CXCR5 to be highly expressed by PCa tissues and cell lines and its specific ligand, CXL13, is significantly elevated in the serum of patients with PCa and differentiated PCa cases with other benign prostatic diseases. CXL13:CXL13 interactions promote PCa cell invasion, migration, and differential matrix metalloproteinase (MMP) expression. Thus, it is important to understand the molecular and cellular processes that mediate these events. In this study, we quantified changes in apoptosis, cell cycle, and cytoskeleton rearrangement of these factors integrate their signal transduction activities and converge into the Ras/MAPK cascade during progression to advanced PCa [5].

Introduction

Prostate cancer (PCa) represents a major cause of cancer related morbidity and mortality worldwide and it is now recognized as one of the most important medical problems facing the male population. Complex signal transduction pathways exist in prostate carcinomas relative to normal prostate epithelial cells [1]. Prostate tumors have extensive morphological heterogeneity, which underlies their molecular and biological complexity [2,3]. However, the mechanisms triggering aberrations in PCa cell signal transduction remain largely unknown. In general, cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. Weinberg et al. described prostate tumorigenesis from observations of broader systemic structure of cancers. They established that six essential alterations in PCa cell physiology ultimately mandate malignant growth: i) self-sufficiency in growth signals, ii) insensitivity to growth-inhibitory (antigrowth) signals, iii) evasion of programmed cell death (apoptosis), iv) limitless replicative potential, v) sustained angiogenesis, vi) and tissue invasion and metastasis [4]. As PCa progresses, it exhibits increased expression of growth factors (epidermal growth factor, transforming growth factor α, keratinocyte growth factor, basic fibroblast growth factor, insulin-like growth factor-I) and/or their cognate receptors [5]. Additionally, these factors integrate their signal transduction activities and converge into the Ras/MAPK cascade during progression to advanced PCa [5].

Androgen deprivation is an initial treatment option for PCa [6]. However, this therapy becomes ineffective when the tumor progresses to androgen-independent [7,8]. PC3 cell lines are extensively used models to study of cellular signaling that may occur during androgen-independent (advance) PCa progression [9,10]. PC3 cells are androgen-independent and the acquired hormone-refractory properties of this cell line have been linked to its high skeletal metastatic potential. Hence, this model cell line has been used to provide important insights into the cellular events involved in advance PCa. The mechanisms underlying hormone-independent growth of PCa cells involve alterations in the androgen receptor, related regulators of transcription, and the emergence of growth factors that replace signals normally regulated by androgens in the prostatic epithelium [11]. Potentially, hormone refractory PCa cells use chemokines as growth factors to survive and proliferate in the absence of androgens [11]. In recent years, chemokines are among the most cited molecules in cancer research, most likely because they play pivotal roles in homing and directional migration of chemokine receptor-bearing tumor cells to target organs where corresponding ligands are expressed [12,13].

Chemokine receptors are known to feed into the Ras/MAPK signaling pathway by transactivating growth factor receptors, which are members of the receptor tyrosine kinase family [14]. Collectively, these signaling events lead to tumor survival and proliferation. We previously demonstrated that CXCR5 is expressed by PCa cell lines and highly correlated with advanced disease [15] and CXL13 is significantly

Keywords: Akt; Cdk; CDKN1B; Integrin-β3; Fak; Src; Paxillin

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Materials and Methods

Cell lines and culture

The PCa PC3 cell line (ATCC CRL-1435) was derived from a bone metastasis of a grade IV prostatic adenocarcinoma patient, and was cultured in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and maintained in a cell culture incubator at 37°C in a humidified atmosphere with 5% CO₂. Cells were serum starved overnight prior to stimulation with human recombinant CXCL13 (Peprotech, NJ, USA).

