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TITLE: Tyronsine Kinase Gene Expression Profiling in Prostate Cancer

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**13. ABSTRACT (Maximum 200 Words)**
The expression of protein kinases genes if often altered in tumors. The aberrant expression of several of these genes typically parallels the progression toward a more malignant phenotype. We developed a cDNA micro-array-based screening system to mearuser the level of expression of tyronsine kinase (tk) genes. The hardware for preparation of cDNA micro-arrays and basic protocols for hybridization were developed in year 1. In the second year, we finished cDNA synthesis from prostate cancer cell lines and 6 frozen tissue specimens. We continued our DNA sequencing effort and added additional targets to our micro-arrays. Using prostate cancer cell lines, the system delivered reproducible results about tk gene expression during cell transformation and progression toward a more malignant phenotype. Comparing the absolute expression levels form cDNA micro-arrays with data from Northern blot analyses suggested that our initial approach using mixed-based oligonucleotide primers led to lowered representation of highly abundant transcript. This problem has been addressed with a new primer design to be better suited to investigate the tk gene expression in small samples. We then applied our protocols to the gene expression analysis of micro-dissected tissues, and found reliable detection of abl gene overexpression with an RNA equivalent of 3 cells.

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## Table of Contents

## INTRODUCTION:

Aberrant expression of receptor or cytosolic tyrosine kinase genes and, in particular, their hyper-expression are common phenomena in prostate cancer and 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 developed a rapid assay using innovative cDNA 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. Four years of research and development have lead to a simple device and an optimized protocol to perform expression profiling of RNA marker for prostate cancer progression using RNA isolated from as little as three cells.

## BODY:

Here, we report our progress as it relates to the approved 'Statement of Work'.

### *Task 1.* Identify tyrosine kinase (tk) genes expressed in normal and neoplastic prostate tissues

#### *1.1 Prepare cDNAs from six cell lines and ten frozen tissue specimens (months 1-18)*

In collaboration with the Cancer Tissue Core facility at the Comprehensive Cancer Center, University of California, San Francisco (UCSF) (directed by Dr. B.M Ljung) and Dr. S. Hayward, now at the Vanderbilt University Medical Center, Nashville, TN, we completed this task by isolating RNA from 15 cell lines (Table I) and 10 frozen tissues as described in our progress report covering the period of March 1, 2001 – February 28, 2002.

**Table I. Prostate specific cell lines used in the experiments.**

| | | |
|---|---|---|
| DU145 | ND-1 | |
| PC-3 | LNCAP | |
| BPH-1 CAFTD-01 | BPH-1 CAFTD-02 | |
| BPH-1 CAFTD-03 | BPH-1 CAFTD-04 | |
| BPH-1 CAFTD-05 | BPH-1 CAFTD-06 | |
| BPH-1 CAFTD-07 | BPH-1 CAFTD-08 | |
| BPH-1 TETD-A | BPH-1 TETD-B | BPH-1 |

RNA was isolated using a commercial kit (Qiagen) and transcribed into cDNA immediately. Remaining RNAs were stored at –80 degrees. We prepared cDNAs from the RNA specimens by oligo-dT or random priming followed by reverse transcription. Commercial kits (Qiagen, Roche, Ambion) were used for all steps. Typically, 1 μg of total RNA produced sufficient quantities of cDNA for gel electrophoritic quality control, cloning and/or repeated micro-array analyses.

#### *1.2 Perform RT-PCR reactions and clone PCR products in plasmids (months 3-18)*

Initially, we followed an in vitro DNA amplification protocol using mixed-base oligonucleotide primers and about 100 ng of cDNA in PCR reactions to amplify tk-specific cDNA fragments as described in the two progress reports covering the periods of March 1, 2000 – February 28, 2001 and March 1, 2001 – February 28, 2002. PCR products were cloned in plasmid vectors (pAMP, Invitrogen, Carlsbad, CA) using directional cloning.

#### *1.3 Perform pre-screening with known tk fragments, cDNA sequencing and database searches (months 5-22)*

### *1.4 Add novel clones to the panel of expressed tk gene fragments (months 6-24)*

These two tasks (i.e. tasks1.3, 1.4) were completed as described in our progress report covering the period of March 1, 2001 – February 28, 2002.

