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TITLE: A Genomic Approach to Identifying Novel Targets for Early Detection and Intervention of Prostate Cancer

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A Genomic Approach to Identifying Novel Targets for Early Detection and Intervention of Prostate Cancer

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13. ABSTRACT (Maximum 200 Words)
Early detection and intervention is key to a favorable prognosis in prostate cancer. Despite advances in the detection and treatment of prostate cancer, the mortality rate remains high. To improve survival, early detection and treatment strategies tailored to pre-invasive prostate cancer are required. We propose to catalog genetic alterations associated with the developmental stages of disease for use as diagnostic tools and to identify the critical genes that drive the transformation of premalignant lesions to tumors for use as molecular targets for novel treatment design. The combination of laser capture microdissection (efficient isolation of specific cell types form hundreds of specimens) and SMAL DNA fingerprinting technology (high-throughput analysis of genomic targets using minute quantities of DNA yielded form the microdissected cells) will facilitate systematic comparison of samples in various stages of disease development. By the end of this work, we will have identified a set of genetic loci (and genes) by virtues of their frequency of alteration in premalignant lesions and subsequent in low-grade tumors. We will have established a publicly accessible “genetic alterations in prostate cancer” database which catalogs somatic changes present in the various stages of cancer progression. Such information contribute to the fundamental understanding of prostate cancer pathogenesis.

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Annual Report for Award Number DAMD17-01-1-0028

Project Title: A Genomic Approach to Identifying Novel Targets For Early Detection and Intervention of Prostate Cancer

Authors: Wan L. Lam and Juergen R. Vielkind

1. Introduction

Accumulation of key genetic alterations is thought to be the underlying mechanism that drive the development of prostate cancer, and such changes have not been well defined. The goal of this project is to apply high-resolution DNA fingerprinting technology to identify key genetic alterations associated with the progression stages of prostate cancer.

1.1. Background

Early detection and intervention is key to a favorable prognosis in prostate cancer (CaP), as in many types of cancers. Disease progression is thought to be driven by cumulative genetic alterations affecting a small number of genes (see Kinzler and Vogelstein, 1996). Identification of these genes would provide novel targets for diagnosis and intervention. Despite many efforts these genes have not yet been identified. One possibility to discover these genes is by identifying genetic alterations that parallel the histopathological progression stages in prostate cancer. This can only be achieved by a genome-wide screen for alterations.

CaP consists of a mixture of normal epithelial cells, stromal cells, benign hyperplastic cells, PIN, and various clones of invasive carcinoma. To date, due to the difficulty in obtaining sufficient material from the small early progression stages and the lack of analytical methods applicable for genome-wide scanning of minute clinical specimens, much of our knowledge on genetic changes in CaP has been derived from analyses of advanced tumors and cell lines, which are characterized by complex genetic changes many of which may be random, due to generalized genetic instability, rather than being etiologic. Thus, genetic alterations relevant to disease progression may be more easily identified by examining stages earlier in the development of a tumor, before the accumulation of randomly altered changes occurs. We therefore focus on identifying genetic changes in premalignant lesions which subsequently appear in the early invasive stages of CaP. “Normal epithelium” versus “PIN” comparison will reveal the spectrum of genetic changes in the early stages of CaP development, while the “PIN” to “tumor” comparison will identify the subset of changes critical to disease progression.

The objective of this proposal is to identify the progressive genetic alterations that cause normal prostate epithelial cells to transform into precursor prostatic intraepithelial neoplasia and invasive cancer cells of low Gleason grade. This will be achieved by systematically comparing high density DNA fingerprints of microdissected samples in various stages of disease development.

Over a 3 year period, we proposed to compare DNA samples extracted from normal cells, from precancerous cells and from tumor cells in a variety of patient biopsies, in order to identify key genetic changes in the stages of disease development. Our results in the second year as well as progress in relation to the original Statement of Work are summarized in this Annual Report.
2. Body

We have proposed to use laser capture microdissection to selectively isolate pure cell populations representing normal epithelium, benign hyperplasia, high grade PIN and invasive cells of low Gleason grades 1-3. We have couple this cell isolation approach with SMAL-PCR DNA fingerprinting technology in order to analyze these various CaP progression stages at thousands of randomly distributed genetic loci for frequent alterations. The specific aims are:

1. To generate SMAL DNA fingerprints from normal epithelium, PIN and invasive carcinomas of early stages.
2. To identify recurring alterations that are present in the early progression stages.
3. To establish an expandable database of recurring changes for each stage of disease development.
4. To assign recurring alterations to specific chromosomal regions.
5. To identify candidate genes in the mapped regions for future mutation scanning.
6. To begin validation of candidate genes.

