AD_____

Award Number: DAMD17-03-1-0154

TITLE: Analysis of Preneoplasia Associated with Progression to Prostatic Cancer

PRINCIPAL INVESTIGATOR: Dr. Maisa Yoshimoto
Jeremy Squire, Ph.D.

CONTRACTING ORGANIZATION: Ontario Cancer Institute
Toronto, Ontario, Canada M5G 2M9

REPORT DATE: March 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2005	Annual Summary (1 Mar 2003 - 28 Feb 2005)

4. TITLE AND SUBTITLE
Analysis of Preneoplasia Associated with Progression to Prostatic Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0154

6. AUTHOR(S)
Dr. Maisa Yoshimoto
Jeremy Squire, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Ontario Cancer Institute
Toronto, Ontario, Canada M5G 2M9

E-Mail: Jeremy.squire@utoronto.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

PURPOSE: To examine the topographical variation in expression levels of genes associated with prostate cancer, telomere dysfunction and/or chromosomal instability. **SCOPE:** To show that telomere erosion observed in prostatic epithelium may involve DNA damage response/repair pathways at the onset of preneoplasia (HPIN) and cancer in men. **MAJOR FINDINGS:** Our working hypothesis is that cells that undergo telomere loss as part of the normal aging process in the prostate are more susceptible to undergo chromosome end-fusion thus triggering genomic instability. Our initial progress showed that loss of telomere length occurred in preneoplastic HPIN lesions that were located close to small, localized microfoci of newly diagnosed prostate cancer. We have optimized whole genome and RNA amplification techniques and shown that there is high fidelity and reproducibility of dissected amplified PCR product. **RESULTS:** The first phase of gene expression profiling in HPIN, and cancer foci using repair/damage response array has been successfully performed. We are developing topographical maps of telomere loass, genomic instability and concomitant changes in gene expression. **SIGNIFICANCE:** These results will form the first direct link between telomere-dependent alteration, DNA repair and damage response signaling in porstate cancer.

14. SUBJECT TERMS
Preneoplasia, Chromosomal instability, DNA damage, DNA repair, telomere erosion

15. NUMBER OF PAGES
101

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....7

Reportable Outcomes.....7

Conclusions.....8

References.....8

Appendices.....9

INTRODUCTION

Premalignant Lesions and Cancer

The development of solid tumors is generally thought to be a multistep process, whereby successive genetic events occur in a normal cell to render it increasingly malignant. The accumulation of many such changes is widely assumed to result from an underlying genetic instability [1]. In prostate cancer (CaP), the genetic and epigenetic phenomena that result in cancer are not well understood, but there is evidence that premalignant lesions exist in the prostate may precede the development of cancer by many years. Understanding the molecular mechanisms underlying the early steps of CaP tumourigenesis will contribute toward the identification of molecular or recurrent genetic biomarker(s) crucial for signaling early transformation changes that lead to CaP. High grade prostatic intraepithelial neoplasia (HPIN) is regarded as the most likely precursor of prostatic adenocarcinoma and its histological detection in biopsy is considered a risk factor for CaP development [2]-[4] HPIN can be found in up to 70-80% of glands that show presence of carcinoma. It is considered that in about 1/3 of patients HPIN will progress to invasive carcinoma within 10 years. **Thus, early genetic events in HPIN are most likely to be key in driving the tumorigenic process. Our progress whilst being supported by this grant allowed us to develop a coherent biological model that addresses the early genetic steps in the transition from HPIN to neoplasia.**

BODY

Chromosomal Instability

Chromosomal instability (CIN) arises as a result of aberrations in the cell's mitotic machinery and/or in the structural integrity of the chromosomes such as telomere dysfunction and is thought to be a major mechanism underlying tumorigenesis. Our progress in CaP before the start of this work was centered on the analysis of telomere erosion associated with CIN in HPIN (Vukovic *et al.*, 2003) [4]. **The purpose of this project funded by Award DAMD17-03-1-0154 was to follow up on this observation and to study gene expression profiles in the context of genetic instability, telomere erosion and CaP.** Because of the limited numbers of cells present in tiny HPIN lesions and microfoci of CaP it was necessary to optimize methods of representative amplification of nucleic acid following dissection (**tasks a and b in SOW**). In Hughes *et al.* (2004 – **Appendices 5** and **6**) we optimized array comparative genomic hybridization (aCGH) analysis of DNA amplified by strand displacement primed PCR from dissected homogeneous tissue samples. In follow-up work discrete, individual tumor microfoci and HPIN were dissected from radical prostatectomy samples with unfavorable outcome. Microfoci of epithelial acini from the same tumor, separated by no more than 3mm, exhibit significantly different aCGH profiles indicating high extent of CIN within CaP tumors and underscored the intrinsic genotypic heterogeneity of early lesions of CaP. Significantly, losses of chromosomes 8p, 13q and 16q were recurrently observed, as were

