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13. ABSTRACT *(Maximum 200 Words)*

There is strong evidence that the expression of genes involved in signal transduction such as protein kinases is altered in tumor cells and that the aberrant expression of one or several of these genes parallels the progression of tumors to a more malignant phenotype. We developed a DNA micro-array based screening system to monitor the level of expression of tyrosine kinase (tk) genes and to derive quantitative information to support prognostication and therapeutic decisions. In the first year of this effort, we finished the development and testing of hardware necessary to prepare the DNA micro-arrays, performed RNA extraction, cDNA preparation and labelling reactions, and defined a working protocol for array hybridization and quantitative analysis. Our prototye design simultaneously measures the expression of 63 different tk genes. Additional targets for investigation will be defined by the ongoing molecular cloning and sequencing of gene transcripts found in prostate cancer cell lines and clinical specimens. Using a set of phenotypically well characterized prostate cancer cell lines, the system has proven to be able to deliver reproducible data regarding changes in tk gene expression during cell transformation and progression towards a more malignant phenotype.

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INTRODUCTION:

Aberrant expression of receptor or cytosolic tyrosine kinase genes and, in particular, their hyper-expression are common phenomena in prostate cancer, which are believed to alter cell growth and response to external signals such as growth factors, hormones etc. Knowledge about the relative levels of expression of many tyrosine kinase genes, all at the same time, might contribute significantly to a better understanding of the processes of tumor development and progression. We are developing a rapid assay that will use innovative DNA micro-arrays carrying small amounts of individual tyrosine kinase gene-specific targets to simultaneously determine the expression levels of up to 100 tyrosine kinase genes using a small number of cells. Three years of research and development will lead to discovery of a set of gene-specific markers associated with prostate cancer progression and a simple device capable of performing inexpensive expression profiling of these markers. The research and development efforts in the first year of this 3-year project focused on the design and testing of robotic instruments to prepare DNA micro-arrays and the preparation of prototype arrays carrying sets of more than 50 gene-specific tyrosine kinase fragments. Additional effort was directed towards the optimization of cDNA preparation, labeling, hybridization and detection protocols as well as the molecular cloning and sequencing of prostate cancer-specific tyrosine kinase gene transcripts.

BODY:

To extend our pre-existing panel of tyrosine kinase (tk) genes and to identify tk genes expressed in normal, benign hyperplastic and neoplastic prostate tissues, we isolated RNA's and synthesized cDNA's from four different prostate cancer cell lines: DU145, ND-1, PC-3 and LNCaP (cell lines provided by 2 different sources). The cDNA's were prepared by reverse transcription using a random oligonucleotide primer and a commercial kit (Ambion Inc.).

In addition, we collaborated with Drs. Simon Hayward and Jerry Cunha at the University of California, San Francisco (UCSF), who provided cultures of nine phenotypically well characterized cell lines representing different stages of malignant transformation of prostate epithelium. The relation between individual cell lines in their model system of prostate tumor initiation and progression is shown in Table I below.

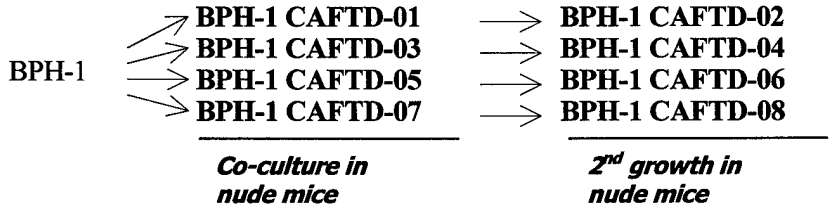

Table I: The relation between individual cell lines in the BPH-1 lineage.

Briefly, this model system is based on prostatic epithelial cells immortalized by transformation with SV40 large T antigen (BHP-1, Table I). The BPH-1 cells were immortalized, but did not form tumors in mice. When they were mixed with cancer associated fibroblasts (CAF) and injected in the kidney capsule of nude mice, they formed tumors. Four independent tumor-derived cell lines were labeled 'BPH-1 CAFTD-01, -03, -05 and –07'. These tumor cell cultures were harvested, expanded and re-injected into nude mice giving rise to tumors free of CAF cells. Cells harvested from these

second generation tumors were brought into culture. These cultures (cell lines 'BPH-1 CAFTD-02, -04, -06 and –08') were labeled according to the scheme shown in Table I.