Phosphorylation-specific antibody microarrays

Phosphorylation-specific antibody microarrays (Fullmoon Biosystems Inc., Sunnyvale, CA, USA) were used to monitor the up-and-down-regulated proteins in PC3 cells following CXCL13 stimulation. One array was printed with 95 antibodies related to proteins involved in cytoskeletal pathways. A second array was customized to include 248 antibodies related to proteins involved in tumorigenesis. The array layout consisted of antibodies against phosphorylated- and unphosphorylated-proteins, each replicated six times, where actin and GAPDH served as controls. Microarrays were hybridized with biotinylated proteins from PC3 cell lysates, treated with CXCL13 (100 ng/ml for 5 minutes); untreated cells served as controls. Following protein conjugation to antibody, Cy3-streptavidin was added and slides were processed following manufacturer's instructions, and scanned using Axiom GenePix 400B microarray scanner (Molecular Devices). Ratios of phosphorylated to unphosphorylated proteins were calculated from the intensity values obtained. Results were divided into datasets and uploaded into Ingenuity Pathways Knowledge Database as text files containing GenBank accession numbers.

Data analysis

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems; www.ingenuity.com) was used to analyze datasets. This application condensed the large dataset generated from the microarrays into networks, enabling visualization of overall molecular events, and determined the connectivity of molecules and their relevant association within each identified network. Scores for each network were computed based on the fit of the dataset. Score was derived from a p-value and indicates the probability of the involvement of focus molecule(s) in a given network. These networks were then presented as a graph indicating how molecules interact. Biological functions were calculated and assigned to each network in order of significance. Canonical pathways analysis identified pathways from IPA libraries. Fisher's Exact test was used to calculate p-values and determine the probability that the association between the protein in the dataset and the canonical pathway did not occur by chance alone.

FACE assay

Fast Activated Cell-based ELISA was performed to quantify and confirm phosphorylated-total proteins (Src, ERK, FAK, and PI3K) in PC3 cells following CXCL13 stimulation. The assay was performed using FACE kit (Active Motif, Carlsbad, CA, USA) following manufacturer’s instructions. Briefly, PC3 cells cultured in 96 well plate (5 × 10⁵ cells in each well) were serum-starved overnight and stimulated with 100 ng/ml of CXCL13 (Peprotech, NJ, USA) at two different time points (5 min and 10 min). Untreated wells served as negative control. Following stimulation, the cells were rapidly fixed in 4% paraformaldehyde (prepared in phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄, pH 7.4) for 20 min to preserve activation-specific protein modifications. Each well was then incubated with antibody blocking buffer for 1 h followed by incubation with primary antibody phospho-c-Src/ERK/FAK/PI3K that recognizes the respective phosphorylated proteins. The same procedure was repeated using antibodies against total-c-Src, ERK, FAK, and PI3K to quantitate the respective total proteins irrespective of phosphorylation. Following washing, the cells were incubated with HRP-conjugated secondary antibody for 1 h. Chemiluminescence was read using a SpectraMax luminometer (Molecular Devices, Downingtown, PA, USA). Once the chemiluminescence was recorded, the relative number of cells in each well was then determined using the provided Crystal Violet solution. Cells were stained for 30 min and the absorbance was read using a microplate spectrophotometer at 595 nm. The OD₅₉₅ value obtained indicated the relative number of cells in each well, which were later used to normalize results relative to the number of cells. Experiments were performed in triplicate and results show the ratio of active (phosphorylated) to total proteins normalized to cell density per well.

Results and Discussion

Quantitative analysis of regulatory proteins differentially phosphorylated following CXCL13 treatment of PC3 cell lines

To understand functional relationships and mechanisms of differential alteration in protein phosphorylation in response to CXCL13 and to derive probable CXCL13-modulated signaling pathways in CXCR5-bearing PCa cells, we used IPA software. The analyzed antibody microarrays contained 343 different antibodies representing markers for biological pathways including apoptosis, cell cycle, signal transduction, cytoskeleton and cancer. These arrays were used to monitor changes in protein expression and activation patterns of prostate tumor cells following stimulation with CXCL13. Results indicated that the proteins analyzed from CXCL13-treated PC3 cells clustered into 8 different networks. Networks that received the highest scores are listed in Table 1. These networks mostly correlated with functions relevant to cancer, cell cycle, cellular movement, cell morphology, and reproductive system diseases. Focus molecules in each network represent proteins on the array exhibiting increases in phosphorylation. The networks are displayed graphically as nodes (individual proteins) and edges (biologic relationships between the nodes). All edges are supported by at least one literature reference of direct physical, transcriptional and enzymatic interactions or from canonical information stored in the Ingenuity Pathways Knowledge Base.
CXCL13-mediated Akt1/2-Cdk1/2-CDKN1B pathway in PCA cells

