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ARTÍCULO ORIGINAL

DNA microarray analysis reveals metastasis-associated genes in rat prostate cancer cell lines

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Introduction. The molecular and cellular mechanisms involved in prostate cancer progression towards a hormone-independent and highly invasive, metastatic phenotype, are not well understood. Cell lines with different metastatic potential, when analyzed by microarray techniques, offer valuable tools for identifying genes associated with the metastatic phenotype.
Objectives. Gene expression profiles were compared for two rat prostate cancer cell lines with differing metastatic abilities in order to better characterized molecular underpinnings of the prostate cancer metastatic process.
Materials and methods. Affymetrix arrays were used to analyze gene expression of two rat prostate cancer cell lines, MAT-LyLu and G. Microarray data were analyzed using pathway and functional group analysis. A selected set of genes was subjected to real-time polymerase chain reaction for validating the microarray data.
Results. Microarray data analysis revealed differential expression of genes from a number of signaling and metabolic pathways. Overexpression was detected in 48 genes and underexpression in 59 genes of the MAT-LyLu line compared to the standard G line. Genes were grouped into functional categories, including epithelial-extracellular matrix interaction, cell motility, cell proliferation, and transporters, among others. Many of these genes were not previously associated to prostate cancer metastasis.
Conclusions. Many genes with altered expression associated with a metastatic prostate cancer phenotype were identified. Further validation of these genes in human prostate samples will determine their usefulness as biomarkers for early diagnosis of recurrence or metastasis of prostate cancer, as well as potential therapeutic targets for this disease.

Key words: carcinoma, neoplasm metastasis, gene expression, biological markers, extracellular matrix, microarray analysis.

Análisis de micromatrices de ADN revela genes asociados a metástasis en líneas celulares de cáncer de próstata de rata

Introducción. Los mecanismos moleculares y celulares involucrados en la progresión del cáncer de próstata hacia un fenotipo metastásico no son bien entendidos. El análisis molecular de líneas celulares con diferentes potenciales metastásicos ofrece un instrumento valioso para identificar genes asociados al fenotipo metastásico.
Objetivos. Comparar los perfiles de expresión genética de dos líneas celulares de cáncer de próstata de rata con diferentes capacidades metastásicas para un mejor entendimiento del proceso metastásico.
Materiales y métodos. Se utilizaron micromatrices de Affymetrix para analizar la expresión génica de dos líneas celulares de próstata de rata con diferentes propiedades metastásicas, MAT-LyLu y G. Los datos fueron analizados en el contexto de vías y grupos funcionales. Se utilizó reacción en cadena de la polimerasa en tiempo real para validación de genes seleccionados.
Resultados. Además de la expresión diferencial de genes en un número de vías de señalización y metabólicas, se detectó sobre-expresión de 48 genes y expresión disminuida de 59 genes en la línea MAT-LyLu comparado con la línea G. Los genes fueron agrupados en

categorías funcionales, tales como interacción epitelial-matriz extracelular, motilidad celular, proliferación celular, y transportadores, entre otros. Muchos de estos genes no han sido asociados previamente a la metástasis del cáncer de próstata.
Conclusiones. Se identificaron genes con expresión alterada asociados al fenotipo metastásico del cáncer de próstata. La subsiguiente validación de estos genes en tejido prostático humano pudiera revelar su utilidad como marcadores biológicos para esta enfermedad.

Palabras clave: carcinoma, metástasis de la neoplasia, expresión génica, marcadores biológicos, matriz extracelular, análisis de micromatrices.

The American Cancer Society estimates that 234,460 new cases of prostate cancer were expected in the United States in 2006, representing the most common cancer in men and the third leading cause of male cancer-related deaths (1). Treatments for prostate cancer including surgical and hormonal therapies, have shown beneficial effects only for early-stage, hormone-dependent disease. However, for more advanced stages, the cancer cells become hormone-independent and highly invasive, until they reach an incurable clinical stage associated with an increased incidence of metastases, especially to the bone. The molecular and cellular mechanisms involved in this process are not well understood (2).

Dunning rat tumor cell sublines of variable metastatic potential: G (non metastatic) and MAT-LyLu (highly metastatic) were used to investigate the differences in gene expression associated to metastatic prostate cancer. These two cell lines were derived from the same parental tumor, the Dunning R-3327 from Copenhagen rats (3). The R3327-G, or simply G, cell line is a poorly differentiated, androgen-dependent tumor line obtained in Dunning's laboratory after *in vitro* passaging of the original tumor. The MAT-LyLu (Metastatic At-Lymph Node and Lung) cell line was developed at Johns Hopkins School of Medicine and characterized by Isaacs et al (4,5). The G and MAT-LyLu cell lines exhibit many characteristics seen during the progression of human prostate cancer, including androgen responsiveness and differential metastatic ability, representing a well characterized model to study the metastatic process (6,7).

In the current study, the gene expression profiles of the two rat cell lines were determined with Affymetrix DNA microarrays (GeneChip Rat Genome U34A array). There was altered expression (overexpression and underexpression) of 107 genes in the MAT-LyLu cell line was compared to the G cell line. Several of the genes were not previously associated with prostate cancer metastasis. Validation of these metastasis-associated genes in human prostate samples have a potential application as biomarkers for early diagnosis of recurrence or metastasis or as therapeutic targets for this disease.

