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Identification of Metastasis-associated Genes in Prostate Cancer by Genetic Profiling of Human Prostate Cancer Cell Lines

LUTZ TROJAN[1], AXEL SCHAAF[1], ANNETTE STEIDLER[1], MARKUS HAAK[2], GEORG THALMANN[3], THOMAS KNOLL[1], NORBERT GRETZ[2], PETER ALKEN[1] and MAURICE STEPHAN MICHEL[1]

[1]*Department of Urology and*
[2]*Center for Medical Research, University Hospital Mannheim, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany;*
[3]*Department of Urology, University Hospital, University of Bern, Anna-Seiler Haus, Inselspital, Bern, Switzerland*

Abstract. *Objectives: Prostate cancer (PCa) is a heterogeneous tumour entity with known interindividual differences in biological behaviour regarding tumour aggressiveness and metastatic potential. To date, the prediction of the metastatic status of patients with PCa has not been possible. To identify the molecular causes behind these differences, the gene expression profiles of two cell lines (LNCaP and LNCaP C4-2) with different metastatic potentials were examined using DNA microarray technology. Materials and Methods: LNCaP and LNCaP C4-2 cells were cultured under standard conditions. RNA was isolated using Trizol® extraction. After processing the total RNA according to the manufacturer's instructions, we performed Affymetrix GeneChip analysis with HG-U133A chips. Data analysis was performed using NetAffx, dChip, GenMAPP and OMIM software. Results: After statistical evaluation of the raw data, we obtained a set of 158 differently expressed probe sets in the LNCaP and LNCaP C4-2 cells. The search for genes associated with proliferation, cell metabolism, growth factors, metastatic potential and tumour progression in this list revealed a number of 42 differently expressed probe sets. The comparison of this list of probe sets with the literature resulted in a list of 14 differently expressed genes which could well contribute to the metastatic potential and progression of PCa. Of these 14 genes only 6 (Cip1, IGF-1, NK4, CXCL 12, ILGF2R, RHOE) have already been associated with PCa, whereas the other 8 genes (FSTL-1, SOCS-2, Midkine, Thrombospondin 1, Secretory leukocyte protease inhibitor, Desmoglein 2, MLT 1, PTPRF) had not been previously related to PCa. Conclusion: DNA microarray technology offers the possibility of screening a large number of genes with regard to alterations in the expression level or mutations. In this study, we identified 14 genes that are most probably associated with the higher metastatic potential of LNCaP C4-2 cells as compared to LNCaP cells. Eight of these 14 genes are potential new molecular markers for assessing the metastatic potential of PCa, or may serve as therapeutical targets.*

Correspondence to: Lutz Trojan, MD, MSc, Department of Urology, University Hospital Mannheim, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany. Tel: +49-621-3834080, Fax: +49-621-3831452, e-mail: lutz.trojan@uro.ma.uni-heidelberg.de

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Prostate cancer (PCa) is the second leading cause of male cancer death and the most common malignancy in men in Western Europe. PCa is a heterogenous tumour entity with known interindividual differences in biological behaviour regarding tumour aggressiveness and metastatic potential. To date, the time of metastatic spread *via* blood vessels or lymphatics determines the point of incurable disease.

Several prognostic factors for disease progression and metastatic potential have been identified including tumour staging and grading, PSA level, as well as experimental factors like angiogenesis and proliferation markers like Ki-67 (8). Yet, it remains impossible to determine the metastatic potential and the metastatic status of an individual tumour.

We, therefore, investigated two human cancer cell lines, LNCaP and LNCaP-C4-2, which are known to inherit a different metastatic potential in established animal models (7, 9, 10). The aim of this work was to identify potential new markers to open up the possibility of performing a multifactorial analysis of individual PCa specimens in order to estimate the individual metastatic potential of each patient in the future. With the knowledge of specific tumour markers, an assessment of the metastatic potential and progression with the help of alignment charts can be made. Molecular genetic profiling for differences in the gene expression of the two cell lines was performed with Affymetrix GeneChip technology.