The graphical display of networks allows for visual assessment of function, biological mechanism, and relevant interactions among proteins. Proteins that occupied focal positions in the top regulatory networks included Akt, Cdc2 (Cdk1), Cdk2, and CDKN1B in network 1 (Figure 1). These proteins belong to networks of cell-division cycle and growth, cytoskeleton, and cancer. Importantly, CXCL13 was found to regulate cyclins, Cdns, and Cdk inhibitors. These molecules are key modulators of the tightly controlled cell-division cycle and are known to interact and form stable (inactivated) complexes [18]. The

Network ID | Molecules in Network | Scores | Focus Molecules | Top Functions
--- | --- | --- | --- | ---
1 | AKT1, AKT2, RTK, AMPK, ATM/ATR, BRCA1, CDC2, CDC25A, Cdc25B/C, CDC25C, CDK2, CDKN1B, CHEK1, CHEK2, Cyclin A, Cyclin B, Cyclin D, Cyclin E, E2F, FoxO1, FoxO3, IGF, Laminin, LIMK1, MAP2K2, MAP2K3, MEK2, MEK6, mTOR, PRKAA1, RAF1, RB1, Rb, SRC, SRC1, STK11 | 31 | 19 | Cancer, Cell cycle
2 | Actin, Alpha Actinin, Beta Arrestin, Calpain, CAV1, CFL1, Cofilin, Collagen(s), CTNNB1, Dynamin, Erm, EZR, F Actin, FAK-Src, FGFR1/2, GSK3B, Integrin alpha V beta 3, KRT18, MAP2K1/2, NF2, NTRK2, Pak, phosphatase, PTEN, PTK2, PXN, Rac, Ras homolog, Rock, SRC, Talin, VASP | 26 | 20 | Cellular Movement, Cell Morphology
3 | AKT1, ALP, Calmodulin, Calmodulin II, Caskase, CDK1, CK2, CREB1, CTNNB1, Cytochrome c, ERBB2, ESRR1, FSH, GLRX2, HDAC8, Histone h3, Histone h4, Hsp70, HSP60-2, HSP90AB1, ICAM1, JUN, Nfat, PDPK1, Pp2b, Proteasome, RNA polymerase II, Rxr, Smad, SYN1, TFIIH, Tubulin, YWHAZ | 21 | 14 | Cancer, Reproductive system disease

Table 1: Highest scoring networks involved in CXCL13-treated metastatic prostate cancer.

Phosphorylation-specific antibody microarrays were used to monitor active and inactive protein levels in PC3 cells following CXCL13 stimulation. The resulting profiles were analyzed and clustered in eight different networks and the focus molecules of each network are written in bold. Score was derived from a p-value and indicates the probability of the involvement of focus molecule(s) in a given network. Focus molecules in each network represent proteins on the array exhibited increases in phosphorylation.