Materials and methods

Cell lines

Dunning rat tumor cell sublines, G (non metastatic) and MAT-LyLu (highly metastatic) were grown using RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown in T-75 culture flasks with 25 cc of growth media. All tissue culture work was done in a Class IIA cell culture hood with 20 min of UV light and ethanol wash between handling of the different cell lines.

Total RNA isolation

Total RNA was prepared from MAT-LyLu and G cells grown to approximately 75% confluence using Trizol solution (Invitrogen) and stored as an ethanol precipitate. The resulting RNA concentration was measured spectrophotometrically, and quality was assessed by the 260/280 ratio and agarose gel electrophoresis. Two biological replicates (duplicates)consisted of RNA samples ob-

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tained from cells grown at different dates and processed independently for microarray experiments (no sample pooling was done before microarray hibridizations). In this manner, microarray hybridization for each cell line was performed in duplicate.

Microarrays

To study the gene expression profile of these rat cell lines, the Rat Genome U34A Array (Affymetrix, Santa Clara, CA) was used. This array represented approximately 7,000 full-length gene sequences and approximately 1,000 EST clusters consisting of expressed sequence tags-transcribed sequences of unknown function. *Microarray procedures*

Total RNA was processed at the University DNA Microarray Facility of the Center for Molecular Medicine at the SUNY Stony Brook School of Medicine, according to protocols described in Affymetrix GeneChip Expression Analysis technical manual (Affymetrix, Santa Clara, CA). The manual can be downloaded from the Affymetrix website (http://www.affymetrix.com/ support/technical/manual/expression_manual.affx). RNA quality was first assessed by electrophoresis using the Agilent Bioanalyzer 2100 and spectrophotometric analysis. Then, total RNA from each sample was used to generate high fidelity cDNA. Briefly, first-strand cDNA was reverse transcribed from 15 µg of total RNA using 600 U of SuperScript II RT (Invitrogen) in a 20-µl total volume containing T7-(dT)24 primer, 10 mM DTT, 500 µM of each dNTP, and 1x first-strand cDNA buffer for 1 h at 42°C. Second-strand cDNA was synthesized in 1x reaction buffer containing 200 µM of each dNTP, 10 U of DNA ligase, 40 U of DNA polymerase I, and 2 U of RNase H (final volume = 150 µl) for 2 h at 16°C. Samples were then treated with 20 U of T4 DNA polymerase for 5 min at 16°C and incubated in 10 µl of 0.5 M EDTA. Double-stranded cDNA was purified with phase lock gel electrophoresis (Eppendorf Scientific, Westbury, NY) and phenol-chloroform extraction, followed by ethanol precipitation. Upon completion of cDNA synthesis, 1µg of product was converted to biotinylated cRNA using the Enzo BioArray HighYield RNA Transcript Labeling Kit. Labeled cRNA was fragmented and hybridized to the Affymetrix oligonucleotide GeneChip rat U34A arrays. Twelve micrograms of biotinylated target cRNA were hybridized per microarray. Then, the gene chips were automatically washed, to remove non-specifically bound cRNA, and stained with streptavidin-phycoerythrin conjugate in the GeneChip Fluidics Station. Washed arrays were automatically scanned using Hewlett-Packard GeneArray Scanner equipped with an argon ion laser. The methodology used for the microarray procedure follows the MIAME (Minimum Information About a Microarray Experiment) guidelines (www.mged.org/ Workgroups /MIAME/miame.html).

Microarray data analysis

Scanned output files were analyzed with dChip v1.3 software (www.dchip.org) and Affymetrix MicroArray Suite 5.0 (MAS 5.0) software. First, arrays were normalized by dChip v1.3 using the invariant set normalization method (8). After normalization, model-based gene expression estimates and outlier detection algorithm were obtained according to the perfect-match-only model performed by Li-Wong (9); transcripts regarded as outliers were excluded for further analysis. Affymetrix MAS 5.0 software was used to determine if the transcripts were detected as present, absent, marginal, or no call. For significant expression level, a cut off value of 500 units was used. DNA microarray data generated from MAT-LyLu samples were compared to microarray data from G samples using dChip v1.3 software and the resulting expression analysis files were subjected to biological pathway and functional group analysis to determine the significance of changes at the biological context.

Pathway analysis

To identify biological pathways playing a role in the metastatic process, the microarray data were analyzed with the GenMAPP (Gene Map Annotator and Pathway Profiler) and MAPPFinder programs developed at the Gladstone Institutes at the University of California at San Francisco (10) (www.genmapp.org). Criteria used for GenMAPP/ MAPPFinder analysis for increased expression were a minimum average intensity of 500 units, a % present (P) call of 100, and a fold change $\geq$1.8

in the MAT-LyLu cell line compared to the G cell line. Criteria for decreased expression were a minimum intensity of 500 units, a % P call of 100, and a fold change of ≤-1.8 in the MAT-LyLu cell line compared to the G cell line. The programs generated a Z score based on the hypergeometric distribution.