We began to clone and characterize the tk genes expressed in the four prostate cancer cell lines (DU145, ND-1, PC-3 and LNCaP) and all nine of the BHP-1 derived cell lines. Using mixed-base oligonucleotides specific for conserved sequences in tk genes, we amplified ~160-170 bp fragments encoding the catalytic domain of expressed tk genes in a PCR reaction. We then size selected and cloned the PCR products into a suitable plasmid vector (pAmp1) using commercially available kit (UDG cloning kit; Gibco/Life Technologies). Ampicillin resistant clones were picked from agar plates and their insert sizes were determined by agarose gel analysis of PCR products generated with vector-specific PCR primers. As of March 2001, we cloned and partially characterized 336 tk fragment containing plasmid clones derived from prostate cancer and BPH-1 derived cell lines. These clones join more than 400 tk fragment containing clones which were previously isolated from thyroid and breast tumors. From all these clones, DNA is isolated, bound to nylon filters and prescreened with probes prepared from known tk (Figure 2 in Appendix 1). Following this pre-screening step, we perform cDNA sequencing and database searches and add novel clones to the panel of expressed tk gene fragments. DNA isolated from this panel of tk gene fragments is then arrayed and printed onto glass slides to measure tk gene expression in cell lines, normal and tumor tissues.

During the report period, we finished the construction and test of a robotic system capable of printing DNA microarrays with about 100 different sequences on glass slides. The arrayer (Figure 1 in Appendix 1) can print on up to 91 slides in a single run. Typically, we print approximately 50 slides per run, with duplicate arrays on each slide. The stainless steel printing pin has an open slit in the tip cut by 0.001 inch Electrical Discharge Machining (EDM) wire - a tool commonly employed in precision semiconductor manufacturing. Each loading takes up approximately 1 μl and allows continuous printing of more than 100 dots due to the capillary function. A full 384-well plate of DNA samples can be arrayed onto 50 slides (in duplicate) in 8 hrs, including the time needed for repeated wash and drying steps in each printing cycle. This arrayer can print more complex DNA arrays in the same time frame if more printing pins are installed. For example, 12,288 clones can be printed in the same time interval with 32 pins loaded in the print head. When the DNA is dissolved at a concentration of 250ng/μl in 50% dimethyl sulfoxide (DMSO) printing solution, spot diameters range from 80-125 μm printed on poly-L-lysine coated slides. The arrayer can print to a precision of approximately 15μm. Therefore, if the spots are positioned 200μm apart (center-to-center), they are sufficiently spaced.

The fabrication of tyrosine kinase (tk) cDNA arrays on glass slides was done in-house with our DNA micro-arrayer. Standard microscope glass slides were thoroughly cleaned with concentrated sodium hydroxide and ethanol before coating with poly-L-lysine. These two chemical steps provided a positively charged layer to bind DNA to the slides. The coated slides were stored in closed slide boxes two weeks before they were used for printing. This "aging" process proved to promote surface chemistry of the slides for better array spot morphology. We also evaluated surface-treated slides for DNA micro-array printing from different vendors, but concluded that in-house prepared poly-L-lysine coated slides showed superior DNA binding and lower levels of autofluorescence.

Array quality control was monitored throughout the fabrication process. Since dust can be a major source of background fluorescence and can clog slotted steel pins, we enclosed the arrayer and

installed an air filter. All reagents used for printing and coating of slides were prepared fresh and filtered. Before post-printing processing of the arrays, morphology of the spot as well as DNA distribution within the spots were examined with DAPI staining and visualized in a Zeiss fluorescence microscope. After post-printing processing, arrays were stained with POPO-3 dye (10 μM) and scanned at Cy3 channel to check the retention of DNA on the arrays for hybridization.

A Zeiss Axioplan II Mot fluorescence microscope was programmed to acquire an fluorescence image of arrays of 10x10 spots in 9 image frames. Image acquisition and processing were automated by macros to show details of spots with 1 micron resolution. While being less sensitive than commercial DNA microarray scanners, the system is being used routinely for quality control of each batch of new arrays.

After printing, the locations of arrays were marked on the reverse side of the slide with diamond marker pen since the array will become invisible after processing. The slides were placed up side down in re-hydration chamber filled with 1x SSC. Between 5 and 15 minutes (depending on the size of the array) allowed the arrays to re-hydrate, but care was taken not to allow spots to swell too much and run into each other. Hydrated slides were placed on a heat plate pre-set to 70 ^{0}C for 3 seconds to snap-dry the array. These slides were then cross-linked by an UV cross linker (such as Stratalinker 1800 or 2400, Stratagene Inc.) at 65 mJ. Cross-linking was optional, however, it did promote the bonding of DNA onto the poly-L-lysine coating. After cross-linking, slides were incubated in a blocking solution consisting of 0.21M succinic anhydride in 1-methyl-2-pyrrolidinone, 0.06M sodium borate for 20 minutes (pH 8.0). The slides were then denatured in a boiling water for 2 minutes. The denatured arrays were submerged in a 95% ethanol bath for 1 minute. This step de-hydrated the denatured DNAs and allowed them to remain single-stranded. An immediate centrifugation at 500 rpm for 5 minutes removed any trace ethanol or water. The arrays were then ready for hybridization.