Figure 1: CXCL13 regulates key molecules involved in cell cycle. Phospho-specific antibody microarrays were separately hybridized with CXCL13-treated or untreated PC3 cell lysates. Ratios of phosphorylated to unphosphorylated molecules were calculated and the datasets uploaded into the Ingenuity Pathways Analysis application. Networks were algorithmically generated based on molecules’ connectivity. Results were normalized to GAPDH levels. Colors represent fold changes in phosphorylation. Gray indicates no change in phosphorylation status, green indicates decreased phosphorylation, pink indicates baseline phosphorylation, and red indicates increased phosphorylation relative to baseline.
phosphorylation of Cdk inhibitors causes their dissociation and triggers their ubiquitination and degradation by proteasomes. This mechanism releases cyclins and Cdns enabling them to be activated through phosphorylation that results in the initiation of DNA replication [19]. The progression from G1 to S phase for initiation of DNA synthesis is principally regulated by the cyclin-E/Cdk2 complex [20]. This complex is inactivated by the Cdk inhibitor, CDKN1B (also known as p27/Kip 1), which is inactivated by phosphorylation [21]. Our results show that both Cdk2 and CDKN1B are phosphorylated following CXCL13 stimulation of PC3 cells, and that they constitute a focal point in the cell cycle network. This suggests that CXCL13 potentially regulates cell cycle progression from G1 to S phase in PCa cells by inactivating CDKN1B and activating Cdk2. Furthermore, Cdk1 was shown to be phosphorylated following stimulation of PC3 cells with CXCL13. Besides its major role in cell division cycle, Cdk1 has been implicated in PCa cell invasion and correlated with a motile phenotype of cancer cells promoted by integrin activation [22]. Indeed, our array results established CXCL13-driven integrin and Cdk1 phosphorylation implying that CXCL13 could support motility and/or proliferation of PCa cells.

**Integrinβ3-Src-PXN pathway mediated by CXCL13 PC3 cells**

Integrin-β3, Src, Paxillin, and Rac protein occupied focal positions in network 2 (Figure 2). The phosphorylation of kinases and adaptor molecules central to cell migration includes FAK, Src, integrins, paxillin, and Rac. Cell motility is a complex process requiring the integration of diverse signaling cascades. The initial step of transducing extracellular signals through integrins to the cytoskeleton requires activation of the tyrosine kinases – Src and FAK [23]. The recruitment of FAK to integrins leads to its autophosphorylation and enables it to bind the SH2 domain of Src. In turn, Src phosphorylates FAK on multiple residues that increase FAK kinase activity. The active Src/FAK complex can now phosphorylate several downstream binding partners including PXN. Phosphorylated PXN is implicated in Rac activation and stimulation of cell motility [24]. Thus, the ability of CXCL13 to activate molecules critical for cell motility indicates that CXCL13/CXCR5 interactions can contribute to the metastatic process by regulating PCa cell migration. Besides its central role in cell motility, the integrinβ3/Src/FAK complex also regulates tumor cell growth by activating the JNK signal transduction pathway [25]. JNK phosphorylates several members of the JNK family, including p38 and Elk-1, which play important roles in the regulation of cell proliferation, differentiation, and apoptosis.

Figure 2: CXCL13 regulates key molecules involved in cell migration. Phospho-specific antibody microarrays were separately hybridized with CXCL13-treated or untreated PC3 cell lysates. Ratios of phosphorylated to unphosphorylated molecules were calculated and the datasets uploaded into the Ingenuity Pathways Analysis application. Networks were algorithmically generated based on molecules’ connectivity. Results were normalized to GAPDH levels. Colors represent fold changes in phosphorylation. Gray indicates no change in phosphorylation status, green indicates decreased phosphorylation, pink indicates baseline phosphorylation, and red indicates increased phosphorylation relative to baseline.
of the jun/fos family, causing increased transcriptional activity and reduced degradation of AP-1 proteins, which are important for the induction of cell cycle progression [26]. Our results show that CXCL13 stimulation of PC3 cells results in c-jun phosphorylation in addition to activation of integrin signaling components suggesting a potential role in PCa cell growth.

**CXCL13-mediated Akt-Jun-CREB1 signaling cascade by PC3 cells**

Proteins that comprised focal positions in the regulatory networks also included Akt, Jun, and CREB1 in network 3 (Figure 3). Indeed, our data confirms inactivation of phosphatase and tensin homolog (PTEN) in PC3 cells. PTEN point mutations or deletions are extensively present in PCa and contribute to tumorigenesis [27,28]. Loss of PTEN leads to uncontrolled phosphatidylinositol (3,4,5) triphosphate (PIP3) formation catalyzed by PI3K in response to stimulus. This causes sustained activation of Akt which phosphorylates substrates involved in oncogenesis including CREB [29]. CXCL13 was a potent activator of Akt and subsequently CREB in PC3 cells. However, it has been recently reported that the oncogenic phenotypes of PTEN inactivation are not attributed to active Akt alone [30]. JNK belonging to the mitogen-activated protein kinase (MAPK) signaling pathway family has been identified to function in parallel with but independent of Akt and its activation highly correlates with PCa progression [31]. Our results point to CXCL13-mediated JNK phosphorylation in PC3 cells. Understanding the important function of MAPK signaling pathways in tumor growth and proliferation, one could speculate that CXCL13 also acts as a growth factor to regulate PCa cell survival and expansion. Indeed, a role for CXCL13 in promoting malignant cell survival and growth has been previously described by Husson et al. [32].