Materials and Methods

Cell culture and total RNA isolation. LNCaP and its derivate LNCaP C4-2 were used for the genetic profiling of human PCa cell lines with a high and a low metastatic potential in orthotopic nude mice cancer models. LNCaP (supplied by the American Tissue and Cell Collection, ATCC, Wesel, Germany) PCa cells were cultured in

Figure 1. *Table of statistically significantly expressed genes in LNCaP C4-2 cells identified by NetAffx and Gene Ontology.*

GO Function Name	Number of identified genes (Percentage of total number)
Biological process	110 (0,9 % of 12,304)
Hemostasis	7 (5.6 % of 124)
Digestion	2 (4.0 % of 50)
Extracellular matrix organization and biogenesis	1 (8.3 % of 12)
Circulation	1 (0.7 % of 140)
Response to external stimulus	17 (1.1 % of 1,521)
Homeostasis	1 (1.1 % of 86)
Pathogenesis	4 (3.5 % of 113)
Secretion	1 (2 % of 50)
Death	5 (0.8 % of 568)
Histogenesis and organogenesis	2 (1.6 % of 121)
Drug resistance	1 (5.2 % of 19)
Small molecule transport	1 (1.4 % of 69)
Oncogenesis	2 (1.1 % of 174)
Cell growth and/or maintenance	44 (1.0 % of 4,047)
Cell motility	7 (1.7 % of 404)
Cell communication	43 (1.1 % of 3677)
Cell differentiation	15 (1.3 % of 1,077)
Reproduction	2 (1.1 % of 180)
Cellular component	78 (0.7 % of 10,453)
Collagen	2 (3.3 % of 63)
Basement membrane	2 (2.7 % of 72)
Synaptic junction	1 (2.3 % of 42)
Integral to membrane	22 (0.6 % of 3,174)
Plasma membrane	15 (0.6 % of 2,180)
Nucleus	13 (0.4 % of 3,248)
Cytoplasm	24 (0.6 % of 3,562)
Ribonucleoprotein complex	2 (0.5 % of 362)
Membrane fraction	8 (1.0 % of 736)
Soluble fraction	2 (0.7 % of 276)
Molecular function	115 (0.8 % of 13,690)
Kinase regulator activity	1 (1.4 % of 69)
Enzyme inhibitor activity	10 (3.8 % of 263)
GTPase regulator activity	1 (0.3 % of 284)
Enzyme activator activity	1 (0.4 % of 239)
Antiviral response protein	1 (2.2 % of 44)
Metal ion binding	10 (0.7 % of 1,309)
Protein binding	8 (0.4 % of 1,753)
Nucleotide binding	13 (0.8 % of 1,549)
Nucleic acid binding	19 (0.5 % of 3,347)
Carbohydrate binding	1 (0.7 % of 136)
Glycosaminoglycan binding	6 (5.4 % of 110)
Kinase activity	7 (0.7 % of 995)
Transferase activity	13 (0.7 % of 1,684)
Oxidoreductase activity	10 (1.4 % of 677)
Lysase activity	1 (0.6 % of 165)
Hydrolase activity	21 (1.0 % of 2,091)
Cell cycle regulator	1 (100 % of 1)
Peptide hormone	4 (8.8 % of 45)
Collagen	1 (6.4 % of 16)
Tumour antigen	3 (13.6 % of 22)
Transmembrane ephrin	2 (50.0 % of 4)
Ephrin	2 (66.6 % of 3)
Tumour suppressor	1 (1.1 % of 85)
Apoptosis inhibitor activity	3 (4.0 % of 74)
Structural constitutent of ribosome	2 (0.8 % of 237)
Extracellular matrix structural constituent	1 (1.0 % of 96)
Transporter activity	22 (1.1 % of 1,876)

75-cm^2 cell culture flasks as monolayers in a humidified atmosphere of 5% CO_2 in RPMI medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum and 4 mM glutamine. LNCaP C4-2 (kindly supplied by Dr. Thalmann, Bern, Switzerland) PCa cells were cultivated under the same conditions using T4 medium as described by Thalmann *et al.* (1). Cells were grown to 80-90% of confluence and then harvested with 2-3 ml 0.25% trypsin/EDTA and thereafter subcultured at a 1:5 ratio. LNCaP cells were used for these experiments between passage numbers 24-34 along with LNCaP C4-2 cells between passage numbers 54-68.