gains of chromosome arm 8q (with concomitant loss of 8p). This latter observation implicates isochromome 8q formation. These findings were consistent with existing cytogenetic literature based on metaphase CGH studies. This study is in preparation for publication (**Appendix 1**).

Damage Response Pathways and Application of Molecular Profiling in the Analysis of HPIN and CaP

Certain components of DNA damage response pathways, originally defined by their roles in the repair of chromosomal DNA breaks, are found at telomeres and are necessary for normal telomere maintenance and functions. Breaks in chromosomal DNA can be repaired by nonhomologous end-joining (NHEJ) or by homologous recombination. Many of the components of DNA damage response needed for telomere maintenance or cognate downstream repair proteins are represented on pathway-specific GEArrays (SuperArray, Frederick, MD, USA).

Molecular profiling to interrogate the transcriptome in the different stages of prostate tumourigenesis (including preneoplastic HPIN) has been hampered by the microscopic size of these premalignant lesions and the infiltrating nature of the disease process within the gland. Thus traditional tissue RNA extraction techniques are not applicable, as the contaminating cells that constitute the majority of a clinical sample compromise the resulting gene expression data. The use of microdissection and T7 amplification with pathway-specific macroarrays (see below) has provided a more focused hypothesis-driven approach to profiling subsets of genes that are mechanistically related to telomere-dependent genomic instability and preneoplasia.

Identification of Topographical Variation of Differentially Expressed Genes in CaP Associated with Telomere Dysfunction and/or CIN

According to the rationale for the experiments, the end of the first year of funding is to evaluate the role of damage response and repair signaling pathways associated with telomere erosion and acquisition of CIN in HPIN. In the present proposal, we have chosen to evaluate the DNA damage pathway in CaP insteed of HPIN (as originally proposed), as our previous study exhibited significantly different aCGH profiles indicating high extent of CIN within CaP tumors and intrinsic genotypic heterogeneity of HPIN (Appendix 1). Wedges of tissue parallel to the archived sample comprising ten 'cancer specimens from radical prostatectomy' were snapped frozen tissue and embedded in optimal cutting temperature solution for analysis of expression differences in DNA damage signaling and genome stability pathways. In this study the tissue was sectioned into six 10μm thick slides for RNA extraction and four 4μm thick slides for immunohistochemistry (IHC), flanked by 6μm thick slides for hematoxilin and eosin (H&E) staining. Microdissection was performed only on samples that had relatively high tumor content (>75%) but lack the wide spread of tumor using adjacent H&E-stained sections as guidance. RNA was extracted using the Absolutely RNA micro-isolation kit (Stratagene, La Jolla, CA) and then subjected to T7 linear amplification. In the present proposal, we have chosen to use the Amino Allyl MessageAmp aRNA amplification kit (Ambion, Austin, TX), rather than Clonetech SMART PCR amplification (as originally proposed) as our pilot data demonstrated superior yield and fidelity. Amplified RNA was

labeled and hybridized to pathway-specific GEArray following the manufacturer's instructions (SuperArray, Frederick, MD, USA). We used chemoluminescence-based labeling and detection methods and the level of expression of each gene was quantified using the GenePix software (Axon, Union City, CA). The quantified values were normalized with cyplophilin A (present on each of the GEArray) and analyzed using the GEArrayAnalyzer software. Our data suggest that the genome of CaP cells display mutation mechanisms in DNA mismatch repair gene associated with genetic instability. Significantly, RAD51 dysfunction suggests a molecular mechanism to account for the most frequent chromosomal aberration in CaP: isochromosome 8q (see Appendix 1). It is suggested that failure in the fidelity of homologous recombination within the repetitive sequences, that comprise the kinetochore complex, could lead to recuurent loss of 8p and gain of 8q by rearrangement of chromosome 8-specific alphoid centromeric sequences. Thus, the high fidelity process of homologous recombination can be the major DNA repair pathway, which is indispensable for the maintenance of genetic stability (**task e**). This study is in preparation for publication (**Appendix 2**).