To facilitate printing of DNA on nylon filters for recombinant clone screening, we designed a printing tool that can be mounted on the Beckman Biomek 1000 robot in our laboratory. The tool (Figure 2) can hold up to 32 steel pins arranged so that they fit directly into the wells of a 384-well microtiter plate. The slotted pins (purchased from V&P Scientific, San Diego, CA) carry about 50 nl of liquid. The entire volume is deposited in one spot on a positively charged nylon filter. The pins needs to be refilled prior to each print step. In between DNA samples, the print pins are washed thoroughly and blot dried. The system can array a 96 well microtiter plate in a little more than 1 hour spotting all 96 wells in duplicate as well as printing three such micro-arrays. We are presently comparing the sensitivity of hybridization to the filter bound arrays with the results obtained with glass bound arrays. Sample prints are shown in Figure 2 (Appendix 1) following hybridization and washes at low and high stringency.

It was necessary to adjust optimize hybridization and wash conditions to obtain specificity and reduce background fluorescence to a level where quantitative information could be obtained. The slides were placed inside hybridization chambers with a cover fastened tightly by clamps. Multiple drops of 3X SSC were added around the arrays on the slide to maintain the humidity and prevent drying of hybridization solution. Hybridization was performed under glass coverslips overnight at 65 ^{0}C. The arrays were then washed in 3 changes of wash solution (1x SSC+ 0.03% SDS) at room temperature for 5 minutes, 0.2x SSC at room temperature for 1 minute, and then 0.2x SSC at 50 ^{0}C for 1 minute. After the washes, the slides were dried immediately by centrifugation at 500 rpm for 5 min before scanning. Current sample preparation and hybridization procedures have produced signal to background (noise) ratios of more than 100 (in the green channel) to more than 200 (in the

red channel). This dynamic range can be further improved by a reduction of background fluorescence.

We also investigated algorithms for array readout comparisons between measurements and data mining. The Axon GenePix 4000 (Axon Inc.) array scanner used to acquire all images has a preview resolution of 40 μm and a scanning resolution of 10 μm. The photomultiplier sensitivity can be adjusted by the user during preview to optimize the signal intensity. GenePix 3.0 (Axon Inc.), an image acquisition and analysis software, was used to analyze the images acquired from our tk arrays and provided numerical data that was imported into spreadsheets for further analysis. For display purposes, the images were saved in standard formats and imported into common graphics programs such as Adobe Photoshop.

The performance of the system was tested by hybridization of fluorochrome-labeled tk gene-specific PCR fragments onto our tk-specific DNA micro-arrays. In preliminary experiments, we used a first generation of DNA micro-arrays comprised of genes previously identified to be expressed in thyroid tumors (Table II, Appendix 2). This allowed us to optimize hybridization and wash conditions, and to generate data regarding the relative level of tk gene expression in various cancer cell lines. We then prepared DNA micro-arrays carrying a more extensive panel of tk genes (Table III, Appendix 2) and found that hybridization to these arrays provide information relevant to tumor progression and differences between cell lines (Figure 5). For example, we hybridized a combination of Cy5-labelled tk fragments prepared from cell line BPH-1 (shown in red in Appendix 3, Figure 4) and Cy3-labelled tk fragments prepared from cell line BPH-1 CAFTD-02 (shown in green in Appendix 3, Figure 4) to our 'new' DNA micro-array carrying more than 60 tk gene fragments (Table III, Appendix 2). Scanning of the array revealed specific differences between the parental and the tumorigenic cell lines. Genes expressed at a higher level in BPH-1 CAFTD-02 cells lead to increased green signals on the array, while genes with expression levels lower in BPH-1 CAFTD-02 cells than in BPH-1 cells generated spots that exhibited stronger red fluorescence (Figure 4, Appendix 3). The Genepix software provided quantitative information about Cy3 and Cy5 fluorescence intensities for each of the spots, which allowed us to calculate fluorescence intensity ratios comparing test and reference cDNA probes. All spots have a tk fragment clone number associated with them, which are specific for particular tyrosine kinase genes. In our preliminary analysis, we defined a gene as being up-regulated or down-regulated in the tumorigenic cell line when the average Cy3/Cy5 ratio exceeded 2.0 or fell under 0.4, respectively. Using these arbitrary cut-off values, we identified several genes with altered expression in the tumorigenic lines. The most drastic change in BPH-1 CAFTD-02 cells compared to the parental BPH-1 cell lines appeared to be an up-regulation in the expression of Ephrin B1 (arrows in Figure 4 (TK5B), Appendix 3), a gene whose product is likely to be involved in the interactions between cells or between cells and the extra-cellular matrix. Other genes expressed in higher levels in the tumorigenic line BPH-1 CAFTD-02 compared to the parental line included c-mer, Ephrin A2 and $p56^{lck}$.