Briefly, the DNA from plasmid clones with inserts of about 125-190 bp was isolated, fingerprinted or screened against known tk genes and 'novel' clones were sequenced at the U.C. Berkeley, Biochemical Core Facility. The list of genes cloned and identified so far contained several sequences reported to have transforming activity, such as trk, axl/ufo or to be overexpressed in various types of cancer (like the EGF receptor). As of June 2001, we had cloned and characterized about 240 tk fragment containing plasmid clones derived from prostate or breast cancer tissues and cell lines. These clones join more than 500 tk fragments containing clones that were previously isolated from thyroid tumors. Our present panel of kinase gene tags used to prepare cDNA micro-arrays contains 60 tk or serine-threonine kinase genes plus HLA-A, which happened to be amplified by our first generation–mixed base PCR primers.

## *Task 2.* Measure tk gene expression in cell lines, normal and tumor tissues

### *2.1 Prepare DNA microarrays carrying about 100 different sequences (months 3-24)*

As described in our progress report covering the period of March 1, 2001 – February 28, 2002, the fabrication of tyrosine kinase (tk) cDNA arrays on glass slides was done in house using our laboratory-built robotic system to print cDNA micro-arrays with about 124 target spots per array. Array analysis revealed a heterogeneous distribution of spot diameters

We recently modified the printing equipment to prepare more homogeneous spots sizes. We worked with scientists at Telechem, Sunnyvale, CA (manufacturer of the 'ARRAY IT' reagents) and, following test prints, installed their proprietary print pin on our system (Fig.1). Using the new pin, we printed 100 micro-arrays. Inspection of the arrays showed that the spot diameters were essentially homogeneous, but the spots were printed in irregular positions due to motion of the pin. Motion of the print pin was minimized by installation of a new holder (manufactured by Telechem for our arraying equipment)(Fig. 2).

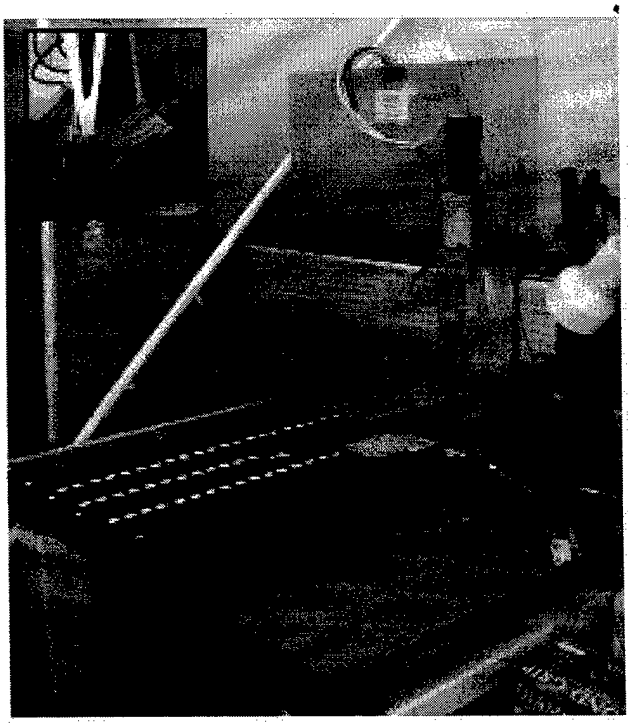


**Figure 1 (Left). Modifications made to the DNA micro-arraying equipment.** The picture shows the Telechem print head installed on our arrayer just prior to printing a set of genomic DNA micro-arrays. The insert shows an enlarged view of the print head with one Telechem pin installed.

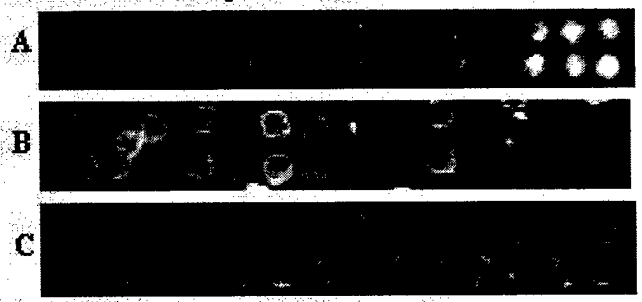



**Figure 2. Technical improvements.** A) Arrays printed with the old (DiTech) pins; B) arrays printed with a Telechem pin on the old holder; C) arrays printed with the same pin as in B installed on the Telechem print head.