Consequently, we anticipate performing real-time RT-PCR validation on several important genes. For the purpose of this validation, appropriate primers for the genes of interest will be designed using the guidelines provided by Applied Biosystems (Streetsville, ON). The real-time RT-PCR will be carried out in two separate steps, reverse transcription using Superscript II RNase H^- Reverse Transcriptase (Invitrogen, Burlington, Ontario, Canada) followed by quantitative PCR using the SYBR Green reagents. Incorporation of the SYBR Green dye into PCR products will be monitored in real time with the ABI PRISM 7700 sequence detection system (Applied Biosystems), thereby allowing determination of the threshold cycle (C_T), the point at which exponential amplification of PCR products begins. The C_T values for the housekeeping gene cyclophilin A will be used to calculate the relative transcript abundance for the genes of interest.

Immunohistochemistry/Expression Level of Proteins using Tissue Microarrays

The experimental design of protein validations of differential expression is investigating qualitative and quantitative variation in proteins such as TP53, hTERT, PTEN and other proteins in HPIN that exhibit a significant association with telomere erosion, acquisition of CIN, ploidy change etc. Our rationale for this component of the study is that some of the established molecular pathways suggested by our expression studies may involve alterations in protein phosphorylation and kinase signaling detectable at the protein levels (as described below). It is clear that many such activating covalent modifications will alter protein function, but they may not be detectable by conventional analysis of RNA expression levels. We are currently selecting well-characterized antibodies suitable for detailed investigating of pathways of interest.

Fluorescence *in situ* Hybridization (FISH) and Immunohistochemistry of PTEN using Tissue Microarrays

Phosphatase and tensin homolog (PTEN), is a tumor suppressor protein that inhibits the phosphatidylinosital-3-kinase (PI3K)/Akt/protein kinase B (PKB) pathway and is one of the most frequently deregulated genes in a wide range of cancers. Although somatic

PTEN alterations have been reported in prostate cancer, including loss of heterozygosity or homozygous deletions, point mutations, and promoter hypermethylation, the relationship between genomic alterations of PTEN and prostatic neoplasia remains unclear. Archival formalin-fixed, paraffin embedded tissues from 25 radical prostatectomy specimens were used for tissue microarray assembly. Arrayed tissues were examined by H&E staining and scored according to Gleason grading criteria. Standard dual-color FISH was performed using commercially-available DNA probes (Vysis Inc., Markham, ON, Canada) for band 10q23 (PTEN locus) and band region 10p11.1-q11.1 (centromere of chromosome 10). At least 100 nonoverlapped intact interphase nuclei were scored in areas of cancer, HPIN, benign glandular epithelium and stroma. The signal counts for areas of neoplasia were compared against those obtained from benign and stromal cell nuclei using standard statistical methods. Groups of 6/6 (100%) cases of benign hyperplasia and 16/16 cases (100%) of PIN were found to be PTEN deletion-negative while 17/25 (68%) of cancer samples were PTEN deletion-positive ($P < 0.01$). In addition, a correlation was observed between PTEN deletion status and immunohistochemical PTEN analysis. These observations support the hypothesis that a relative imbalance of PTEN is likely to be an important factor in the PIN progression to prostate cancer. This current study has been prepared to publication. See illustration of PTEN expression analysis by IHC in **Appendix 3** (Figure 1) and PTEN gene copy number analysis by FISH in **Appendix 4** (Figure 2).