When investigating expression pattern in other BPH-1 derived cell lines, we found additional characteristic changes. For example, cell line BPH-1 CAFTD-07 showed high levels of expression of HLA-A, which was not observed in BPH-1 or BPH-1 CAFTD-02 cells (arrows in Figure 5 (TK16B), Appendix 3). While it showed relatively high levels of expression of Ephrin A2 and $p56^{lck}$, this cell line differed from BPH-1 CAFTD-02 in its up-regulated expression of c-fms and two novel kinases for which we did not find matches in the Genbank database (provisionally labeled 'no match' and 'STE20-like kinase'). Several genes were found down-regulated in the tumorigenic lines, among them erbB2, the Ephrin receptors A4 and B4, fer (which mediates cross-talk between N-cadherin and β1-integrins), c-yes1 as well as the EGF receptor (in some lines). Further studies using in situ hybridization will be applied to independently verify these findings prior to publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Finished the construction and test of a robotic system to prepare DNA micro-arrays on glass slides
- Finished the design, assembly, programming and test of a robotic system to prepare DNA micro-arrays on nylon filters
- Finished the design and programming of a microscope-based digital imaging system to acquire high resolution images for quality control of fluorescently stained DNA micro-arrays
- Prepared plasmid libraries of cDNA fragments highly enriched in tyrosine kinase gene fragments found expressed in prostate cancer cell lines, commenced insert sequencing and expanded the panel of tyrosine kinase genes used for expression profiling
- Demonstrated the feasibility of tk gene expression profiling after RT-PCR using established and prostate-derived cancer cell lines
- Demonstrated tk gene expression changes as prostate epithelial cells become tumorigenic and grow anchorage-independent

REPORTABLE OUTCOMES:

- **manuscripts**

1. Heinz-Ulrich G. Weier, Horst F. Zitzelsberger, H.-Ben Hsieh, Melita V. Sun, Mariwil Wong, Robert A. Lersch, Paul Yaswen, Jan Smida, Christine Kuschnick, Orlo H. Clark (2001) Monitoring Signal Transduction in Cancer: Tyrosine Kinase Gene Expression Profiling. J. Histochem. Cytochem. 49: 673-674 (scheduled to appear in the May 2001 issue)

- **presentations**

1. Hsieh, H.B., Weier, H.-U.G. "Kinase Gene Expression Profiling-Instrumentation & Prototype Setup", Oral presentation, Corning Inc., Corning, NY, May 8, 2000.
2. Hsieh, H.B., Weier, H.-U.G. "Kinase Gene Expression Profiling in Human Tumors", Subcellular Structure Department Seminar, Life Science Division, E.O. Lawrence Berkeley National Laboratory, May 10, 2000.
3. Weier, H.-U.G. (2000) Towards a Full Karyotype Screening: SKY, chip technology. Oral presentation. Third International Symposium on Pre-Implantation Genetic Analysis. Palazzo di Congressi, Bologna, Italy, June 22-23, 2000.
4. Weier H-UG, Zitzelsberger HF, Hsieh H-B, Sun MV, Wong M, Lersch RA, Yaswen P, Smida J, Kuschnick C, Clark OH (2001) Poster Presentation. Monitoring signal transduction in cancer: tyrosine kinase gene expression profiling. Joint Meeting of the Histochemical Society and the International Society for Analytical and Molecular Morphology, Santa Fe, NM, February 2-7, 2001.
5. Hsieh H-B, Lersch RA, Callahan DE, Hayward S, Wong M, Clark OH, Weier H-UG (2001) Monitoring signal transduction in cancer: DNA microarray for semi-quantitative analysis.

Poster Presentation. Joint Meeting of the Histochemical Society and and the International Society for Analytical and Molecular Morphology, Santa Fe, NM, February 2-7, 2001.