Aims 1-4 started in Year 1 of the project extending into Years 2 and 3, while aims 5 and 6 are Year 2-3 activities. A comparing our progress against the original Statement of Work is presented in the Reportable Outcomes section below.

2.1. Experimental design and methodology

There is no deviation from the design proposed in the original research plan. As mentioned in the previous annual report, the methodologies were described in detail in the original proposal. This is a brief description of the two key technologies used in this project.

Laser Capture Microdissection.

To overcome tissue heterogeneity in the prostate biopsy specimens, each sample is evaluated histopathologically and then subjected to microdissection. Serial 5mm sections are prepared and each placed in the centre of an uncoated glass slide facilitating laser capture of the target cells. Slide 1 to 5 are stained with toluidine blue. Slides 1-4 will be kept desiccated until used while slide #5 is be coverslipped and used as the reference slide for pathological evaluation. The remaining slides are kept in reserve for verification of experimental result and/or used if not enough cells can be microdissected from slides 1-4. Under direct microscopic observation, a vial cap, which carries a thermoplastic film on its undersurface, is placed over sections from paraffin-embedded or frozen biopsy material. A laser is then aimed over the desired cells in the sections. Upon activation of the laser, the targeted cells are selectively adhered onto the film and can be removed for further analysis (for review of LCM see: Pappalardo et al., 1998; Simone et al., 1998). DNA is extracted from the captured cells. DNA concentrations are determined by quantitative PCR comparing against a standard curve generated from the amplification of known DNA quantities of genomic DNA.
SMAL-PCR DNA fingerprinting. This technique is designed for systematic high density scanning for alterations in cancer cells. Due to the minute quantities of DNA available in microdissected samples, we have modified the conventional Arbitrarily Primed-PCR (AP-PCR) DNA fingerprinting (Peinado et al., 1992; Ionov et al., 1993) technology for analyzing microdissected cells. We have named the new technique Scanning of Microdissected Archival Lesions, SMAL-PCR. As in AP-PCR, SMAL-PCR utilizes multiples of short, arbitrary primer pairs of 10 nucleotides to simultaneously amplify a large number of targets randomly distributed throughout the genome. The resulting PCR fragments are separated on acrylamide gels allowing the identification of polymorphic genetic alterations between, e.g. normal and cancerous tissue.

This technique has yielded much more genetic information than that provided by the conventional assays for detecting chromosomal alterations. We have shown that highly reproducible DNA fingerprints could be generated from < 2 ng of DNA (300 cell equivalents) extracted from cells of various archived tumor specimens.

2.2. Progress in Specific Aims

Aim 1: Generate SMAL DNA fingerprints from normal epithelium, PIN and tumors

Procurement of archival tissue:
Tissue acquisition continues. A large number of paraffin embedded CaP biopsies have been evaluated histopathologically in order to identify specimens containing pre-cancerous and cancerous lesions (cells) suitable for microdissection. Under the supervision of 2 pathologist, areas of normal epithelium, PIN and invasive carcinoma were identified (Figure 1).

![A.](image1.png) ![B.](image2.png) ![C.](image3.png)

Figure 1. Partial images of a cross-section through a formalin-fixed paraffin-embedded whole-mount prostate gland showing (yellow arrows) the histology of (A) normal, (B) prostatic intraepithelial neoplasia (PIN) and (C) early carcinoma cells of Gleason grade 3.
**LCM, DNA extraction and SMAL fingerprinting:**

The optimization of LCM was described in detail in the previous report and will not be addressed here, except that one example is provided in Figure 2.

**Figure 2.** Microdissection of PIN from toluidine blue stained sections of formalin-fixed paraffin-embedded prostate archival biopsies by Laser Capture Microdissection (LCM).