Functional group analysis

The catalog of characterized genes is incomplete in the context of biological pathways, and many genes are of unknown function. Therefore a functional group analysis was performed in order to have a more complete picture of genes associated to the metastatic phenotype and to increase the possibilities of finding genes not previously associated to prostate cancer metastasis. For functional group analysis, the dChip v1.3 software provided a means for comparing the MAT-LyLu microarray data vs. G microarray data., It generated a gene list that contained genes with $P \leq 0.05$ (from t-test), a fold change $\geq \pm 2.0$, and a % P call of 100.These genes were thereby identified as having significantly altered expression (either overexpression or underexpression). This set of genes was subjected to hierarchical clustering (dChip v1.3) using the Pearson correlation metric. Before clustering, the expression value for a gene across all samples was standardized to have a mean of 0 and standard deviation of 1. dChip v1.3 also determined in all branches the presence of local clusters enriched by genes having a particular function (functional clusters), according to gene ontology (GO) terms (11). In this way, co-expressed genes were detected that shared similar functions relevant to prostate cancer metastasis. dChip v1.3 also generated a p value for functional clusters with significance set at $p<0.01$.

Real time PCR

Quantitative real time PCR (qRT-PCR) was performed for selected genes with the same individual RNAs used in microarray experiments in order to validate the technique (12). Gene-specific primers were designed with *Primer3* software for kelch repeat 1 (krp1) (forward primer = AAGGAGATGGCGCTA GACAA; reverse primer = CACGGCCTCAAAT ACCACTT), endothelial cell-specific molecule 1(ESM-1) (forward primer = CTACTTTG GACGGCTGCTTC; reverse primer = CGTCACC GAAATCATCTTCC) and caveolin-1 (CAV-1) (forward primer = TGAAGATGTGAT TGCGG AAC; reverse primer = ATCTCTT CCTGCGTGCTGAT).

One microgram total RNA was reverse transcribed using oligo-dT and the Reverse Transcription System kit (Promega). Two microliters of reverse transcribed product was subjected in triplicate to qRT-PCR using the LightCycler DNA Master SYBR Green system (Roche Molecular Biochemicals, Mannheim, Germany). PCR was performed in a LightCycler (Roche, Mannheim, Germany) with a 2 min pre-incubation at 95°C followed by 40 cycles of amplification steps. PCR products were subjected to melting curve analysis to verify that no amplification of non-specific products had occurred. In each qRT-PCR run, a standard curve was generated using a serial dilution of a cDNA standard identical in size and sequence to the target gene fragment to be amplified. Serial dilution ranged from 0.01 to 100 pg of the standard cDNA fragment. The standard curve was calculated by the Light Cycler 5.32 software (LC-Run Version 5.32, Roche) by regression of the crossing points of the PCR curves from the dilution series of the standard cDNA fragment. Changes in gene expression were quantified by calculating the average value of the triplicate reactions from the MAT-LyLu cell line and comparing that to the average derived from the triplicate reactions from the G cell line. The GAPDH gene was chosen as a control housekeeping gene for normalization, since no change in the GAPDH mRNA levels occurred across the entire sample set (MAT-LyLu and G samples). Statistical significance of changes was determined by t-test.

Statistical methods

For DNA microarray and qRT-PCR analyses, a $p<0.05$ from t-test (two-sided) was considered statistically significant. For hierarchical clustering, significant functional cluster p values were calculated using the hyper-geometric distribution. For pathways analysis with GenMAPP/MAPPFinder programs, the Z score based on the hypergeometric distribution was used. The Z score reflected the significance of changes and it falls in a range of positive to negative values. A cut-off value of

2.0 was selected for the Z score, representing the equivalent of p= 0.05 for the hypergeometric distribution. Z scores were generated automatically by the programs.

Results

Many differentially expressed genes were identified from several signaling and metabolic pathways in MAT-LyLu (Table 1). Among the signaling pathways, differential expression was found in the androgen receptor signaling pathway, focal adhesion, Wnt- signaling, hepatocyte growth factor receptor (HGF), epidermal growth factor (EGF) receptor 1, MAPK, integrin mediated cell adhesion, TGF beta, and Insulin signaling pathways (Table 1). In addition to signaling pathways, a number of genes were overexpressed in the cholesterol biosynthesis and nucleotide metabolic pathways (Table 1). Overexpression of these metabolic pathways may account for the high metabolic activity required for the MAT-LyLu cell line to maintain its characteristic high level of cell proliferation compared to the non-metastatic G line, which has a much lower doubling time (4,13). On the other hand, functional group analysis of the DNA microarray data revealed the significantly altered expression of 107 well-characterized genes (Table 2), 48 overexpressed and 59 underexpressed in MAT-LyLu compared to G cell lines, according to the criteria described in materials and methods.

Hierarchical clustering of the significantly expressed genes clearly showed the gene expression differences between MAT-LyLu and G cell lines (Figure 1). Significant functional clustering of the differentially expressed genes included the following GO categories: extracellular region/extracellular matrix (20% of the genes), development (5%), defense response (7%), proteolysis and peptidolysis (5%) and magnesium ion binding (5%), among others.

Some of these functional clusters reflected the changes believed to occur during the metastatic process, such as alteration of extracellular matrix components, neovascularization, changes in cell structure and increased motility (14). Thus, there is a strong concordance of our results with the scientific literature about the metastatic process.

We also compared our microarray data with other microarray analysis of prostate cancer metastasis (15,16). For example, in the study by LaTulippe et al, they reported the altered expression of several genes that correlate with the metastatic phenotype in prostate cancer and those genes were grouped into functional categories, such as cell cycle regulation, DNA repair, cell structure, and motility among others. Although we cannot correlate directly the results presented by LaTulippe et al (clinical samples) with our results (rat cell lines samples), those results by LaTulippe et al showed a similar trend of changes in the metastatic prostate cancer than our data (Table 2), demonstrating the validity of our approach and the significance of our results.