6. Lersch RA, Fung J, Hsieh H-B, Smida J, Weier H-UG(2001) Monitoring signal transduction in cancer: from chips to FISH. Joint Meeting of the Histochemical Society and and the Poster Presentation. International Society for Analytical and Molecular Morphology, Santa Fe, NM, February 2-7, 2001.

- **funding obtained**

none

CONCLUSIONS:

This 3-year New Investigator project is well on track and, although most of its initial milestones were to be met after 18 months, the preliminary results have already proven the hypothesis that changes in tk gene expression can be monitored by a combination of RT-PCR using tk gene family-specific primers and DNA micro-arrays. The soft- and hardware components necessary for these studies were put as scheduled. Concordant with the timeline presented in the original proposal, research and development in the second year will focus on finishing the definition of the panel of genes to be profiled, thorough studies of assays reproducibility and sensitivity, and application of the technology to clinical specimens. Hybridization of labeled cDNA preparations to the DNA micro-array seems to possess the required specificity, and second year research will address the issues of hybridization background reduction, detection sensitivity and definition of a suitable reference DNA probe.

REFERENCES:

None

APPENDICES:

1. Figures 1 and 2
2. Table 1 and 2
3. Figures 3, 4 and 5

Appendix 1

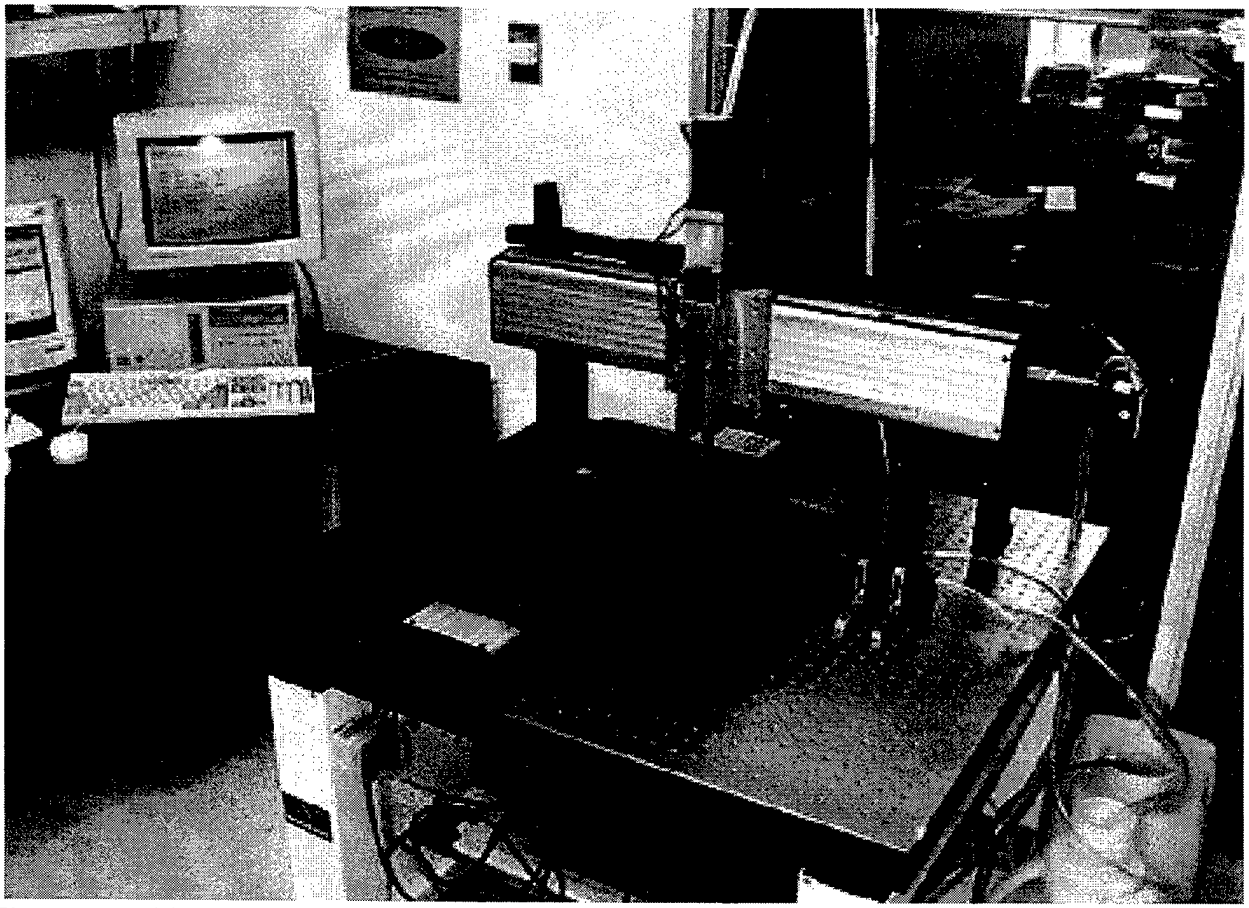

Figure 1: This DNA micro-arrayer built in our laboratory allows to print duplicate arrays on up to 91 slides. When in operation, the instrument is enclosed to maintain a mostly dust-free environment with controlled humidity.