For total RNA isolation, the cells were washed twice with ice-cold PBS. After removing the PBS, the cells were covered with 2 ml Trizol®LS (Gibco BRL, Invitrogen, Karlsruhe, Germany) reagent and immediately harvested by scraping. The lysate was transferred to a 15ml Falcon tube. The subsequent procedure was carried out according to the protocol supplied by the manufacturer.

cDNA synthesis and array expression analysis. The technique of array analysis was introduced by Fodor *et al.* in 1993 (2) and has previously been described (3, 4). Microarray analysis of the used human genome (HG) U133A GeneChip arrays was conducted in accordance with the instructions of the manufacturer. Briefly, 5 to 10 μg of total RNA were used to prepare biotinylated cRNA's for hybridisation using the standard Affymetrix protocol (Affymetrix, Santa Clara, CA, USA). For this purpose, RNA was converted to first-strand cDNA using T7-linked oligodeoxythymidylic acid primer, followed by a second-strand synthesis. The double-stranded cDNA was then used as a template for labelled transcription reactions using biotinylated ribonucleotides. Fifteen μg of each labelled cRNA was then hybridised to Affymetrix HG-U133A GeneChips using standard conditions in an Affymetrix fluidics station.

Microarray analysis of LNCaP-mRNA was conducted at five seperate times and LNCaP C4-2-mRNA was analysed three times. Each time, mRNA was obtained from a different set of cell culture experiments.

Data analysis and biocomputational tools. After scanning with the GeneChip® Scanner 3000, the Affymetrix data were imported to the Affymetrix Data Mining Tool (Version 3.0) and a primary analysis of data was performed. The data mining tool was used to calculate change, change in *p*-value and signal log ratio. For normalisation purposes, the Affymetrix probe mask scaling procedure was used. On the basis of these values, statistically differently expressed genes were identified. Fold changes of $\geq |2|$, corresponding to a signal log ratio (SLR) of $\geq |1|$, with a change in *p*-values of $0.0045 \geq p \geq 0.9955$ for every single comparison were defined as statistically significant. With the help of the NetAffx analysis software (www.affametrix.com/analysis/index.affx), dChip (Version 1.3 www.dchip.org), GenMAPP (Version 1.0 www.genmapp.org) software and the OMIM database (www.ncbi.nlm.nih.gov), the genes related to increased metastatic potential were identified by secondary analysis.