Optimization of DNA extraction protocol chived in Year 2 increased DNA yield for SMAL PCR experiments. Figure 3 shows the effects of various digestion buffers on DNA extraction and yield and Figure 4 shows the evaluation of DNA yield using a multiplex PCR approach.
**Figure 3.** Effects of various digestion buffers on DNA extraction and yield.
Lane 1 = 10mM Tris/1mM EDTA (pH 8.0), 1% Tween 20; Lane 2 = 50mM Tris/1mM EDTA (pH 8.0), 1% Tween 20; Lane 3 = 50mM Tris/1mM EDTA (pH 7.5), 1% Tween 20; Lane 4 = 10mM Tris/2mM EDTA (pH 8.0), 1% Tween 20; Lane 5 = 10mM Tris/2mM EDTA (pH 8.0), 0.5% SDS; Lane 6 = 10mM Tris/1mM EDTA (pH 8.0), 0.5% SDS and 50mM NaCl [ +0.1% Proteinase K Digestion overnight at 42°C]

**Figure 4.** Assessing DNA quantity and quality of microdissected prostate samples by multiplex PCR. PCR amplification (30 cycles) of 250bp and 157bp LEN gene fragment of microdissected samples (A-G) of unknown quantity and quality compared to amplification of a decreasing genomic DNA concentration standard.
Genomic DNA was extracted from 73 laser capture microdissected prostate samples that comprised 28 samples of normal cells, 15 samples representing cells from prostatic intraepithelial neoplasia (PIN) and 30 samples of early invasive carcinoma of varying degrees (Gleason grades 2 to 4) from 28 formalin-fixed, paraffin-embedded radical prostatectomy specimens. Sections from 28 formalin-fixed, paraffin-embedded radical prostatectomy specimens were microdissected by laser capture microdissection (LCM) to obtain matched samples of normal, prostatic intraepithelial neoplasia (PIN) and early invasive carcinoma cells (Gleason grades 3). Genomic DNA was extracted from these cell samples and pairs of normal and tumor DNA samples (at 2 nanogram each) were subjected to SMAL-PCR DNA fingerprinting that scans microdissected archival lesions for genetic alterations.

Aim 2: Identify recurring alterations that are present in PINs and tumors

Comparison of DNA fingerprints between normal cells, PIN and/or tumors from individual patients revealed genetic alterations (gain or loss of PCR signal). Figure 5 is a schematic diagram of this technology.

A.

B.

Figure 5. Principle of SMAL-PCR DNA fingerprinting: (A) A single arbitrary primer pair (red arrows) binds to multiple targets and is used to amplify genomic DNA on specific regions of a chromosome. Alterations in DNA in patients B to F do not allow primer binding (blue bar) and thus (B) result in loss of a polymorphic fragment in patients B to F.
Comparison amongst a panel of patients revealed recurring genetic alterations (for example, Figure 6). The criterion for the initial screen is that an alteration has to occur more than once in >10% of cases. More than 25 recurring polymorphic fragments have been cloned, sequenced. Localization of these sequences to specific chromosome regions (see Aim 5 below) have been performed in Year 2 of this project as scheduled.

**Figure 6.** (A) SMAL fingerprints from paired prostate normal (N) and early tumor (T) cells from 3 CaP patients. (B) An example of a 250bp recurrent gain in tumor samples, identified in 3 of 14 patients screened. Closed arrows indicate examples of polymorphisms between patients and open arrows indicate differences between normal and tumor samples.

Selected DNA fragment, such as those show in Figure 6 above, were excised from the dried gel guided by the autoradiograph. Re-amplification yielded sufficient quantities of material for cloning into plasmid vectors. Figure 7 the raw data of re-amplification for two of the recurring SMAL-PCR fragments (see next page). Colony PCR is used to confirm the cloning of the correct size fragments (Figure 8).
Figure 7. 1% Agarose gel of re-amplification of (A) SMAL alteration 1 (150bp) in two patients A and B; and (B) alteration 13 (500bp) in three patients C, D and E with primer 1 alone, primer 2 alone and both primers combined.

Figure 8. Colony PCR of patient C in alteration 13 (primers 1 alone); 8 of 9 transformed clones were confirmed to contain the expected size (500bp insert + 168bp vector = 668bp).
The identity of each clone is determined by DNA sequencing (Figure 9).

**Figure 9.** DNA sequence of alteration 13. Sequence determined at the Nucleotide Acid Protein Services (NAPS) Unit at the University of British Columbia.

**Aim 3: To establish an expandable database of recurring genetic changes**

A database of recurring genetic alterations has been established. Information on increasing number of recurring changes, such as frequency of occurrence, histopathological stage of sample, sequence of DNA fragment have been entered into this database.

The database is continually being updated with chromosomal locations of the alterations as the information appears (see Aim 4 below). The genes situated within these regions will be added once determined (Aim 5). The depth of this expandable database will grow as increasing numbers of samples are analyzed.