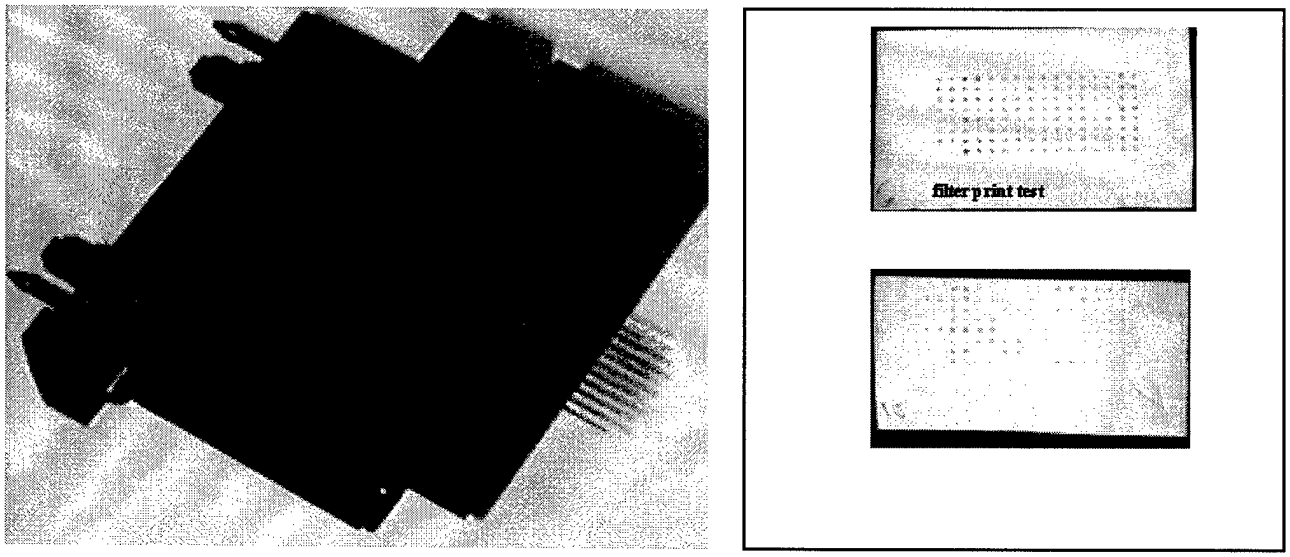

Figure 2: Left: The DNA micro-arrayer tool to fit onto our Beckman Biomek 1000 laboratory robot. Its 32 pin capacity allows rapid printing on glass or nylon membranes directly out of 384 well micro-titer plates. Each pins carries about 50 nanoliters of liquid. Right: Tyrosine kinase (tk) gene fragment containing nylon filters (shown at twice the original size) prepared to the test system were hybridized with biotinylated tk probes and washed at low (top) or high stringency (bottom). Bound probes were detected with avidin-alkaline phosphatase and a chromogenic substrate. All fragments were printed in duplicates (pairs).