For specific gene validation, two approaches were used-correlation with gene expression database results and with qRT-PCR analysis. In this way, the data were matched with the Cancer Genome Anatomy Project database (cgap.nci.nih.gov) and with the Prostate Expression database (17). Each gene was first matched with the corresponding human gene using the *Entrez Gene* identification number, since the databases are for *Homo sapiens* species. Table 2 indicates the many genes

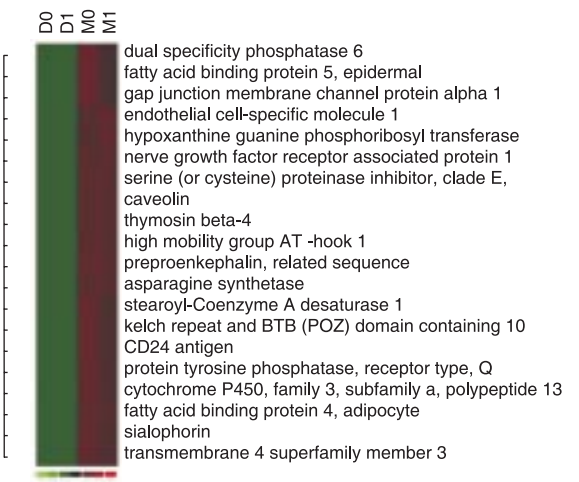

Figure 1. Hierarchical clustering: overexpressed genes (red color) in MAT-LyLu (MLL).The figure representing the hierarchical clustering was automatically generated by dChip v1.3 software (www.dchip.org) using the Pearson correlation metric. The 20 most overexpressed genes are shown.

Table 1. GenMAPP/MAPPFinder pathway analysis of differentially expressed genes in the MAT-LyLu cell line compared to the G cell line. Signaling and Metabolic Pathways that showed significant differential expression in the MAT-LyLu cell line compared to the G cell line (Z score ≥ 2.0). After GenMAPP/ MappFinder analysis, pathways were ranked based on the Z score and percentage of genes changed. The cut-off value chosen for Z score was 2.0 (equivalent to p =0.05 for the hypergeometric distribution). The number and percentage of genes changed in each pathway and the corresponding Z score were calculated with the MAPPFinder program.

A. Signaling pathway/ individual genes	***Entrez gene***	**Fold change**	**% changed**	**Z score**
Androgen receptor			**17**	**3.3**
Cav (Caveolin 1)	25404	31.05		
Pten (phosphatase and tensin homolog)	50557	6.64		
Mdm2_predicted	314856	1.81		
Pxn (paxillin)	360820	2.92		
Cyclin D1	58919	7.95		
Caspase 1	25166	-11		
Focal Adhesion			**14**	**3.3**
fibronectin 1	25661	3.4		
laminin, alpha 3	286966	2.47		
Spp1 (secreted phosphoprotein 1)	25353	4.1		
Cav (Caveolin 1)	25404	31.05		
Cav3 (caveolin 3)	29161	-2.3		
Ptk2 (protein tyrosine kinase 2, Focal adhesion kinase 1, FAK)	25614	-2.0		
Pxn (paxillin)	360820	2.92		
Pten (phosphatase and tensin homolog)	50557	6.64		
Rock2 (Rho-associated coiled-coil forming kinase 2)	25537	2.2		
Cyclin D1	58919	7.95		
Ccnd3 (cyclin D3)	25193	-4.7		
Sepp1 (selenoprotein P)	29360	-23.0		
Mapk6(mitogen-activated protein kinase 6)	58840	3.1		
Wnt signaling			**25**	**3.6**
Prkcd (protein kinase C, delta)	170538	2.28		
Fzd1 (frizzled homolog 1)	58868	-2.57		
Fzd2 (frizzled homolog 2)	64512	-3.77		
Cyclin D1	58919	7.95		
Ccnd3 (cyclin D3)	25193	-4.7		
Fosl1 (fos-like antigen 1)	25445	14.5		
Plau (plasminogen activator, urokinase)	25619	4.68		
Hepatocyte growth factor receptor			**24**	**3.5**
Met (met proto-oncogene:Hepatocyte growth factor receptor)	24553	2.57		
Pten (phosphatase and tensin homolog)	50557	6.64		
Pxn (paxillin)	360820	2.92		
Ptk2 (protein tyrosine kinase 2)	25614	-2.0		
Map2k2 (mitogen activated protein kinase kinase 2)	58960	-1.8		
Epidermal growth factor receptor 1			**13**	**2.6**
Arf4 (ADP-ribosylation factor 4)	79120	1.71		
Cav (Caveolin 1)	25404	31.05		
Gja1(gap junction membrane channel protein alpha 1)	24392	10.53		
Kras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog)	24525	-1.8		
Ddef1_predicted (development and differentiation enhancing,	314961	-2.7		
predicted) Krt2-8 (keratin complex 2, basic, gene 8)	25626	22.6		
Pxn (paxillin)	360820	2.92		
Dnm1(dynamin 1)	140694	-1.84		
Ptpn12 (protein tyrosine phosphatase, non-receptor type 12)	114856	1.85		
Dusp1 (dual specificity phosphatase 1)	114856	-2.3		
Stat1(signal transducer and activator of transcription 1)	25124	-3.23		
Stat2 (signal transducer and activator of transcription 2)	288774	-2.3		
MAPK signaling pathway			**12.6**	**2.5**
Kras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog)	24525	-1.8		
Map2k2 (mitogen activated protein kinase kinase 2)	58960	-1.8		
Map3k2	25579	1.93		