KEY RESEARCH ACCOMPLISHMENTS

- Integration of telomere erosion, genomic alterations and DNA damage response expression analysis to investigate mechanisms of early progression in prostate cancer. Detection of PTEN regional deletion and implication that isochromosome 8q is the most frequent event in CaP and HPIN (**Appendix 1**)
- Identification of topographical variation of differentially expressed genes in CaP associated with telomere dysfunction and/or CIN and identification of RAD51 as potential mechanism leading to 8q isochromsome formation (**Appendix 2**)
- Beheshti B, Vukovic B, Hughes S, Watson SK, Ishkanian AS, Lam WL, and Squire JS. High resolution genomic analysis of prostate cancer using CGH arrays and molecular cytogenetic methods. 95th Annual Meeting of the American Association for Cancer Research, Orlando, FL, USA, March 2004 (poster presentation).
- Yoshimoto M, Cutz J-C, Bayani J, Evans AJ, Zielenska M and Squire JA. Detection of PTEN Deletion in Prostate Cancer but not Prostatic Intra-epithelial Neoplasia (PIN) by Fluoresecence In-situ Hybridization (FISH) on Tissue Microarrays. 94th Annual Meeting of the United States and Canadian Academy of Pathology, San Antonio, TX, February-March, 2005 (poster presentation).

REPORTABLE OUTCOMES

Hughes S, Yoshimoto M, Evans A, Beheshti B, Squire JA. The use of whole genome amplification to study genomic instability in prostate cancer (*In preparation, SEE APPENDIX 1*)

Yoshimoto M, Albert M, Wong L, Hughes S, Macgregor P, Squire J, Evans A. Pathway-specific array analysis of DNA Damage Signaling in Prostate Cancer (*In preparation, SEE APPENDIX 2*)

Yoshimoto M, Cutz J-C, Nuin PAS, Bayani J, Zielenska M, Evans AJ, Squire JA. Detection of PTEN Deletion in Prostate Cancer but not Prostatic Intra-epithelial Neoplasia by Fluoresecence *in situ* Hybridization on Tissue Microarrays (*In preparation*)

Hughes S, Lim G, Beheshti B, Bayani J, Marrano P, Huang A, Squire JA. Use of whole genome amplification and comparative genomic hybridisation to detect chromosomal copy number alterations in cell line material and tumour tissue. Cytogenet Genome Res. 2004 (*SEE APPENDIX 5*)

Hughes S, Arneson N, Done S, Squire JA. The use of whole genome amplification in the study of human diseases. Progress in Biophysics & Molecular Biology. 2004 (*SEE APPENDIX 6*)

CONCLUSIONS

We have determined patterns of genomic alterations and expression findings derived from pathways-specific DNA damage signaling array (**task d**). We have optimized RNA amplification technique with high fidelity and reproducibility since our analysis is based on a few thousand cells microdissected tissue (**task c**). We are rapidly developing topographical maps of telomere loss, genomic instability and concomitant changes in DNA repair and damage response signaling. In addition one gene PTEN that has emerged from these analyses is being validated at the copy number and protein level (**task f**). Our next phase is to expand validation of our expression findings at the RNA and protein level, and to determine which molecular pathways are the most predictive of prostate cancer onset (**task g**).

REFERENCES

1. Loeb, LA. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res, 1991; 51(12):3075-9.
2. McNeal, JE and DG Bostwic. Intraductal dysplasia: a premalignant lesion of the prostate. Hum Pathol, 1986; 17(1):64-71.
3. Haggman, MJ et al. The relationship between prostatic intraepithelial neoplasia and prostate cancer: critical issues. J Urol, 1997; 158(1):12-22.
4. Vukovic B, Park PC, Al-Maghrabi J, Beheshti B, Sweet J, Evans A, Trachtenberg J, Squire J. Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. Oncogene.2003;22(13):1978-87

APPENDIX 1

The use of whole genome amplification to study genomic instability in prostate cancer.

S. Hughes[1]*, M. Yoshimoto[2]*, Andy Evans[4,5], B. Beheshti[3], J.A. Squire[2,4,6].

Institute of Cancer Research, UK[1], Ontario Cancer Institute, Princess Margaret Hospital[2], University of Toronto, Faculty of Medicine[3], University of Toronto Department of Medical Biophysics[4], Division of Pathology, Faculty of Medicine[5], Department of Laboratory Medicine and Pathology[6]

Key Words: prostate cancer, genomic instability, whole genome amplification, comparative genomic hybridization, genomic DNA arrays

Corresponding author: Jeremy Squire

Princess Margaret Hospital

Ontario Cancer Institute

University Health Network

610 University Avenue, Room 9-717

Toronto, Ontario

M5G 2M9, Canada

Email: jeremy.squire@utoronto.ca

* Both authors contributed equally to this work.