**Aim 4: To assign recurring alterations to specific chromosomal regions**

The sequence of each SMAL-PCR alteration is being used to identify specific human bacterial artificial chromosome (BAC) clones containing the sequence. In turn, the location of the BAC clone in the human genome informs the chromosomal location of the recurring genetic alteration. This effort will continue in Year 3.
Genetic alterations were discovered at the following chromosomal regions: 2p23.1, 8q22.3 and 18q21.31 were unique to PIN while changes at 3p26.3, 4q35.2, 8q24.13, 11p13, and 16q12.2 were detected only in cancer cells; alterations at 1p32.1, 1q44, 5q23.2, 9q22.32 and at 12p12.1 were found in both stages.

Figure 10. Illustration of the chromosome regions that display instability in CaP as identified by SMAL-PCR.

A detailed summary of the recurrent alterations identified is shown in Table 1. Chromosomal location, assigned patient number, histological stage of lesions, as well as information on the identified fragments are listed.

In addition, DNA probes generated from selected BACs have been used in fluorescent in situ hybridization experiments on tissue sections from radical prostatectomy specimens to validate the alterations detected by SMAL. This activity will also continue in Year 3.
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<th>Assigned Patient No</th>
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<th>Gain or Loss of Polymorphic Fragment</th>
<th>Frequency</th>
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Aim 5: To identify candidate genes in the mapped regions for future mutation scanning

We have identified candidate genes in all of the chromosomal regions of alteration, described in Aim 4 above, by computational searches of all known cDNA, EST and microarray and SAGE expression data bases. 11 candidate genes have been implicated so far. This information (gene list) is continually being added to the database described in Aim 3 above.

Aim 6: To begin validation of candidate genes

In the original proposal, we anticipated to begin validation of candidate genes (the best two genes) near the end of the project in Year 3. We have initiated this process by assembling the necessary reagents and performing “test runs” using known genes at the end Year 2. However, in order to select the best candidates for validation, this activity will not be implemented on candidate genes until the scheduled time in Year 3.

3. Key Research Accomplishments

While some of the accomplishments are incremental (accumulation of data ), there have been exciting scientific discoveries made in the second year of this project.

The key research accomplishments in Year 2 of this project is listed below:

1. Procurement of a growing panel of histopathologically graded prostate specimens and the identification of >75 lesions suitable for microdissection.

2. Laser capture microdissection of >75 samples. The LCM technology is well established in Dr. Vielkind’s (co-applicant) laboratory. In addition to the Arcturus PixCell II laser capture microdissection device described in the previous annual report, 2 additional laser assisted microdissection devices have been established based on funds provided by other granting Agencies. These equipments have allowed us to tailor dissection strategy for specific tissues. This activity (isolation of pure cell populations) will continue in Year 3.

3. Identification of >24 recurring genetic alterations in PIN and/or prostatic carcinoma. We have observed that some of the alterations identified so far do map to chromosomal regions previously unknown to be involved in prostate cancer development.

4. Discovery of 11 candidate genes in 14 altered chromosomal regions. (Analysis of the rest of the novel regions is in progress.)

5. Work on laser capture microdissection has been presented at 4 conferences to date.

Stephanie Ma, Hans Adomat, Terry Bainbridge, Doug Webber, Wan Lam, Juergen Vielkind. A concerted genomic and proteomic approach to understand the early steps in the genesis of prostate cancer. American Association for Cancer Research, San Francisco, CA (April 2002)

Stephanie Ma, Hans Adomat, Terry Bainbridge, Doug Webber, Wan Lam, Juergen Vielkind. A concerted genomic and proteomic approach to understand the early steps in prostate carcinogenesis. British Columbia Cancer Agency Conference, Vancouver, Canada (Nov 2001)


6. Two Abstract have been published and 2 have been submitted based on our early results. One manuscript is in preparation.


4. Reportable Outcomes

The scientific accomplishments for Year 2 is listed in section 3 above. This section measures our progress (reportable outcomes) against the original Statement of Work (Task 1-6).

Task 1. To generate DNA fingerprints from normal epithelium, PIN and early invasive carcinomas (months 1-30)
   • case selection and pathology review of 300 cases (months 1-30)
   • laser capture microdissection of 150 samples for each stage (months 1-30)
• DNA preparation, quantitation and quality control (months 1-30)
• generate 90,000 DNA fingerprints using 20 primer pairs for each of the 450 samples (months 4-30)
• collect and process fingerprint images for analysis (months 4-30)

This Task spans Year 1 and 2. For month 1-24, the following reportable outcomes have been achieved.
• >150 cases have been reviewed by collaborating pathologists to identify 75 suitable for microdissection
• >75 samples (ie. Normal, PIN, cancer) were microdissected
• DNA was extracted from all samples
• Hundreds of SMAL-PCR profiles were generated using 1 primer pair; on average, 30 fingerprint bands are produced per profile giving an estimate of 10,000 fingerprint bands.