*2.2 Optimize hybridization conditions to provide quantitative information (months 6-24)*

*2.3 Optimize PCR parameters for quantitative amplification of target genes (months 12-18)*

As described in our progress report covering the period of March 1, 2001 – February 28, 2002, we completed these two tasks by first isolating total RNA from prostate cancer cell lines or frozen tissues sections. We then prepared cDNAs from the RNA by random priming and reverse transcription. Next, we applied a two-step in vitro DNA amplification protocol. A size selection step helped to reduce the amplification of non-kinase derived sequences to less than 10% as verified by DNA sequencing.

*2.4 Develop algorithms for array readout and comparisions between measurements (months 9-24)*

A Axon GenePix 4000 (Axon Inc.) array scanner was used to acquire all images (see progress Report covering the period of (March 1, 2001 – February 28, 2002). 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 (Microsoft Excel) for further analysis. For display purposes, the images were saved in standard formats and imported into common graphics programs such as Adobe Photoshop. Given the relatively small numbers of arrays that have been hybridized so far, it was sufficient to normalize the data and compare numeric values in spreadsheets.

***Task 3.* Validate assays for multigene expression profiling in small amounts of tissue**

*3.1 Develop software for databasing, automated analysis of expression profile datasets and their annotation (months 18-24)*

The GenePix software provides the results as numeric data that can be inported into spreadsheets or databases. We installed Microsoft Access software which is sufficient to handle most or all of our databasing needs. We also installed BRB ArrayTools Version 1.03 recently released by the Biometric Research Branch of the Division of Cancer Treatment and Diagnosis at NCI. This package developed by Richard Simon and Amy Peng allows for a more comprehensive analysis of DNA microarrays and their annotation. The software is presently being tested using the datasets obtained with our second generation tk cDNA arrays.

*Task 3.1. Develop software for databasing, automated analysis of expression profile datasets and their annotation (months 18-24)*

Given the rather small number of cDNA samples analyzed to date, we decided to handle all results in form of Microsoft Excel spreadsheets.

*Task 3.2. Test the system with serial dilutions of cells (months 24-30)*

This task is completed as described in the Progress Report for the period March 1, 2002 – February 28, 2003. Briefly, one of our goals was to develop of assays for accurate multigene expression profiling that require only small amounts of tissue. Such assays might ultimately allow the analysis of

specimens obtained by fine needle biopsies or tissue from micro-dissected tumor sections. Using serial dilutions of cDNA samples to determine the assay sensitivity, we noted that cDNA isolated from about 500 cells was needed for reproducible results using our initial set of tyrosine kinase-specific PCR primers.

We therefore redesigned our PCR primers using the published cDNA sequences of known tk genes, and we optimized the PCR amplification scheme carefully balancing the amounts of forward and reverse primers and optimizing primer annealing conditions. Although the new primer set (F-TYRK-4, R-TYRK-4) is similar to the previous set, its complexity was decreased 1.8-fold for the new forward primer and 3-fold for the reverse primer. This lead to increased specificity and amplification efficiency. Initial measurements to test the linearity of the amplification process using artificial mixtures of tk gene fragments had shown superior (i.e., more linear) amplification results and demonstrated a better specificity of the new primers (Progress Report for the period March 1, 2002 – February 28, 2003).