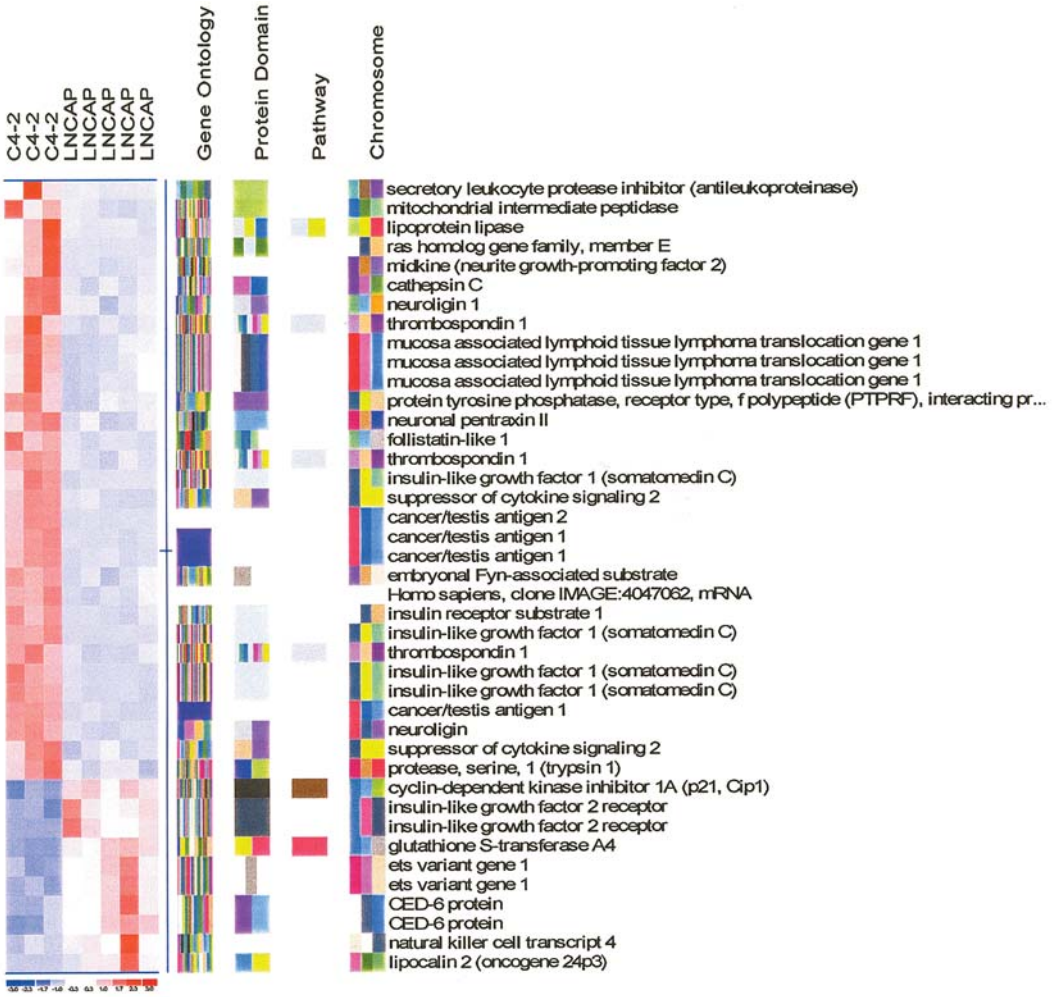

Figure 2. *Hierarchical clustering of samples to determine how the patterns of gene expression (red-increase, blue-decrease in expression) correlated between multiple samples within the groups of LNCaP resp. LNCaP C4-2 cell lines and between the two groups.*

Results

Gene expression profiling. With the help of the GeneChip® expression arrays, we analysed the relative expression of the mRNA in around 22,000 genes of the human genome. The RNA profile of LNCaP cells was used as a baseline and the RNA expression profile of the LNCaP C4-2 cells (high metastatic potential) was compared to these data. Each performed analysis on LNCaP cells (n=5) was compared to each analysis on LNCaP C4-2 cells (n=3), resulting in 15 comparisons.

Changes in *p*-values under 0.0045 were regarded as increases (I) in expression, whereas changes in *p*-values higher than 0.9955 were regarded as decreases (D) in expression. The application of these criteria resulted in a list of 158 statistically different probes. Using the Gene Ontology (GO) classification, the identified probe sets can be subdivided according to their function (Figure 1). According to the genetic function, 42 probe sets associated with cell proliferation, cell metabolism, growth factors and metastatic potential could be identified. Hierarchical clustering was used to determine how the patterns of gene expression correlated between multiple samples within the groups of LNCaP resp. LNCaP C4-2 cell lines and between the two groups (Figure 2). The hierarchical clustering analysis revealed a distinct separation of the two grouped cell lines with regard to the up-/ down-regulation of genes (Figure 2).

The identified probe sets implicate genes responsible for cell adhesion, enzyme inhibition, cell motility, regulation of apoptosis, cell cycle regulation, cell communication and DNA replication.

The detected group of up-regulated genes with enzyme inhibitor activity in LNCaP C4-2 cells comprises secretory leukocyte protease inhibitor (antileukoproteinase), thrombospondin 1 and cyclin-dependent kinase inhibitor (p21, Cip1). Up-regulated genes in LNCaP C4-2 cells associated with cell motility are, in turn, thrombospondin 1 and insulin-like growth factor 1 (somatomedin C), which is also responsible for DNA replication. Homologue E of the RAS homologue gene family, neuroligin 1, chemokine ligand 12, poliovirus receptor ligand 3, desmoglein 2, thrombospondin 1, protein tyrosine phosphatase (receptor type) f polypeptide (PTPRF), neuronal pentraxin II, cancer/testis antigen 1 and 2 as well as embryonal FYN-associated substrate are overexpressed members of the group of genes responsible for cell adhesion in LNCaP C4-2 cells, whereas the natural killer cell transcript 4 shows a lower expression compared to LNCaP cells. In the group of genes which are associated with cell communication and growth factors, insulin-like growth factor 2 receptor and natural killer cell transcript 4 show a lower expression in LNCaP C4-2 cells. However, the expression of midkine (neurite growth-promoting factor 2), mucosa-associated lymphoid tissue lymphoma translocation gene 1, suppressor of cytokine signalling 2 and the already described thrombospondin 1, as well as insulin-like growth factor 1 (somatomedin C), RAS homologue gene family member E and embryonal FYN-associated substrate are up-regulated. In addition to insulin-like growth factor 2, the expression of CED-6 protein, which is also responsible for vesicle-mediated transport, is down-regulated in LNCaP C4-2 cells. In addition to thrombospondin 1 and midkine, the expression of the genes encoding for lipoprotein lipase and follistatin-like 1, which all belong to the group of heparin binding proteins, are up-regulated. The group of LNCaP C4-2 up-regulated genes responsible for proteolysis and peptidolysis contains mitochondrial intermediate peptidase, cathepsin C, mucosa-associated lymphoid tissue lymphoma translocation gene 1 and serine protease 1. The gene of glutathione S-transferase A4, an enzyme with alkyl- or aryl-transferase activity, is also up-regulated in LNCaP C4-2 cells. Ets variant gene 1, a gene responsible for the regulation of transcription, shows a significantly lower expression in LNCaP C4-2 cells compared to LNCaP cells. In the group of genes differentially expressed in LNCaP C4-2 cells related to apoptosis, we identified 3 members, one of which is up-regulated (mucosa-associated lymphoid tissue lymphoma translocation gene 1). In contrast, the expression of the other two members of this group, PTB domain adaptor protein CED-6 and cyclin-dependent kinase inhibitor 1A (p21, Cip1), are down-regulated.