**Canonical pathway analysis and interpretation of biologically and disease relevant functions**

At least 50 different proteins from PC3 samples showed a notable increase in phosphorylation in response to CXCL13 stimulation compared to untreated samples. These proteins were parsed into biologically relevant functions and diseases and were found to be most significantly associated with tumor growth (Table 2). A detailed canonical pathway analysis followed, and revealed the major signaling pathways regulated by CXCL13 in PC3 cells. Our results identified PI3K/Akt and SAPK/JNK as pathways to be largely modulated by CXCL13 (Figure 4). These two signaling pathways were merged and overlaid with the analyzed microarray data from CXCL13-treated and untreated PC3 cells and show common molecules belonging to PI3K/
Phosphorylation-specific antibody microarrays were used to monitor active and inactive protein levels in PC3 cells following CXCL13 stimulation. The resulting profiles were analyzed and clustered into known biological functions and diseases to better understand the underlying mechanisms involved in CXCL13-induced signaling molecules in prostate cancer. The p-values were calculated using Fisher’s exact test and indicate the probability of the involvement of these molecules in a given network associated with biological functions and diseases.

### Table 2: Proteins regulated by CXCL13 and their relevant biological functions in PC3 cells.

| Function                               | Proteins                                                                 |
|----------------------------------------|--------------------------------------------------------------------------|
| PI3K/Akt Signaling                     | AKT1, AKT2, BAD, BCL2, BCL2L1, CAV1, CDC2, CDK2, ELK1, JUN, MAPK3, MAPK8, NF2, PTK2, RAF1, SRC, STMN1 |
| SAPK/JNK Signaling                     | GJAR1, ITGB3, JUN, JUNB, LIMK1, MAPK3, MAPK8, PDPK1, PTB2, RAF1, SRC     |
| ERK/MAPK Signaling                     | AKT1, AKT2, BAD, CAV1, GJAR1, ITGB3, JUN, JUNB, LIMK1, MAPK3, MAPK8, PDPK1, PTB2, RAF1, SRC, STMN1, VAV1 |
| Anti-Apoptosis                          | AKT1, AKT2, BAD, BCL2, BCL2L1, CAV1, CDC2, CDK2, ITGB3, JUN, MAPK3, MAPK8, PDPK1, PTB2, RAF1, SRC, STMN1, VAV1 |
| Prostate carcinoma                     | AKT1, AKT2, CDC2, CDK2, ITGB3, JUN, RAF2, SRC                            |
| Metastasis                             | AKT1, ITGB3, NF2, PTK2, RELA, SRC                                         |
| Cell cycle progression                 | BCL2, CAV1, CDC25C, CDK2, MAPK8, RAF1, VAV1                              |
| Survival of tumor cell lines           | AKT1, AKT2, BCL2, BCL2L1, CAV1, CDK2, CDKN1A, CDKN1B, CHEK1, CHEK2, CEB1, EGFR, ERBB2, FRAP1, JAK1, MET, NFKB1, NFKB2, NTRK2, PDGFRB, PRKAA1, PTB2, RELA, RELB, SRC, STAT3 |

Akt and SAPK/JNK signaling cascades (Figure 5). To this end, Src, Akt, Jnk, Bcl, and Jun phosphorylation were significantly elevated in CXCL13-treated PC3 cells, when compared to untreated controls. In addition, activated PI3K/Akt, Integrin-β3/Src/FAK, and DOCK2/JNK signaling pathways were combined into a single diagram to identify their connectivity to CXCL13 signaling, regulation, and integration downstream of CXCR5 (Figure 6). These results indicate that CXCL13 contributed to cellular signaling that regulates PCa metastasis, and could serve as a growth and/or cell survival factors for PCa cells.