Abstract

Prostate cancer is a multifatorial disease with genetic and environmental components involved in its etiology. The knowledge of genetic basis of CaP has increased over the past years, mainly in the pathways that underlie tumorigenesis and growth and drug resistance for each clinical stage. Histologically prostate cancer is a heterogeneous disease consisting of cancerous and pre-malignant lesions interspersed by normal non-cancerous tissue. This heterogeneity can complicate genomic analysis, as when DNA is bulk extracted from such tissue the genetic information obtained represents an average for all of the cells within the sample. To minimize this problem we obtained DNA from individual regions of High-grade prostatic intraepithelial neoplasia (HPIN) and prostate cancer (CaP) by laser capture microdissection. The small quantities of DNA material thus obtained were then amplified by means of strand displacement amplification (SDA) for use in genomic DNA array comparative genomic hybridisation (gaCGH). Recurrent chromosome copy number abnormalities (CNAs) where observed in both HPIN and CaP. In HPIN, chromosomal imbalances involving chromosome 8 where common, whilst in CaP additional chromosomal changes involving chromosomes 6, 10, 13 and 16 where also frequently observed. An overall increase in chromosomal changes was seen in CaP compared to HPIN suggesting a universal breakdown in chromosomal stability. The recurrent observation of 8p loss and concomitant 8q gain implicates isochromosome 8q as a frequent event in HPIN and CaP. The combination of LCM, SDA and gaCGH is ideally suited for the identification of CNAs from small cell clusters and may help to discover potential genomic markers for early diagnosis or identify the location of tumour suppressor genes (TSG) or oncogenes previously unreported in

prostate tumourigenesis, as well as to investigate the potential role of chromosomal instability (CIN) in preneoplasia associated with progression in prostatic cancer.

Introduction

The worldwide occurrence of CaP is on the increase, with it now having overtaken lung cancer as the most commonly diagnosed malignancy of men in the Western World. In spite of significant progress in its clinical management comparatively little is known about the aetiology of the disease and predicting outcome is still very complicated for most patients. Histopathologically, CaP displays considerable heterogeneity and can contain several premalignant as well as cancerous foci. The presence of HPIN, a putative precursor of CaP, is currently considered the most likely precursor to invasive prostatic adenocarcinoma (van der Kwast *et al.*, 1999). However, most of these foci are latent and rarely develop into clinical cancer. Unfortunately a significant number (3%) do progress and can become aggressive and lethal (Al-Maghrabi *et al.*, 2002). The major difficulty facing clinicians is the identification of those patients presenting at an early stage who are likely to develop life-threatening disease. As a result, intensive research is currently underway to identify the key alterations that may prove to be important for both classification and prognosis prediction.

The advent and development of CGH, initially described in the early 1990's (Kallioniemi *et al.*, 1992), has revolutionised cytogenetics and allowed for genome-wide screening of numerous cancer types and the identification of genomic copy number alterations (CNA) associated with specific tumour types, stages or outcomes (Squire *et al.*, 2002; Cleton-Jansen *et al.*, 2004; Kimura *et al.*, 2004). When first introduced, CGH used metaphase chromosomes targets to identify CNAs, however, recent advance have substituted these with arrayed DNA sequences that provide higher resolution, 1Mb versus

10Mb, and greater ease of analysis. (Hughes *et al*, 2004a) Whole genome scans of CaP patients and cell lines have identified consistent, chromosomal alterations, which include recurrent loss of chromosomal regions from 5p, 6q, 8p, 10q, 13, 16q and 17p, in addition to gain on 1q, 3q 7p, 7q, 8q 11p, 17q, Xp and Xq (Wolf *et al.*, 2004; van Dekken *et al.*, 2003; Clark *et al.*, 2003). In addition, genotypic heterogeneity and multiple foci have been suggested to be a likely mechanism underlying clonal evolution of PIN (Bostwick *et al.*, 1998). The accumulation of genetic changes that occurs during the stepwise evolution from normal tissue to metastasis, although likely due to increased genetic instability, may indicate the chromosomal locations of TSG or oncogenes that are important in tumourigenesis.