A. Signaling pathway/ individual genes	*Entrez gene*	Fold change	% changed	Z score
Prkcd (protein kinase C, delta)	170538	2.28		
Casp1 (caspase 1)	25166	-11.1		
Mapk6(mitogen-activated protein kinase 6)	58840	3.1		
Arrb1 (arrestin, beta 1)	25387	1.74		
Ppm1a (protein phosphatase 1A, magnesium dependent, alpha isoform)	24666	2.52		
Hspb1 (heat shock 27kDa protein 1)	24471	-3.75		
Ddit3 (DNA-damage inducible transcript 3)	29467	-2.45		
Dusp5 (dual specificity phosphatase 5)	171109	2.35		
Dusp6 (dual specificity phosphatase 6)	116663	11.08		
Integrin mediated cell adhesion			**14.2**	**2.3**
Pxn (paxillin)	360820	2.92		
Cav1(caveolin 1)	25404	31.05		
Cav3 (caveolin 3)	29161	-2.3		
Ptk2 (protein tyrosine kinase 2)	25614	-2.0		
Rock2 (Rho-associated coiled-coil forming kinase 2)	25537	2.2		
Mapk6(mitogen-activated protein kinase 6)	58840	3.1		
Map2k2 (mitogen activated protein kinase kinase 2)	58960	-1.8		
Sepp1 (selenoprotein P)	29360	-23.0		
Itga7 (integrin alpha 7)	81008	2.93		
Capn2 (calpain 2)	29154	2.34		
TGFbeta signaling			**16**	**2.1**
Tgfb1 (transforming growth factor, beta 1)	59086	2.53		
Ltbp1 (latent transforming growth factor beta binding protein)	59107	4.79		
Fst (follistatin)	24373	5.91		
Stat1 (signal transducer and activator of transcription 1)	25124	-3.23		
Serpine2 (serine (or cysteine) peptidase inhibitor, clade E, member 2)	29366	8.61		
Sepp1 (selenoprotein P)	29360	-23.0		
Insulin signaling			**11.4**	**2.0**
Slc2a1 (solute carrier family 2 (facilitated glucose transporter), member 1)	24778	1.93		
Stx4a (syntaxin 4A (placental)	81803	2.37		
Arf6 (ADP-ribosylation factor 6)	79121	4.53		
Rhoj (ras homolog gene family, member J)	299145	-1.92		
Egr1 (early growth response 1)	24330	2.39		
Pten | phosphatase and tensin homolog	50557	6.64		
Prkcd (protein kinase C, delta)	170538	2.28		
Prkaa2 (protein kinase, AMP-activated, alpha 2 catalytic subunit)	78975	-7.7		
Mapk6(mitogen-activated protein kinase 6)	58840	3.1		
Map3k12 (mitogen activated protein kinase kinase kinase 12)	25579	1.93		

B. Metabolicpathway/ individual genes	*Entrez gene*	Fold change	% genes changed	Z score
Cholesterol biosynthesis			**53**	**6.8**
Hmgcs1 (3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1)	29637	6.65		
Hmgcr (3-hydroxy-3-methylglutaryl-Coenzyme A reductase)	25675	3.49		
Fdps (farnesyl diphosphate synthase)	83791	4.06		
Sc4mol (sterol-C4-methyl oxidase-like)	140910	16.15		
Cyp51 (cytochrome P450, subfamily 51)	25427	3.84		
Sqle (squalene epoxidase)	29230	3.23		
Sterol biosynthesis			**45**	**5.1**
Hmgcr (3-hydroxy-3-methylglutaryl-Coenzyme A reductase)	25675	3.49		
Fdps (farnesyl diphosphate synthase)	83791	4.06		
Nqo1(NAD(P)H dehydrogenase, quinone 1)	24314	2.14		
Sqle (squalene epoxidase)	29230	3.23		
Nucleotide metabolism			**30**	**2.9**
Prps1 (phosphoribosyl pyrophosphate synthetase 1)	29562	8.25		
Prps2(phosphoribosyl pyrophosphate synthetase 2)	24689	5.37		
Hprt (hypoxanthine guanine phosphoribosyl transferase)	24465	16.53		

were expressed in cancer and/or in prostate samples.

For qRT-PCR analysis, we chose the krp1, ESM-1 and CAV-1 genes were chosen because they represented overexpressed genes relevant to the current study. These genes were validated by qRT-PCR with statistically significant p values (< 0.05) (Table 3). The levels of gene expression determined by qRT-PCR analyses were consistent with those observed in the DNA microarray experiments. For example, the 36- fold increase in expression of krp1 determined by DNA microarray analysis was validated by the 48-fold increase observed by qRT-PCR. These results validated our DNA microarray analysis.

Discussion

Prostate cancer progression from a localized form to a metastatic state seems to be regulated by several oncogenic signals in the prostate epithelial cells and by changes in the stromal-epithelial interaction. These include the Hedgehog signaling and Wnt signaling pathways (18,19) as well as overexpression of several growth factors and their receptors, such as the epidermal growth factor, fibroblast growth factor, HGF, insulin-like growth factor-1 and vascular endothelial growth factor (VEGF) (20). In agreement with published observations, the metastatic phenotype of MAT-LyLu cell line was associated to overexpression of genes involved in several of these signaling pathways. Examples of the overexpressed genes included met proto-oncogene (HGF receptor), phosphatase and tensin homologue on chromosome 10 (PTEN), paxillin (Pxn), and caveolin (Cav)-all of which are important components of the HGF receptor, androgen receptor, epidermal growth factor receptor, integrin mediated cell adhesion, and focal adhesion signaling pathways (Table 1).