Discussion

The Human Genome U133A Set assesses the expression of 22,000 genes with at least 11 repetitions each, with the help of the Affymetrix GeneChip technology. This new technique is a powerful tool enabling us to study a large number of genetic characteristics simultaneously and screen for differences on a cellular transcript level. Tumour progression and the formation of tumour metastasis are the main obstacles in the treatment of prostatic cancer diseases. It is, therefore, of great interest to identify alterations in the gene expression profiles of different non-metastatic and metastatic systems in order to find potential new prognostic markers for tumour progression and to identify possible targets for new treatment strategies. Interesting targets on the cellular level for prostatic tumour progression are genes that influence the cell cycle, apoptosis, metabolism, adhesion, angiogenesis and metastasis (5-7). Therefore, the aim of the study was to identify genetic changes in a cell line after it has acquired a high metastatic potential. For this purpose, we used the combination of LNCaP / LNCaP C4-2 cells, which was originally designed and described by Thalmann *et al.* (1,8). LNCaP C4-2 cells are an androgen-independent variation of the androgen-dependent LNCaP cell line with high tumourigenic and metastatic potential (1). Interindividual differences in the gene expression profiles are minimised in this system, thus enabling the identification of tumour progression-relevant alterations in gene expression through the use of two variations of one cell line.

The interpretation of the data obtained by Affymetrix GeneChip analysis led to 158 significantly differentially expressed probes from all areas of cellular function. With the help of the NetAffx analysis software, dChip (Version 1.3), OMIM and GenMAPP software, a selection of 42 genes associated with proliferation, cell metabolism, growth factors, metastatic potential and tumour progression was performed. These genes belong to different Gene Ontology (GO)-categories and their importance for metastasis varies. In the following paragraphs, the function of these 42 genes and their possible relevance for metastasis and progression of PCa are discussed.

Secretory leukocyte protease inhibitor (antileukoproteinase), as a member of the group of serine protease inhibitors (SPI), is often associated with poor prognosis in cancer patients and has a malignancy-promoting effect in different kinds of tumours (12). Ohlson *et al.* have already described its expression in benign prostatic tissue (13). Its up-regulation in LNCaP C4-2 cells might be a reason for its higher metastatic potential over native LNCaP cells, although its role in PCa has so far not been determined.

Thrombospondin 1 has different effects on living cells: on the one hand, it is known to induce apoptosis in different cell systems mediated by CD 36 and caspase-3 (14). Because

Figure 3. *List of statistically significant differently expressed genes in LNCaP C4-2 cells associated with tumour progression or metastasis in comparison to native LNCaP cells.*

Genes crucial for tumour progression and metastasis in PCa	
Decreased expression	Increased expression
• Cyclin-dependent kinase inhibitor • Insulin-like growth factor 2 receptor • Natural killer cell transcript 4	• Mucosa-associated lymphoid tissue lymphoma translocation gene 1 • Protein tyrosine phosphatase, receptor type, f • RAS homolog gene family, Member E • Thrombospondin 1 • Secretory leukocyte protease inhibitor • Desmoglein 2 • Chemokine ligand 12 • Suppressor of cytokine signalling 2 • Insulin-like growth factor 1 • Midkine • Follistatin-like 1