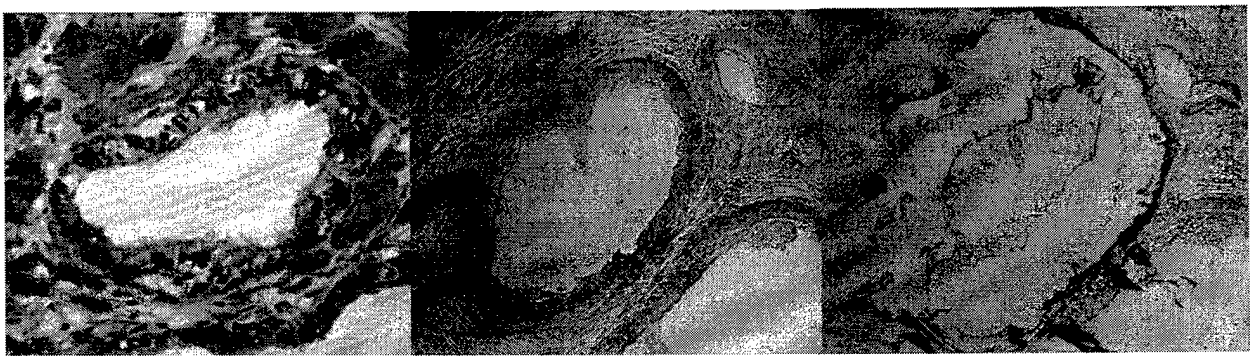
***Task 3.3. Test the assay with microdissected tissue from prostate cancer sections (months 30-48)***

Tests with serial dilutions of cells from several of the cell lines used in the initial stages of this project suggested that the assay was sensitive enough to measure the levels of tk gene expression in as little as 100 cells. We selected frozen tissues blocks from normal ('NL') prostate and tumor tissues and cut sections of about 8-10 μm. The tissue sections were mounted on positively charged microscope slides (Fisher 'Probe on Plus') and stained with 0.5% Methyl Green for 30 sec. After comparing the price, convenience and through-put of tissue microdissection using either the Arcturus laser capture microdissection equipment at the UCSF Comprehensive Cancer Center or a Leica laser capture microdissection equipment at the UCSF Parnassus campus, we decided to set up our own RNAse-free environment and perform the tissue dissections manually. Microdissection was performed using a dissecting microscope and scalpels to collect either 120 or 1200 cells from normal tissues and tumor specimens (Table I, Fig.1). The RNA was isolated from duplicate samples using a Qiagen RNeasy kit and transcribed into cDNA using a Qiagen Sensiscript kit. Aliquots of the cDNA representing an amount of cDNA prepared from the equivalent of 1.5 or 15 cells per microliter were used in PCR reactions using our tk-specific primer set TYRK4.

Following the initial PCR amplification, one half of each PCR reaction was subjected to a second in vitro DNA amplification reaction using the 5-adaptor primer (H3T7). This template DNA was equivalent to TYRK4 PCR products generated from 0.3 or 3 cells. The PCR products from the H3T7 reaction were concentrated 3-fold by alcohol precipitation and resuspension in 10 μl. Three microliter of each of the concentrated H3T7 PCR products was then labeled in 25 μl random priming reactions using Cy3- or Cy5-dUTP. Probes were concentrated 2.5-fold using Microcon-30 columns and 1 μl of each probe was hybridized for 16 hours to the tk arrays. Hybridization results demonstrated consistent tk gene overexpression in all tumor samples. The results for sample sizes equivalent to the amount of cDNA prepared from 15 cell equivalents are shown in Fig. 3 and 4. Most notably, we found high levels of expression of abl (Fig.4B, arrows), which were expressed at much lower levels in normal prostate tissue (Fig. 4A,B). In Fig. 4B the tumor-derived cDNA sample was labeled with Cy5 (shown in red) and was hybridized against the tk gene PCR product from normal tissue (Cy5-labeled, shown in red). We confirmed the overexpression of abl by repeating the experiments with the tumor-derived cDNA labeled with CY3 and the normal control tissue-derived cDNA labeled in Cy3 ('dye swapping' experiment, Fig. 4B). The finding of overexpression of the abl tk gene in prostate cancer agrees with earlier reports (1,2). It is interesting to note that a study by Rijnders et al. (3) could not demonstrate abl expression in prostate cancer cell lines suggesting significant changes in tk gene expression during the establishment of cell lines.