The HGF receptor, also called c-Met, is a heterodimer with tyrosine kinase activity that associates with a multiprotein complex involved in downstream signal transduction (21).

c-Met is activated by the HGF and it has been shown to induce alterations in cell motility, cell shape, adhesion, resistance to apoptosis, and anchorage independent growth. These all contribute to the role of c-Met in cancer and makes this receptor a potential therapeutic target for cancer drugs (22-24). c-Met has also been found to target paxillin (overexpressed in the current study) and focal adhesion kinase (FAK), an activity that can lead to alterations in cell motility and tissue invasion by transformed cells (25,26). The overexpression of several genes in the HGF receptor pathway in the MAT-LyLu cell line (Table1) was also notable.

The clinically aggressive phenotype distinctive of the metastatic cascade (14), has been studied at the gene expression level (15,16). In those studies, the overall gene expression alteration in the metastatic phenotype agrees with the data herein, with similar patterns of altered expression in genes associated with signaling transduction, ECM remodeling, motility, cell cycle regulation, and cell structure. An important validation of the cell line base model used herein are results from studies (15,16) done in human samples which fully agree with the MAT-LyLu data.

Genes relevant for the metastatic phenotype, found with altered expression in the current study, include ESM-1, CD24 antigen, biglycan (BGN), serpine2, fibromodulin, macrophage stimulating 1, alpha-2-macroglobulin, glycoprotein 38, decorin, vascular cell adhesion molecule 1, clusterin, and elastin. These 12 genes are grouped in the ECM interaction functional group. Several of these genes encode proteoglycan (PG) proteins (ESM-1, BGN, fibromodulin, and decorin) which are major components of the ECM that maintain the integrity of structural tissue. However, ESM-1, BGN and fibromodulin have not been associated previously with prostate cancer metastasis. Furthermore, the most overexpressed gene in MAT-LyLu, krp1-which encodes a protein associated with formation of elongated pseudopodia in transformed rat fibroblast (27)-has not been previously associated to prostate cancer metastasis. Additional studies are necessary to determine the association of these genes to prostate cancer metastasis in the human counterpart. Currently experiments are underway in our laboratory to determine the status of expression of ESM-1 gene and protein in human prostate cancer.

Table 2. Significant differentially expressed genes in the Mat-LyLu cell line compared to the G cell line. Positive fold changes indicate overexpression of the gene in Mat-LyLu; negative fold changes indicate underexpression. P value from T-test (two-sided).Genes were grouped into functional categories according to GO classifications.

Functional Group/ individual genes	Entrez gene	Fold change	P value
Extracellular/ECM			
Endothelial cell-specific molecule 1[1]	64536	26.5	0.014186
Protein tyrosine phosphatase, receptor type, Q	360417	19.72	0.011614
CD24 antigen[1,2]	25145	15.56	0.030306
Gap junction membrane channel protein alpha 1[1]	24392	10.53	0.037434
Transmembrane 4 superfamily member 3	171048	10.4	0.016358
Serpine2[1,2]	29366	8.61	0.006009
Activated leukocyte cell adhesion molecule[1,2]	79559	6.65	0.009178
Fibromodulin[1]	64507	2.62	0.010866
Plasminogen activator inhibitor 2 type A[1]	60325	2.11	0.005814
Procollagen, type XII, alpha 1[1]	25683	-2.18	0.005956
Glycoprotein Ib (platelet), beta polypeptide	116727	-2.5	0.017437
Macrophage stimulating 1 (hepatocyte growth factor-like)[1]	24566	-2.61	0.007459
Alpha-2-macroglobulin[1,2]	24153	-3.8	0.007349
Hyaluronan and proteoglycan link protein 1[1]	29331	-3.82	0.011615
Glycoprotein 38	54320	-3.87	0.015588
Decorin	29139	-5.74	0.00339
Vascular cell adhesion molecule 1[1]	25361	-7.82	0.012319
Clusterin[1]	24854	-8.84	0.000569
Elastin[1]	25043	-12.76	0.002621
Biglycan[1,2]	25181	-13.23	0.023713
Selenoprotein P, plasma, 1[1,2]	29360	-23.08	0.025153
Cell motility			
Kelch repeat and BTB (POZ) domain containing 10 (krp1)[1]	117537	36.21	0.031316
Defense response			
Preproenkephalin, related sequence	29237	24.63	0.002009
Sialophorin[1]	24796	24.14	0.020209
T-cell receptor gamma chain[1]	24821	-2.2	0.013275
Myxovirus (influenza virus) resistance 2[1]	286918	-2.22	0.005247
Major histocompatibility complex, class II, DM alpha[1]	294274	-2.37	0.012974
MHC class Ib RT1.S3[1]	294228	-4.8	0.027941
RT1 class Ib, locus Aw2	24737	-5.18	0.025273
Similar to interferon regulatory factor 7 (LOC293624), mRNA	293624	-6.14	0.030563
Development			
Thymosin beta-4[1]	81814	19.82	0.003524
Glutamate receptor interacting protein 1[1]	84016	8.4	0.045733
Synuclein, gamma[1]	64347	6.62	0.01336
Late gestation lung protein 1[1]	171547	-2.26	0.022152
Transgelin[1]	25123	-5.72	0.028973
Magnesium ion binding			
Hypoxanthine guanine phosphoribosyl transferase	24465	11.45	0.021844
Phosphoribosyl pyrophosphate synthetase 1[1,2]	29562	8.25	0.010677
Phosphoribosyl pyrophosphate synthetase 2[1]	24689	5.37	0.000847
UDP-Gal:betaGlcNAc beta1,4-galactosyltransferase, polypeptide 6[1]	65196	3.4	0.009301
Ectonucleoside triphosphate diphosphohydrolase 2[1]	64467	-2.7	0.022148
MAP kinase phosphatase activity			
Dual specificity phosphatase 5[1]	171109	2.35	0.01035
Dual specificity phosphatase 1[1]	114856	-2.3	0.005724
Oxidoreductase activity			
Cytochrome P450, family 3, subfamily a, polypeptide 13[1]	171352	14.28	0.010339