For month 25-30, tissue accrual and DNA fingerprinting experiments continues.

Task 2. To identify recurring alterations that are present in the stages (months 4-30)
• compare pairs of fingerprint images within each histopathological stage to identify recurring alterations (months 4-30)

A total of 24 recurrent changes were identified (66 separate bands, originating from the 24 recurrent changes, were isolated). After cloning and sequencing, 20 recurring genetic alterations have been identified to date.

Task 3. To establish an expandable database of recurring changes for each stage of disease development (months 4-30)
• establish and format database
• data entry as information becomes available (months 4-30)
• identify recurring alterations that occur in PIN and are also present in early invasive carcinomas (12-30)

A database of recurrent genetic changes found in pre-cancerous and cancerous specimens has been established. Information such as pathological grades, frequency of occurrence and DNA sequence are included. Chromosomal location of the genetic alterations as well as candidate genes in these regions will be added as they become available (see Aim 3 in Section 2 above).

Task 4. To assign recurring alterations to specific chromosomal regions (months 12-36)
• clone and sequence selected recurring alterations (months 12-36)
  - Year 1: 10 alterations
  - Year 2: 20 alterations
  - Year 3: 20 alterations
• map sequences to chromosomal locations
- locate alterations by comparing sequences against available human genome sequence (months 12-36)
- locate remaining alterations by hybridization to human BAC library (months 24-36)

Alterations have been mapped to the following chromosomal regions. 2p23.1, 8q22.3 and 18q21.31 were unique to PIN while changes at 3p26.3, 4q35.2, 8q24.13, 11p13, and 16q12.2 were detected only in cancer cells; changes at 1p32.1, 1q44, 5q23.2, 9q22.32 and at 12p12.1 were found in both stages.

Some findings have been confirmed by fluorescent in situ hybridization experiments on tumor tissue sections.

Task 5. To identify candidate genes in the mapped regions for future mutation scanning (months 24-36)
- search cDNA, EST, microarray and SAGE data bases (months 24-36)

Task 5 is schedule for Year 3. However, we have identified 11 candidate genes to date. Altered regions included genes encoding TGF-beta signaling regulator genes, one extracellular matrix gene upregulated by TGF-beta, two genes encoding steroid metabolism enzymes, 5 genes reminiscent of putative tumor suppressor genes and one gene coding for a cleavage stimulation factor.

Task 6. To begin validation of the best 2 candidate genes. (months 18-36)
- dissection of frozen tissue sections from prostate biopsies
- isolate RNA samples (months 18-36)
- mutation detection (months 24-36)

Task 6 was initiated in the middle of Year 2 of this project. Frozen prostate biopsies are being collected. RNA extraction procedure has been optimized. The validation of 2 selected genes will commence in Year 3 on schedule.

5. Conclusions

As mentioned in the previous annual report, operationally, the work accomplished in the first 2 year of this project matched that of the proposed Task in the Statement of Work and the original proposal. Meeting the milestones suggests that the required materials, infrastructure and expertise are available and capable of support the work proposed. The following processes have been established: tissue specimen procurement, histopathological evaluation, laser capture microdissection, DNA extraction and quantitation, SMAL-PCR fingerprint comparison, cloning and sequencing, chromosomal localization, databases and bioinformatics.

Scientifically, our results not only demonstrate the feasibility of this project, but have also yielded novel recurring genetic alterations in microdissected PINs and invasive carcinoma of the prostate, despite the minute size of such samples. Increasing number of genetic alterations is being identified and candidate genes mapped. Regions that contain components (genes) of TGF-
beta signaling and steroid metabolism are among the early changes in prostate cancer development.

As mention in the original proposal, by the end of this work, we will have identified a set of genetic loci by virtue of their frequency of alteration in premalignant lesions and subsequently in low grade tumors. We will have established a "genetic alterations in prostate cancer" database which catalogs somatic changes present in the various stages of cancer progression. This database, when completed, will be made publicly and freely accessible on the BC Cancer Research Center web site. Researchers studying cancer progression (prostate and otherwise) will be able to access information and submit their own data. These data will be extremely valuable in identifying targets for early diagnosis and treatment. Future studies will allow cross-referencing of stage-specific fingerprints against clinical outcome and thus will allow relating genotype to disease risk and behavior.

6. References


7. Appendices

None – figures and diagrams are embedded in the body of text.