| Tube # | Tissue | Type | # Cells | Water (µl) | #Cells/ul |
|---|---|---|---|---|---|
| 1 | PR386-11 | NL | 120 | 12 | 10 |
| 2 | PR386-11 | NL | 1,200 | 12 | 100 |
| 3 | PR386-12 | NL | 120 | 12 | 10 |
| 4 | PR386-12 | NL | 1,200 | 12 | 100 |
| 5 | PR405-21 | NL | 120 | 12 | 10 |
| 6 | PR405-21 | NL | 1,200 | 12 | 100 |
| 7 | PR405-22 | NL | 120 | 12 | 10 |
| 8 | PR405-22 | NL | 1,200 | 12 | 100 |
| 9 | PR385-41 | Tumor | 120 | 12 | 10 |
| 10 | PR385-41 | Tumor | 1,200 | 12 | 100 |
| 11 | PR385-42 | Tumor | 120 | 12 | 10 |
| 12 | PR385-42 | Tumor | 1,200 | 12 | 100 |
| 13 | PR390-41 | Tumor | 120 | 12 | 10 |
| 14 | PR390-41 | Tumor | 1,200 | 12 | 100 |
| 15 | PR390-42 | Tumor | 120 | 12 | 10 |
| 16 | PR390-42 | Tumor | 1,200 | 12 | 100 |
| 17 | PR398-11 | Tumor | 120 | 12 | 10 |
| 18 | PR398-11 | Tumor | 1,200 | 12 | 100 |
| 19 | PR398-12 | Tumor | 120 | 12 | 10 |
| 20 | PR398-12 | Tumor | 1,200 | 12 | 100 |
| 21 | PR395-11 | Tumor | 120 | 12 | 10 |
| 22 | PR395-11 | Tumor | 1,200 | 12 | 100 |
| 23 | PR395-12 | Tumor | 120 | 12 | 10 |
| 24 | PR395-11 | Tumor | 1,200 | 12 | 100 |

**Table I:** The microdissection experiments using normal prostate (NL) and tumor tissue specimens. Using a dissecting microscope and a scalpel, we picked up between 120 and 1,200 (estimated) cells from Methyl Green stained sections. The RNA was extracted and diluted with RNAse–free water to represent the equivalent of 10 or 100 cells per µl ('#cells/µl').



A B C

**Figure 2.** **A:** H&E section of a gland of approximately 120 cells. **B:** Methyl Green section before dissection of approximately 120 cells. **C:** Methyl Green section after dissection of approximately 120 cells.