[1]Genes found in the Cancer Genome Anatomy Project
[2]Genes found in the Prostate Expression database

Functional Group/ individual genes	Entrez genes	Fold change	P value
3-hydroxy-3-methylglutaryl-Coenzyme A reductase[1]	25675	3.49	0.011653
Branched chain keto acid dehydrogenase E1, beta polypeptide	29711	2.14	0.006807
Cytochrome P450, subfamily 24	25279	2.11	0.040331
Cytochrome b-245, alpha polypeptide[1,2]	79129	-3.64	0.024939
3-alpha-hydroxysteroid dehydrogenase	191574	-4.86	0.015007
Proteolysis and peptidolysis			
Plasminogen activator, urokinase[1]	25619	4.68	0.017576
Cathepsin S[1]	50654	-2.33	0.009061
Alanyl (membrane) aminopeptidase[1,2]	81641	-3.18	0.022316
Dipeptidase 1 (renal)	94199	-6.82	0.035748
Caspase 1[1]	25166	-11.12	0.018091
Regulation of cell cycle /proliferation			
Caveolin 1[1,2]	25404	31.05	0.0005
Cyclin D1[1,2]	58919	7.95	0.00532
B-cell translocation gene 3[1]	54230	-17.35	0.039715
CD 81 antigen[1]	25621	-36	0.048536
Regulation of transcription/ DNA-binding			
High mobility group AT-hook 1[1]	117062	15.41	0.001102
Cxxc5	292770	4.25	0.008338
HNF-3/forkhead homolog-1	64826	3.08	0.009154
Similar to RIKEN cDNA 4930415K17 (LOC291670), mRNA	291670	-2.28	0.008314
Nuclear receptor subfamily 4, group A, member 2	54278	-2.62	0.020084
LIM homeobox protein 2[1]	117555	-5.43	0.046292
Transporter activity			
Fatty acid binding protein 4, adipocyte[1]	79451	34.02	0.006713
Fatty acid binding protein 5, epidermal[1]	140868	15.38	0.049212
Rabin 3	29885	6.46	0.023285
Lectin, galactose binding, soluble 9[1]	25476	-2.46	0.017291
apolipoprotein E	25728	-29.52	0.010995
Ubiquitin activity			
Ubiquitin carboxy-terminal hydrolase L1[1]	29545	-16.29	0.006928
Calcium ion binding			
Phospholipase C, delta 4[1]	140693	2.64	0.01117
Parvalbumin	25269	-3.85	0.004735
Purkinje cell protein 4	25510	-6.82	0.036186
Carboxylesterase activity			
Carboxylesterase 1[1]	29225	-2.16	0.038405
DNA repair			
O-6-methylguanine-DNA methyltransferase[1]	25332	-4.62	0.006563
Enzyme inhibitor activity			
Decay-accelarating factor	64036	4.02	0.002607
Glucuronosyltransferase activity			
UDP glycosyltransferase 1 family, polypeptide A6[1]	113992	-3.25	0.018816
Receptor activity			
Coagulation factor II (thrombin) receptor-like 1	116677	4.96	0.017166
Endothelial differentiation, sphingolipid G-protein-coupled receptor, 5	29415	-2.27	0.003533
Growth hormone receptor[1]	25235	-3.12	0.008131
Growth factor			
Insulin-like growth factor 1[1]	24482	-4.85	0.010787
Insulin-like growth factor binding protein 5[1,2]	25285	-5.25	0.028276
Induction of apoptosis			
Nerve growth factor receptor associated protein 1[1]	117089	8.75	0.000319

[1]Genes found in the Cancer Genome Anatomy Project
[2]Genes found in the Prostate Expression database