Appendix 2

	1	2	3	4	5	6	7	8	9	10	11	12
1	Cy3	Cy3	Cy3	K-SAM	c-mer	CLK3	flg	HSIGFIR R	blk	c-src	EphA2	EphA4
2	HSLK/KI AA0204	PTK6	c-syn/FYN	EGFR	HLA-A	HSUO73 49	c-abl	c-yes-1	blk-like	EphA1	EphB4	c-fms
3	pkC-delta	fer	HSEGF01	HUMP4K	HUMPKS CD	JAK3	MKK3	RON	tyk2	EphA4	lck	MLK-3
4	TEK	TYRO3	ZAP-70	LYN	EphB1	UFO	IRR	MEK3	PDGF	tie	arg	JAK1
5	met	PYK2/FA K2	DRR1	MKK3	pra src pp60	clk1	MAPK1	no match	serium inducible	v-abl	Ste20-like	cosmid B1E7
6	G-protein-coupled	GAPDH	HPRT	RPL13A	Cy3	Cy3	Cy3					
7	Cy5	Cy5	Cy5	K-SAM	c-mer	CLK3	flg	HSIGFIR R	blk	c-src	EphA2	EphA4
8	HSLK/KI AA0204	PTK6	c-syn/FYN	EGFR	HLA-A	HSUO73 49	c-abl	c-yes-1	blk-like	EphA1	EphB4	c-fms
9	pkC-delta	fer	HSEGF01	HUMP4K	HUMPKS CD	JAK3	MKK3	RON	tyk2	EphA4	lck	MLK-3
10	TEK	TYRO3	ZAP-70	LYN	EphB1	UFO	IRR	MEK3	PDGF	tie	arg	JAK1
11	met	PYK2/FA K2	DRR1	MKK3	pra src pp60	clk1	MAPK1	no match	serium inducible	v-abl	Ste20-like	cosmid B1E7
12	G-protein-coupled	GAPDH	HPRT	RPL13A	Cy5	Cy5	Cy5					

Table 2: The organization of our tk DNA microarrays used in initial studies to optimize array production and hybridization protocols. 'Cy3' and 'Cy5'indicate positions where we deposited fluorochrome-labeled DNAs as markers.

	1	2	3	4	5	6	7	8	9	10	11	12
1	GAPDH	HPRT	RPL13A	K-SAM	c-mer	CLK3	flg	HSIGFIR R	blk	c-src	EphA2	EphA4
2	HSLK/KI AA0204	PTK6	c-syn/FYN	EGFR	HLA-A	HSUO73 49	c-abl	c-yes-1	blk-like	EphA1	EphB4	c-fms
3	pkC-delta	fer	HSEGF01	HUMP4K	HUMPKS CD	JAK3	MKK3	RON	tyk2	EphA4	lck	MLK-3
4	TEK	TYRO3	ZAP-70	LYN	EphB1	UFO	IRR	MEK3	PDGF	tie	arg	JAK1
5	met	PYK2/FA K2	DRR1	MKK3	pra src pp60	clk1	MAPK1	no match	serium inducible	v-abl	Ste20-like	cosmid B1E7
6	G-protein-coupled	GAPDH	HPRT	RPL13A	TRK	ret (PTC3)	EphA2	ret (PTC3)	ret (HHCC55	TRK	erbB2	EphB2
7	Cy5	Cy5	Cy5	K-SAM	c-mer	CLK3	flg	HSIGFIR R	blk	c-src	EphA2	EphA4
8	HSLK/KI AA0204	PTK6	c-syn/FYN	EGFR	HLA-A	HSUO73 49	c-abl	c-yes-1	blk-like	EphA1	EphB4	c-fms
9	pkC-delta	fer	HSEGF01	HUMP4K	HUMPKS CD	JAK3	MKK3	RON	tyk2	EphA4	lck	MLK-3
10	TEK	TYRO3	ZAP-70	LYN	EphB1	UFO	IRR	MEK3	PDGF	tie	arg	JAK1
11	met	PYK2/FA K2	DRR1	MKK3	pra src pp60	clk1	MAPK1	no match	serium inducible	v-abl	Ste20-like	cosmid B1E7
12	G-protein-coupled	GAPDH	HPRT	RPL13A	TRK	ret (PTC3)	EphA2	ret (PTC3)	ret (HHCC55	TRK	erbB2	EphB2

Table 3: The organization of our new tk DNA microarrays used in studies of gene expression in prostate cancer cell lines, BPH-1 and BPH-1 derived cell lines.