In the present study we describe the use of LCM, SDA and gaCGH to investigate copy number changes occurring in cancer by the analysis of 8 prostatectomy specimens with HPIN and 8 with CaP. Understanding of the molecular mechanisms behind this process will enable identification of molecular and cytogenetic biomarker(s) that are able to predict early transformation into an aggressive phenotype, as well as, fundamental insights concerning the regulatory pathways of the integrity of genome that may lead to multistep field cancerization and preneoplastic HPIN and CaP.

Materials and Methods

Tissue accrual

Fresh prostate tissue was obtained from radical prostatectomies performed at The University Health Network (UHN), Toronto. The tissue was embedded in Frozen Section

Medium (Stephens Scientific, Riverdale NJ, USA). Prostate tissue samples were cut onto microscope slides (8μm thickness) and stained using the HistoGene LCM frozen section staining kit (Arcturus, USA).

Cohort selection

Patient samples for gaCGH analysis were selected dependent on two criteria: the presence of (1) clearly identifiable regions of HPIN or CaP using adjacent hematoxilin and eosin-stained sections as guidance and (2) DNA of high molecular weight provided from 15 patient samples for analysis. DNA quality was assessed by DNA extraction from a single tissue section and visualisation by gel electrophoresis.

Laser capture microdissection and DNA Extraction

A minimum of 1000 cells was removed from foci of HPIN and CaP by laser capture microdissection (LCM) using the Cell Robotics LaserScissors system (Cell Robotics Inc., USA). DNA was extracted from the dissected tissue using the QIAamp DNA Micro Kit (Qiagen, USA) and the DNA concentration was determined using the PicoGreen dsDNA Quantitation kit (Molecular Probes Inc., USA). Both procedures were performed following manufacturers instructions.

Strand displacement amplification

DNA was amplified using the GenomPhi Amplification Kit (Amersham Biosciences, USA) according to manufacturer's instructions (Hughes *et al.*, 2004b). This method of whole genome amplification consistently produced in excess of 5μg of DNA

from a starting concentration of 5-10ng. Briefly, amplification was carried out in two individual steps. The step 1 reaction mixture contained 5-10ng (1000 to 2000 cell equivalents) of DNA (from diluted reference or test DNA) in 1µl of sterile water and 9 µl of Sample Buffer. This mixture was heated at 95°C for 3 minutes and then chilled on ice. The step 2 reaction (amplification) mixture contained 9µl of Reaction Buffer, 1µl of Enzyme Mix and the 10µl from Step 1. The amplification reaction was incubated at 30 °C for 16–18 hours. The enzyme was inactivated by heating at 65 °C for 10 minutes, followed by cooling to 4 °C.

Assessment of DNA quality and strand displacement amplification

Five microlitres of each amplification reaction was electrophoresed through a 1% agarose gel and stained with ethidium bromide in order to assess product yield and product length. All amplification products were purified by phenol-chloroform extraction and DNA concentration and purity were determined by measuring absorbance at A260 and A280.

Spectral Genomic BAC arrays

The genomic DNA arrays used in these experiments were obtained from Spectral Genomics Inc. and consist of 2500 unique BAC and PAC clones, which provide an average genomic resolution of 1 Mb.

CGH experiments were performed using the amplified patient DNA as the "test" and amplified placental DNA as the "normal". In addition, two control experiments were performed, one corresponding to amplified non-neoplastic prostate epithelial cells versus

amplified placental DNA and one corresponding to a comparison of the amplified placental DNA against itself. No CNAs were observed for either of the control experiments. Though CNA information was available for both the X and Y chromosome this data was not included in these analyses.

DNA labeling

Reference DNA (4μg) and test DNA (4μg) were first digested overnight at 37°C using 10 units of *RsaI* (Invitrogen) in a 10μl reaction and then purified using the QIAquick PCR Purification kit (Qiagen). The digested DNA was labeled using the protocol optimized by Spectral Genomics, with separate labeling reactions for Cy3 and Cy5 being set up for both reference and test DNA. Briefly, labeling reaction were set up containing 2μg of DNA, 20μl of 2.5X random primer/reaction buffer mix (Invitrogen) and sterile water up to a final volume of 45μl. The reaction mix was boiled for 5 minutes prior to cooling on ice and the addition of 2.5μl of Spectral labeling buffer (Spectral Genomics, Houston, U.S.A), 1.5μl of either Cy3-dCTP (1mM, Applied Biosystems, Foster City, U.S.A) or Cy5-dCTP (1mM, Applied Biosystems) and 1μl of Klenow Fragment (BioPrime labelling kit, Invitrogen). The reaction was mixed gently and then incubated for 2 hours at 37°C. Following incubation the reaction was stopped by the addition of 5μl 0.5M EDTA (pH8) and heating at 72°C for 10 minutes.