PC3 cells are derived from advanced androgen-independent bone metastasis of PCa. The cells exhibit anchorage-independent growth; hence, these cells are resistant to anoikis, i.e., defined as extracellular matrix detachment-induced form of apoptosis [33]. The underlying mechanisms rendering PC3 cells resistant to cell death requires stimulation of cell survival and anti-apoptotic signals [34]. To this end, the Bcl2 family of proteins is essential in modulating the apoptotic process. Phosphorylation of Bad causes its release from Bcl2 and binding to 14-3-3 protein. Bcl2 is then able to maintain mitochondrial integrity and promote cell survival [35]. Increased expression of anti-apoptotic proteins Bcl2 and Bcl-xl, and decreased expression of pro-apoptotic protein BAD positively correlates with tumor growth and poor prognosis of PCa patients. Together, this is believed to contribute to resistance of hormone-refractory cells to apoptosis induced by androgen ablation [36]. Our results indicate that CXCL13 regulates Bcl2 (Serine 70), Bcl-xl (Serine 62), and BAD (Serine 112 and 136) activity by phosphorylation in PC3 cells demonstrating as such anti-apoptotic properties.

**CXCL13-mediated activation of SRC, FAK, PI3K, and ERK proteins**

Phosphorylated and total SRC, FAK, PI3K, and ERK were quantified by FACE assay in CXCL13 stimulated PCa PC3 cells, to
confirm the results obtained by protein array. The stimulated PC3 cells exhibited an increase in phosphorylated to total SRC, FAK, PI3K, and ERK ratios (Figure 7). Tyrosine kinases play a significant role in cellular signaling involving in PCa progression. Approximately 30 or more tyrosine kinases are known to express in a typical prostate cancer cell and interestingly most of them channel signals through a trio of non-receptor tyrosine kinases, namely Src, Etk, and FAK [37]. Here, CXCL13 signaling through CXCR5 elicited SRC/FAK complex and PI3K activation, which results in a number of signaling cascades, e.g., phosphorylating ERK and Akt, respectively. Importantly, ERK and Akt are necessary for PCa cell invasion and proliferation. A link has been established in previous studies between hyperphosphorylation of Akt and PCa cell survival [38] and hyperphosphorylation of ERK and PCa cell invasion and MMP expression [39,40].

### Concluding Remarks

Chemokines are a family of small, secreted proteins, which are often upregulated in variety of human cancers and PCa cell proliferation, migration, invasion, and survival. In this study, pathway and regulatory network analyses were conducted to understand the mechanistic action of one such chemokine, CXCL13 on the hormone refractive PCa PC3 cell line. The analysis was based on a large-scale phosphorylation-specific antibody microarray data. This procedure allowed for the global characterization of the expression of functional proteins, and offered an advantage over mRNA microarrays in that it accounts for a number of post- translational modifications of proteins that alters cell function [41]. Phosphorylation (catalyzed by kinases) and dephosphorylation (catalyzed by phosphatases) are major modifications a protein can undergo in response to stimuli and thought to regulate almost all aspects of cell life [42]. It is believed that close to 1000 protein kinases and phosphatases are likely to be encoded by the human genome and that reversible phosphorylation of these proteins affects their activity, degradation, and ability to translocate from one subcellular compartment to another [43]. Approximately 30% of cellular proteins contain covalently bound phosphate and abnormal levels of protein phosphorylation are a cause or consequence of major diseases, e.g., cancer [44].

In conclusion, this analysis strongly attributes CXCL13 as an important factor in various steps of the PCa progression. Indeed, cluster analysis was used to identify a group of proteins whose activation patterns attributed to CXCL13: CXCR5 interactions in PCa. This approach defined potential mechanisms involved in PCa development and revealed drug targets to better treat this devastating disease. On the basis of this analysis, we will direct our future studies to establish the biological and physiological relevance of the CXCL13-mediated differential regulation of signaling cascades and their implication in PCa progression.

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