of this function, it is used in experimental studies in combination with cytotoxic agents to treat cancer diseases (15). On the other hand, thrombospondin 1 is a potent anti-angiogenetic factor and has been shown to inhibit tumour growth by preventing endothelial cells from responding to a wide variety of angiogenic stimulators. It has, therefore, been used in several experimental studies (16, 17). In the case of malignant glioma cells, however, it has been shown that a reduction of thrombospondin 1 expression significantly reduces cell motility (18). The role of thrombospondin 1 with regard to tumour progression and metastasis is ambivalent: the first two characteristics make it improbable that thrombospondin 1 is responsible for higher tumour progression, despite its overexpression in LNCaP C4-2 cells. Yet the role of thrombospondin 1 in cell motility makes it an interesting point to consider with regard to the metastatic potential of cells. Jin *et al.* (19) used the thrombospondin 1 gene in their study to suppress angiogenesis and induce necrosis in prostate tumours. Recent studies by Hamano *et al.* (20) showed that the thrombospondin 1 expression level is associated with a susceptibility to therapy with low-dose cyclophosphamide (LDC). For this reason, thrombospondin 1 is an interesting target for further investigations.

The lower expression of the mRNA encoding for the protein cyclin-dependent kinase inhibitor 1 (p21, Cip1) in LNCaP C4-2 cells is of greater interest for tumour progression. Cyclin-dependent kinases (CDKs) have recently raised considerable interest because of their key role in the regulation of the cell cycle progression (21). In proliferating cells, distinct CDKs associated with specific cyclins co-ordinate the different phases of the cell cycle (21). It has been demonstrated, in the past, that cyclin-dependent kinase 1 expression is associated with a poor prognosis in gastric carcinomas (22). In recent phase I and II clinical trials, CDK inhibitors were used as anticancer drugs to treat prostatic cancer along with other malignant diseases (23). These trials have shown that CDK is closely associated with apoptosis in PCas. Based on these facts, the down-regulation of an inhibitor of the CDKs should result in higher proliferation and a lower rate of apoptosis of PCa cells and is therefore of great interest for tumour progression.

Insulin-like growth factor-1 (synonym: IGF-1, somatomedin C) has been made responsible for the induction of cell adhesion and migration in different cancer cells *via* the activation of beta 1-integrin and phosphatidylinositol 3'-kinase/AKT signalling (24). It has been reported, in the past, that a IGF-1 expression is a risk factor for PCa, but not a tumour marker for this disease (25, 26). Nevertheless, its overexpression may play a role in the increased metastatic potential of LNCaP C4-2 cells. Its role for the process of formation and metastasis in PCa has yet to be evaluated.

Neuroligin 1 (NLGN 1) is a plasma membrane protein first isolated from neuronal cell surfaces (27). Together with the neurexin 1 beta protein, it is regarded as being responsible for cell adhesion procedures in neuronal cell networks (28). Although its role in PCa has not yet been described, NLGN 1 might well be a promising target for evaluating the metastatic potential in PCa because of its function in neuronal cells.

It has been reported, in the past, that chemokine ligand 12 (synonym: CXCL 12, stromal cell-derived factor-1) regulates cAMP production and ion transport in intestinal epithelial cells *via* CXCR 4 (29). In PCa, CXCL 12 expression is significantly higher in metastatic tumours compared to normal tissues or non-metastatic tumours (30). *In vitro* a neutralisation of CXCL 12 with antibodies resulted in a decrease in the proliferation of LNCaP cells (30). CXCL 12 seems to be of interest for metastasis and tumour progression. Our results are in accordance with the published data on the role of CXCL 12 for PCa.

The desmosomal cadherins are calcium-dependent transmembrane adhesion molecules and compromise the desmogleins and desmocollins (31). One member of this group of cell adhesion molecules, desmoglein 2 (HDGC), is significantly overexpressed in LNCaP C4-2 cells. The work of Bussemakers *et al.* indicates that desmoglein 2, along with a number of other cadherins and cell adhesion molecules, plays a crucial role in PCa invasiveness and metastasis (32). Recent

studies have affirmed the role of the desmoglein protein family in PCa (31).

It has been suggested that neuronal pentraxin II (synonym: NPTX2) plays an important role in the uptake of extracellular material (33). To our knowledge, this protein is only associated with neuronal cells and its function is not directly related to cell proliferation or metastasis. It is, therefore, probably of no interest for the dissemination of PCa.