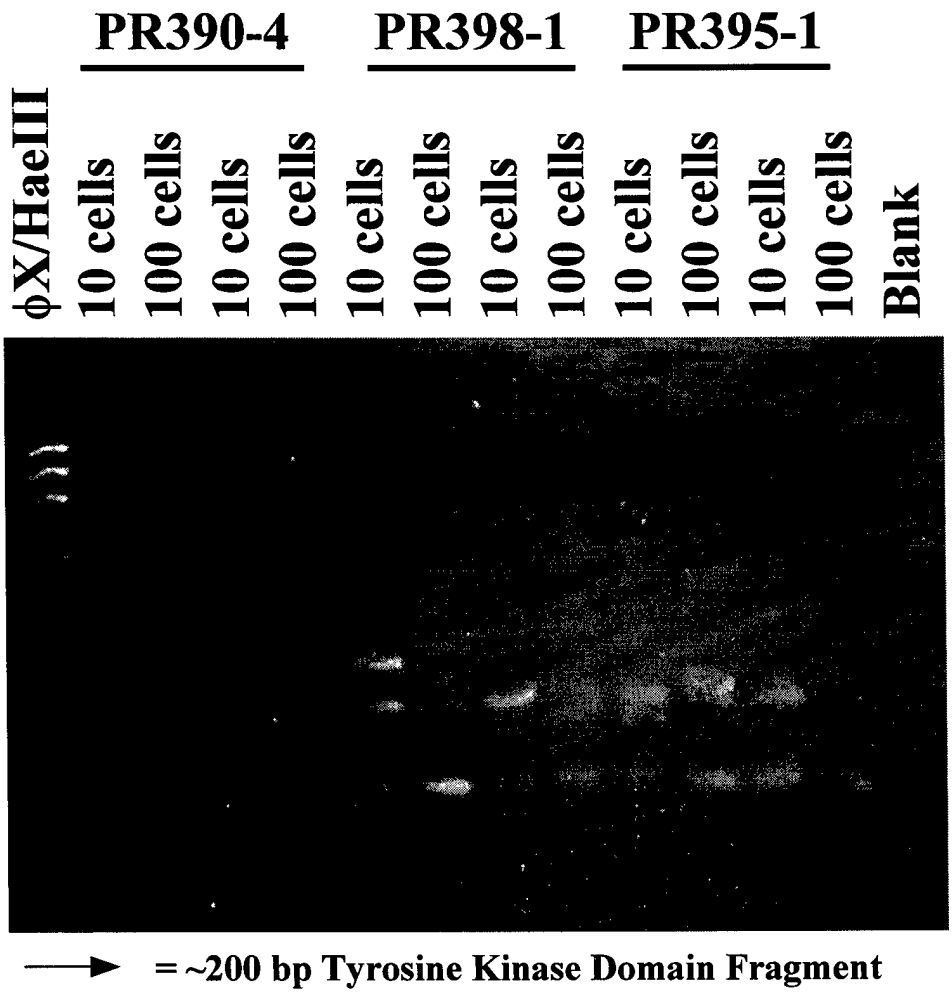



**Figure 3:** PCR Products after 35 PCR cycles with Tyrk4 primers followed by 35 cycles with H3T7 primers.

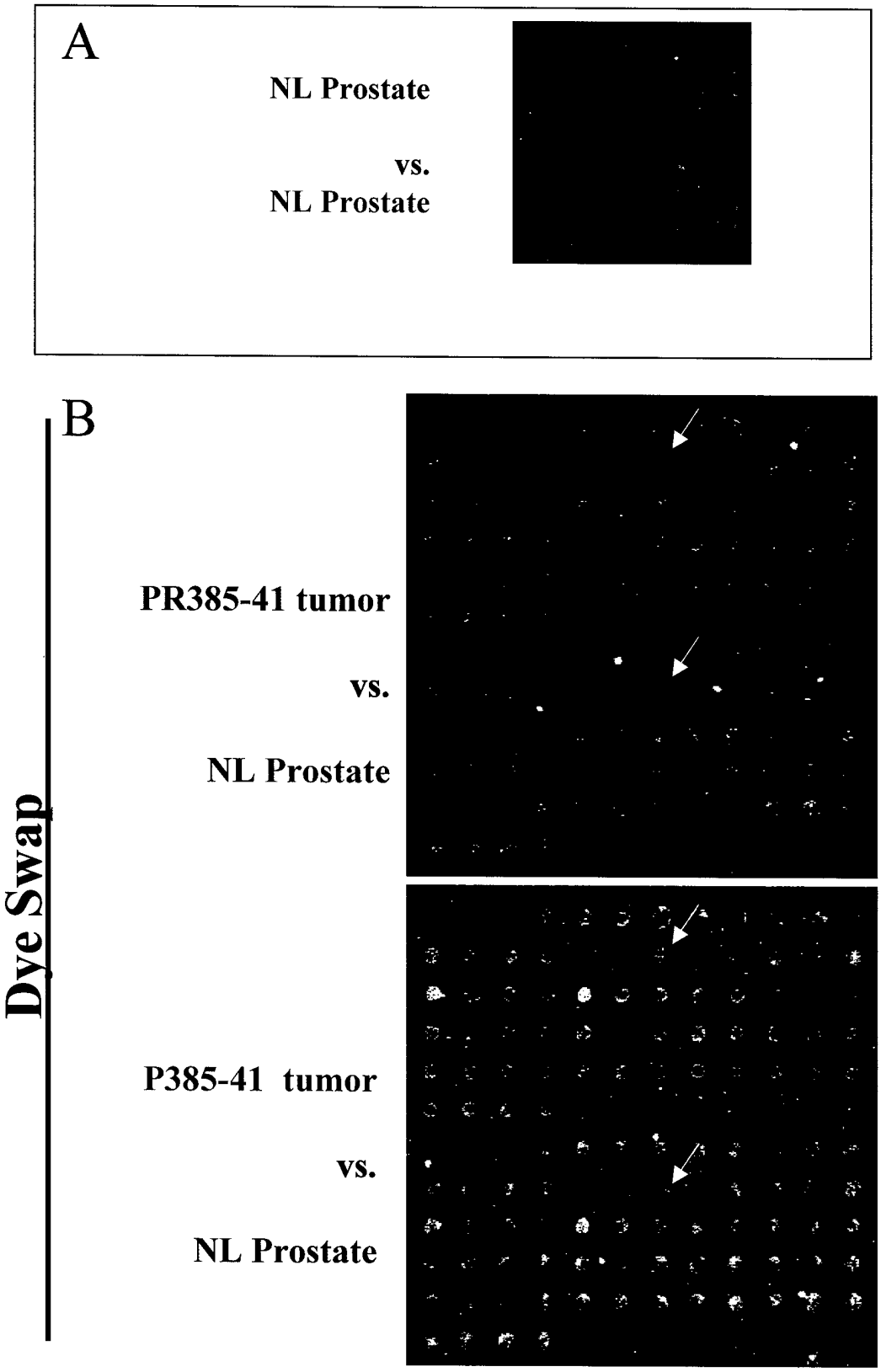



**Figure 4:** Tyrosine Kinase Array Hybridizations of Dissected/PCR-Amplified Prostate Tissues. A: Normal against normal cDNA; B: tumor cDNA against normal cDNA. The arrows point on the spot containing the abl cDNA.

## KEY RESEARCH ACCOMPLISHMENTS:

- Finished the isolation of RNA and preparation of cDNA from 15 prostate cancer cell lines and 10 frozen tissue specimens
- Completed the PCR-amplification of tk-specific DNA fragments and cloned the products into plasmids
- Pre-screening more than 400 cancer cell line-derived tk fragment clones and sequenced an additional 100 clones, database searches identified two clones containing potentially novel tyrosine kinase genes
- Expanded the panel of tyrosine kinase genes used for expression profiling and printed second generation cDNA micro-arrays
- Generated artificial mixtures of tk DNA fragments to be used as reference DNA
- Reconfirmed tk gene expression changes as prostate epithelial cells become tumorigenic using second generation tk micro-arrays
- Initiated search for full length cDNA clones for novel tk genes and screened BAC libraries for genomic clones
- Redesigned oligonucleotide primers to amplify more specifically thus enabling the processing of small amounts of cells or tissue