Functional Group/ individual genes	Entrez genes	Fold change	P value
Kinesin complex			
Peripherin 1	24688	-7.17	0.001239
Ligase activity			
Asparagine synthetase[1]	25612	14.65	0.007699
Arginosuccinate synthetase[1]	25698	-12.75	0.006917
Lactate dehydrogenase activity			
Lactate dehydrogenase B	24534	-15.07	0.013633
Phosphatase activity			
Dual specificity phosphatase 6[1]	116663	11.08	0.025634
Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform[1]	60660	2.31	0.00634
Protein tyrosine phosphatase, receptor type, N[1]	116660	2.22	0.029516
Transcription coactivator activity			
Calcium/calmodulin-dependent serine protein kinase[1]	29647	-4.13	0.005154
Transferase activity			
UDP glycosyltransferase 1 family polypeptide A2[1]	396527	5.93	0.019318
Aminolevulinic acid synthase 1[1]	65155	-2.39	0.015592
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2[1]	24450	-3.17	0.024703
Alcohol dehydrogenase activity			
Alcohol Dehydrogenase 1[1]	24172	-6.38	0.009355
Hydrolase activity			
Adenosine monophosphate deaminase 3[1]	25095	2.14	0.045491
Phosphodiesterase 3B[1,2]	29516	-4.76	0.024876
Fatty acid metabolism			
Stearoyl-Coenzyme A desaturase 1	246074	25.92	0.031324
Other			
Hypothetical LOC290595 (LOC290595), mRNA	290595	8.46	0.002463
Similar to Tubulin alpha-4 chain (Alpha-tubulin 4) (LOC316531), mRNA	316531	4.76	0.017132
Similar to chromosome 14 open reading frame 50 (LOC299153), mRNA	299153	3.58	0.019238
Guanine nucleotide binding protein beta 4 subunit mRNA, partial cds	294962	3.38	0.017726
Similar to kelch domain containing 3; testis intracellular mediator protein (LOC363192), mRNA	363192	-2.17	0.03046
Similar to cornichon-like protein (LOC361705), mRNA	361705	-2.35	0.004193
Similar to RNA-binding protein with multiple splicing (RBP-MS) (LOC361161), mRNA	361161	-2.81	0.024735
Similar to CLN6 protein (LOC315746), mRNA	315746	-3.68	0.00706
Reticulocalbin 3	308580	-3.94	0.027229
Similar to CG31613-PA (LOC364716), mRNA	364716	-8.61	0.047281

[1]Genes found in the Cancer Genome Anatomy Project
[2]Genes found in the Prostate Expression database

Table 3. Genes validated by qRT-PCR. Selected genes were validated by qRT-PCR.

Gene name	Microarray Fold change	qRT-PCR Fold change	qRT-PCR p value
Kelch repeat and BTB (POZ) domain containing 10 (krp1)	36.21	48	0.01816
Endothelial cell-specific molecule 1	26.5	23	0.00995283
Caveolin 1	31.05	45	0.0009527

Overexpression of ESM-1 in the tumor tissue may constitute a negative prognostic factor and a marker for metastasis for cancer patients. This concept has been suggested by others in cases of breast cancer (28), renal cancer (29), and lung cancer (30). However, the current study is the first to link ESM-1 to metastatic prostate cancer.

Fibromodulin, overexpressed, and BGN and decorin, underexpressed, are genes that encode PG proteins that are members of the small leucine-rich PG family. They function, under physiological and pathophysiological conditions, as regulators of matrix assembly, cellular adhesion, migration and transforming growth factor beta (TGF-beta) activity (31,32). In the current study, differential expression of genes in the TGF-beta signaling pathway was found as well (Table 1).

Other genes associated with cell-ECM interaction are CD24 and Serpine 2. CD24 gene, overexpressed in the current study, encodes a small, heavily glycosylated and mucin-like cell surface protein. CD24 has been found overexpressed previousely in prostate cancers (33), and this overexpression has been suggested as predictor of prostate cancer relapse and poor prognosis (34).

Serpine 2 (protease nexin I), found over expressed in the current study, has been implicated in invasive potential of pancreatic cancer cells because it alters ECM production and organization within the tumors (35). Serpine 2 mRNA levels have been found overexpressed in prostate cancer cell lines and possibly interacts negatively with prostasin, an invasion suppressor protein (36).

Another overexpressed gene in the highly metastatic MAT-LyLu cell line, was the Cav-1 gene. Cav-1 was grouped in the regulation of cell proliferation group and encodes a protein that is a component of caveolae membrane coats. In human and animal studies, Cav-1 was overexpressed in metastatic prostate cancer; antisense-mediated down regulation of Cav-1 in prostate cancer cells *in vitro* seems to reduce the metastatic phenotype (37,38). Therefore, Cav-1 is considered a metastasis-related gene in prostate cancers.

Finally, the most underexpressed gene was the CD81 gene that encodes a cell-surface protein and belongs to the tetraspanin superfamily (TM4SF). Loss of CD81 has been found in poorly differentiated hepatocellular carcinoma and extrahepatic metastasis patients (39). CD81 has not been associated with prostate cancer previously; however, another member of the tetraspanin family, KAI1/CD82, has been suggested as a prostate cancer metastasis suppressor gene. The underexpression of the CD81 gene in the current study warrants further investigation to determine its exact function and its potential as a suppressant of metastasis in prostate cancer.

In summary, overexpressed and underexpressed genes were successfully identified in a highly metastatic rat prostate cancer cell line, when compared to a non metastatic rat prostate cancer cell line. The cell lines had been previously established as *in vitro* models for studying prostate cancer. To extrapolate results obtained from this work to the human context, further studies are needed with human samples and detailed clinical characterization of the patients for appropriate clinical correlations. Nonetheless, the identification of genes differentially expressed in these two rat cell lines by DNA microarray analysis is a first logical step in the search for dependable biomarkers for human prostate cancer metastasis.

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Conflict of interests

The authors declare that they have no competing interests.

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