Insulin-like growth factor 2 receptor (synonym: ILGF2R, Mannose 6-Phosphate Receptor, MPRI) functions in the intracellular trafficking of lyosomal enzymes, the activation of the potent growth inhibitor, transforming growth factor beta and the degradation of IGF2, a mitogen often overproduced in tumours (34). ILGF2R shows a lower expression on the mRNA level in LNCaP C4-2 cells than in LNCaP cells. Because of its effect on IGF2, its lack of function might be of importance for prostatic tumour progression. These suggestions are supported by studies of Schaffer *et al.* (35), who were able to show that decreased IGF2R expression partly accounts for the increased growth of lymph node carcinoma of the prostate.

Midkine (synonym: neurite growth-promoting factor 2, NEGF2) is a member of a highly conserved, developmentally regulated human gene family (36). It is a retinoic acid-responsive gene concerned with prenatal development and neurite growth (37). It has been shown that midkine , together with heparin affin regulatory peptide (HARP), comprises a new family of heparin-binding growth/differentiation factors (38). Recently, this family was implicated as being an angiogenic factor and as a tumour growth factor in cancer progression. This insight makes midkine an interesting target to examine.

The gene encoding for mucosa-associated lymphoid tissue lymphoma translocation gene 1 (synonym: MLT, Paracaspase) is identified as a crucial locus for genetic alterations that allow tumour cells of MALT-type lymphomas to evade apoptosis (39). Even though MLT's functions and its mutations may well be of interest for metastasis, there are no investigations concerning its role in PCa and possible effect on the process of metastasis formation.

The up-regulated suppressor of cytokine signalling 2 (synonym: SOCS 2, STAT-induced STAT inhibitor2, SSI2, STATI2, cytokine-inducible SH2 Protein 2, CIS2) has been shown to be a negative regulator of cytokine receptor signalling *via* the Janus kinase (JAK)/signal transducer and activation of transcription (STAT) pathway (40). Cytokines regulate the growth and differentiation of cells by binding to cell-surface receptors and activating intracellular signal transduction cascades (41). By suppressing the activity of the JAK system, an increase in SOCS 2 expression should facilitate the activation of apoptosis. Until now, however, the significance of SOCS 2 for PCa has not been investigated. Its influence on tumour progression and metastasis must, therefore, be determined.

The expression of CED 6 (synonym: engulfment adaptor protein, GULP), a protein associated with the promotion of the phagocytosis of apoptotic cells (42), is down-regulated in LNCaP C4-2 cells as opposed to LNCaP cells. The induction of phagocytosis is an event that takes place after the induction of apoptosis and is of minor interest for cancer cell metastasis, because the death of the cell is already predetermined.

Lipoprotein lipase (LPL) is a protein associated with the clinical picture of hyperlipoproteinemia, type 1 (43). In the case of PCa it has been demonstrated that the loss of LPL expression together with an increase in the expression of the c-myc gene could possibly be associated with a poor patient prognosis (44). Based on this speculation, it is improbable that LPL up-regulation is of importance for the higher metastatic potential of LNCaP C4-2 cells.

The expression of follistatin-like 1 (synonym: FSTL 1, Follistatin-related protein, FRP) in prostatic tissues was reported for the protein level in 2002 (45). Together with follistatin, the follistatin-like protein is responsible for the irreversible binding of activin (46). Activin ligands act as growth and differentiation factors in many cells and tissues. The up-regulation of FSTL gene activity might thus be of interest for PCa progression.

Mitochondrial intermediate peptidase (MIPEP) performs the final step in processing a specific class of nuclear-encoded proteins targeted at the mitochondrial matrix or inner membrane and is related to frataxin, a protein associated with the clinical picture of Freidreich ataxia (47). Even if MIPEP is involved in mitochondrial metabolism, it is improbable that it plays an important role in tumour progression. There is, to date, no study associating MIPEP with any kind of cancer disease.

Cathepsin C (synonym: CTSC, dipeptidyl peptidase I, DPPI) is a lyosomal protease capable of removing dipeptides from the amino terminus of protein substrates, which is involved in the processing of various proenzymes to their active forms (48). The up-regulation of cathepsin C has already been demonstrated for large granular lymphocyte leukemia (49) and solid tumours (50, 51). The exact role of cathepsin C for cancer cell development is not fully understood and its role in tumour progression is therefore hard to determine.