- Demonstrated the feasibility of tk gene expression profiling using cDNA prepared by tissue microdissection from as little as 3 cell equivalents

## REPORTABLE OUTCOMES ( March 1, 2000 – February 28, 2004):

**- peer reviewed publications**

1. Weier H-UG, Greulich-Bode KM, Ito Y, Lersch RA, Fung J (2002) FISH in cancer diagnosis and prognostication: from cause to course of disease. Expert. Rev. Mol. Diagn. 2(2):109-119

2. Weier, H.-U.G. (2002) Quantitative DNA Fiber Mapping. In: FISH Technology. B. Rautenstrauss and T. Liehr (Eds.), Springer Verlag, Heidelberg, pp.226-253

3. Lersch RA, Fung J, Hsieh H-B, Smida J, Weier H-UG (2001) Monitoring signal transduction in cancer: from chips to FISH. J Histochem Cytochem 49:925-926

4. Hsieh H-B, Lersch RA, Callahan DE, Hayward S, Wong M, Clark OH, Weier H-UG (2001) Monitoring signal transduction in cancer: cDNA microarray for semi-quantitative analysis. J Histochem Cytochem 49: 1057-1058

5. Weier H-UG, Zitzelsberger HF, Hsieh H-B, Sun MV, Wong M, Lersch RA, Yaswen P, Smida J, Kuschnick C, Clark OH (2001) Monitoring signal transduction in cancer: tyrosine kinase gene expression profiling. J Histochem Cytochem 49:673-674

- **presentations**

2002 Annual Meeting of the Histochemical Society, Seattle, WA, July 18-21, 2002:

1. Lersch RA, Chu LW, Ito Y, Weier HUG. Toward Tyrosine Kinase Expression Profiling at the Single Cell Level.

2. Weier HUG, Ito Y, Fung J, Lehmann L, Lersch RA, Chu LW, Zitzelsberger HF. Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history.