Appendix 3

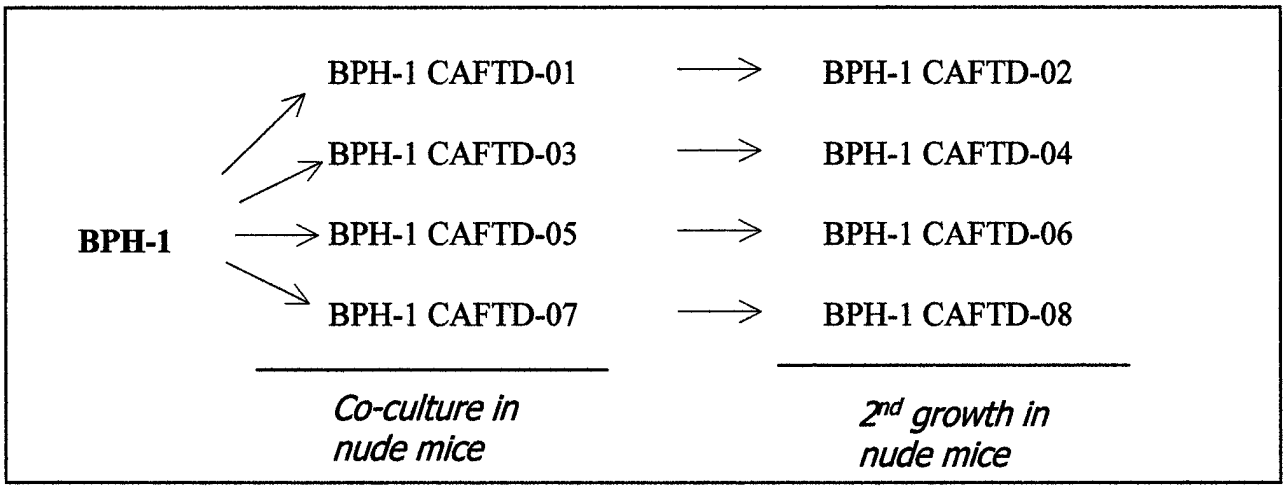

Figure 3: The relation between the immortalized prostate cell line BPH-1 and different lines generated by co-culture with cancer associated fibroblasts.

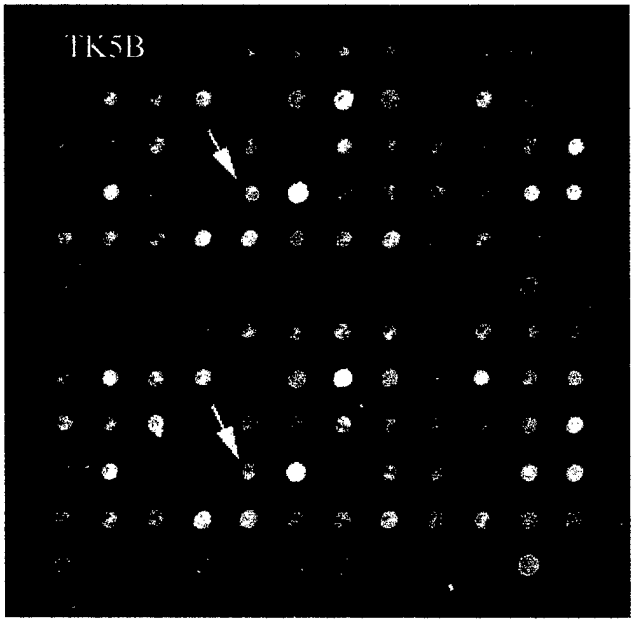

Figure 4: The result of the hybridization of BPH-1 tyrosine kinase transcript (red) and BPH-1 CAFTD02 (green) to the new tk DNA microarrays. The arrays point at duplicate samples of a tk gene expressed at high level in BPH-1 CAFTD02, but not in BPH-1 or BPH-1 CAFTD07.

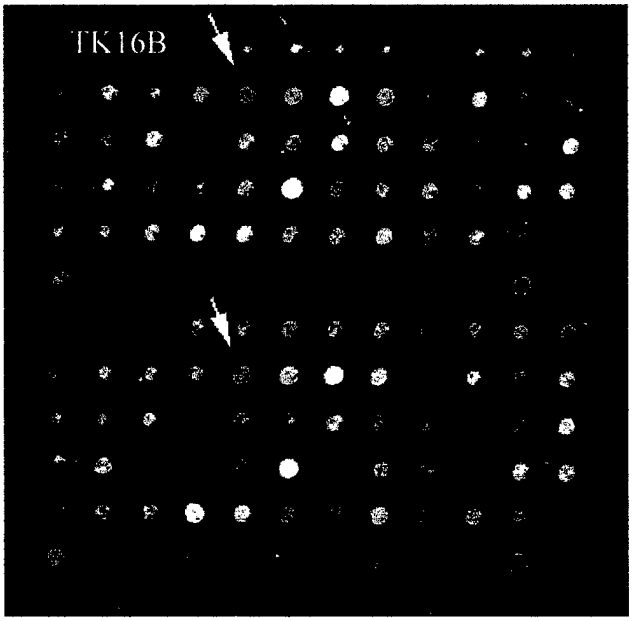

Figure 5: The result of the hybridization of BPH-1 tyrosine kinase transcript (red) and BPH-1 CAFTD07 (green) to the new tk DNA microarrays. The arrays point at duplicate samples of a tk gene expressed at high level in BPH-1 CAFTD07, but not in BPH-1 or BPH-1 CAFTD02.