The glutathione S-transferase A4 (synonym: gluthatione S-transferases alpha 4, GSTA 4) catalyses the conjugation of reduced gluthationes and a variety of eletrophiles. It has been shown that glutathione S-transferases detoxify activated carcinogen metabolites by catalysing their reaction with glutathione (GSH) (52). Therefore the down-regulation of GSTA 4 expression is probably more interesting in terms of carcinogenesis than in terms of cancer progression. The role of glutathione S-transferase A4 in the development of PCa is not clear and needs to be determined in further studies.

Ets variant gene 1 (synonym: ETV 1, ets translocation variant 1) encodes for transcription factors that are involved in tumourigenesis and the developmental process (53). The down-regulation of this protein is probably of minor importance for tumour progression or metastasis.

Homologue E of the RAS homologue gene family (synonym: ARHE, RHOE), a member of the Rho family of Ras-related GTPases, regulates the organisation of the actin cytoskeleton in response to extracellular growth factors together with other proteins of this gene family. In PCa cells, RHOE is associated with cell adhesion and is therefore of great interest in terms of metastasis (54). With regard to prostate tissue, it has recently been shown that RHOE is associated with estrogenic actions in the human prostate and, therefore, with the development of different prostatic diseases (55).

The LAR gene, encoding for protein-tyrosine phosphatase, receptor-type, F (synonym: PTPRF, Receptor-linked protein-tyrosine phosphatase, LAR, Leukocyte antigen-related tyrosine phosphatase) encodes a membrane protein that has a cytoplasmic domain with a homology to protein-tyrosine phosphatase 1B and an extracellular domain homologous with the neural cellular adhesion molecule NCAM. According to the work of Ahmad *et al.* (56), this protein is functionally associated with insulin receptors and, therefore, with cell growth. The up-regulation of the expression of this gene might be of interest for tumour progression and metastasis in PCa.

The cancer/testis antigens 1 (synonym: CTAG 1, NYESO 1) and 2 (synonym: LAGE 1, CTL-recognised antigen on melanoma, CAMEL) are also up-regulated in LNCaP C4-2 cells. Lethe *et al.* (57) noted a correlation between the expression of CTAG 1 and LAGE 1 in a significant fraction of tumour samples, which included melanomas, sarcomas, breast, lung, head, neck and bladder cancers, as well as in benign testis tissue and PCa. Because cancer/testis (CT) antigens are immunogenic in cancer patients and exhibit highly tissue-restricted expression, they are considered promising target molecules for cancer vaccines, but are not of interest in terms of tumour progression or metastasis (58).

Dahl *et al.* (59) identified the cDNA of natural killer cell transcript 4 (NK4) by differential screening of an IL2-activated human natural killer cell (NK) derived cDNA library. In 1998, Bernot *et al.* (60) showed that NK4 is ubiquitously expressed. It has been reported that NK4 acts as an antagonist of the proteins hepatocyte growth factor (HGF) and its receptor, c-Met tyrosine kinase, which play a role in the process of cancer invasion and metastasis in a wide variety of tumour cells (61). With regard to PCa, it has recently been demonstrated in a nude mouse tumour model that NK4 significantly suppressed HGF/SF-induced invasion and migration (62). The down-regulated NK4 expression is therefore of great interest with respect to tumour progression and metastasis.

To recapitule, we can identify 14 different genes, that are most probably responsible for the higher metastatic potential of LNCaP C4-2 cells as compared to LNCaP cells. Of these 14 genes, only 6 (Cip1, IGF-1, NK4, CXCL 12, ILGF2R, RHOE) have already been associated with PCa, while the other 8 genes (FSTL-1, SOCS-2, midkine, thrombospondin 1, secretory leukocyte protease inhibitor, desmoglein 2, MLT 1, PTPRF) have, to our knowledge, not been mentioned in terms of PCa. We have, therefore, detected 8 potential new targets for gene therapeutical purposes or as markers for tumour metastasis and progression.

Conclusion

Our data reveal that there are distinct alterations at the mRNA level between the non-metastatic LNCaP and the metastatic and more aggressive LNCaP C4-2 cell lines. With the help of the Affymetrix GeneChip technology, it was possible to screen a large number of mRNAs of many different genes. Employing modern software and analysis tools, we were able to search for genes responsible for tumour progression and metastasis in the differently expressed genes. This procedure led us to a manageable number of 14 interesting genes associated with tumour that we will have to examine in the future (Fig. 3). Eight of these 14 genes have not yet been mentioned in the literature in terms of prostate tumour progression or metastasis. Further trials will be performed to analyse the exact function of these potential new marker genes in prostate tumour progression.

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