3. Ito Y, Fung J, Hsu J, Katzir N, Lersch RA, Weier HUG. Phenotype analysis of tumor cells with eight color FISH.

4. Chu LW, Troncoso P, Johnston DA, Liang JC. Genetic alterations associated with local prostate cancer progression.

5. Hsieh, H.B., Weier, H.-U.G. "Kinase Gene Expression Profiling-Instrumentation & Prototype Setup", Oral presentation, Corning Inc., Corning, NY, May 8, 2000.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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**

California Cancer Research Program, 'Tyrosine Kinase Gene Expression Profiling in Prostate Cancer', Pilot and Feasibility Study Award, H.-U. Weier (P.I.), 7/01/00-6/30/03

National Institute of Health, 'Spectral Karyotyping for Phenotype Analysis of Cancer Cells', R21/R33 grant, H.-U. Weier (P.I.), 9/01/00-8/31/04

Deartment of Defense Prostate Cancer Research Program, Dietary determinants of prostate cancer progression, Chu, Lisa W. (Post Doctoral Fellow), 01/01/03-12/31/04

## CONCLUSIONS:

This New Investigator project demonstrated the feasibility of performing gene expression profiling in prostate cancer cell lines and tissue specimens using short gene specific DNA fragments, and delivered information about changes in tk gene expression as cell and cell lines progress towards a more malignant phenotype. A modified 2-step in vitro DNA amplification procedure lead to increased sensitivity and minimized amplification bias. While the measurements in the present study were performed with cDNA microarrays on glass, other approaches for DNA quantitation such as electrochemical sensors might lower the cost per assay without sacrificing assay sensitivity.

## REFERENCES:


1. Robinson D, He F, Pretlow T, Kung HJ (1996) A tyrosine kinase profile of prostate carcinoma. Proc Natl Acad Sci U S A 93:5958-5962.
2. George DJ (2002) Receptor tyrosine kinases as rational targets for prostate cancer treatment: platelet-derived growth factor receptor and imatinib mesylate. Urology 60:115-121.
3. Rijnders AW, van der Korput JA, van Steenbrugge GJ, Romijn JC, Trapman J (1985) Expression of cellular oncogenes in human prostatic carcinoma cell lines. Biochem Biophys Res Commun. 132:548-554


## APPENDICES:

1. The approved Statement of Work
2. Personnel Supported

Appendix 1.

## Statement of Work

**Title: Tyrosine Kinase Gene Expression Profiling in Prostate Cancer**

**P.I.: Heinz-Ulrich G. Weier**

*Task 1.* To identify tyrosine kinase (tk) genes expressed in normal and neoplastic prostate tissues (months 1-24)

- prepare cDNAs from six cell lines and ten frozen tissue specimens (months 1-18)
- perform RT-PCR reactions and clone PCR products in plasmids (months 3-18)
- perform pre-screening with known tk fragments, cDNA sequencing and database searches (months 5-22)
- add novel clones to the panel of expressed tk gene fragments (months 6-24)

*Task 2.* To measure tk gene expression in cell lines, normal and tumor tissues (months 3-24)

- prepare DNA microarrays carrying about 100 different sequences (months 3-24)
- optimize hybridization conditions to provide quantitative information (months 6-24)
- optimize PCR parameters for quantitative amplification of target genes (months 12-18)
- develop algorithms for array readout and comparisions between measurements (months 9-24)

*Task 3.* To validate assays for multigene expression profiling in small amounts of tissue (months 18-36)

- develop software for databasing, automated analysis of expression profile data sets and their annotation (months 18-24)
- test the system with serial dilutions of cells (months 24-30)
- test the assay with microdissected tissue from prostate cancer sections (months 30-36)

## Appendix 2

## Personnel

**1. Fiscal Year 2000**

**Weier, H.-U.G.**

**2. Fiscal Year 2001**

**Guan, J.**

**Weier, H.-U.G.**

**3. Fiscal Year 2002**

**Guan, J.**

**Ito, Y.**

**Weier, H.-U.G.**

**4. Fiscal Year 2003**

**Guan, J.**

**Li, Z.**

**Weier, H.-U.G.**

**Zhang, J.**

**5. Fiscal Year 2004**

**Guan, J.**

**Li, Z.**

**Weier, H.-U.G.**

**Zhang, J.**