Hybridisation

The Cy3 labeled test DNA was combined with the Cy5 labeled normal reference DNA and vice versa. Each combined probe was mixed with 45μl of Spectral

Hybridisation Buffer (Spectral Genomics), 11.3µl of 5M NaCl and 110µl of room temperature isopropanol. The samples were incubated in the dark at room temperature for 10-15 minutes and centrifuged at 16,000 x g for 10 minutes and the supernatant discarded. The pellets were then washed with 500µl of 70% ethanol. The supernatant was carefully removed and the pellets air-dried at room temperature in the dark. For hybridisation, the pellets were first resuspended in 10µl of sterile water prior to being mixed with 30µl of Spectral Hybridisation Buffer II (Spectral Genomics) by pipetting. The reconstituted probes were then incubated at 72°C for 10 minutes, placed on ice for 5 minutes and then incubate for 30 minutes at 37°C. The probes were hybridised to BAC arrays, covered with a 22x60mm coverslip and incubated for 12-16 hours at 37°C in a humidified chamber.

Washes

The wash buffers, with the exception of Wash I (2X SSC, 0.5% SDS), were pre-warmed to 50°C. The slides were gently dipped into and out of Wash I until the coverslip detached from the slide. The slides were then washed once in Wash II (2X SSC, 50% deionized Formamide, pH 7.5) for 20 minutes, followed by successive washes in Wash III (2X SSC, 0.1% NP-40, pH 7.5) for 20 minutes and Wash IV (0.2X SSC, pH 7.5) for 10 minutes. All washes were performed at 50°C, with the exception of Wash I. The slides were briefly submerged in distilled deionized water for 5-10 seconds and centrifuged for 5 minutes at 85 x g to dry.

Data collection and Analysis

The slides were scanned using an Axon GenePix 4000A confocal Scanner, each fluorescence signal was collected separately and quantified with the GenePix Pro 3.0 software (Axon Instruments, U.S.A). The data was normalised and analysed using Normalise Suite v2.4 (Beheshti *et al.*, 2003), regions of loss or gain were determined as those that were 2 standard deviations above the mean baseline for each separate sample.

Results

A summary of the chromosomal CNAs detected by CGH for the 8 HPIN and 8 CaP samples is shown in Table 1 and displayed graphically in Figure 1. Redlines to the left of the chromosome indicate loss in the tumour and green lines to the right of the chromosome indicate gain in the tumour. In addition, group a) corresponds to HPIN samples and group b) to CaP samples. The general pattern of loss or gain was very similar in the HPIN and CaP samples, however CaP samples possessed significantly more aberrations than HPIN samples ($p<0.0001$, chi squared test). The average number of CNAs for the 8 HPIN samples was 9.25 (range 3 to 32, median 6.5, although 7 of the 8 samples were in the range 3 to 8, median 6). For the 8 dissected CaP samples an average of 11.25 (range 5 to 18, median 11.5) amplifications or deletions were observed. In dissected HPIN, losses ($\leq$ 25%) were found on 8p (100%), 6q, 10q, 13q, 16q (37.5%, each), 4q, 18q (25%, each), whereas gains ($\leq$ 25%) were found on 8q, 7p (50%, each), 1p, 16p (37.5%, each) and 12q, 20q (25%). For the 8 dissected cancer samples recurrent chromosomal changes, detected in greater than 25% of tumours, were losses on 8p (100%), 10q (75%), 13q (62.5%), 6q (50%), 1p, 16q (37.5%), 5q, 12p, 18q (25%, each) and gains on 7p, 7q, 8q (62.5%), 4p, 16p, 19p (25%)

Small consensus regions of consistent CNA were observed for both HPIN and CaP samples (Table 2), in >25% of samples. For example, 8q23.1 – q23.2 was consistently gained in HPIN (100%), but within a region (8q21.11 – qter) that was commonly gained in >37.5% of the HPIN samples. Similarly, 8q21.3 – q23.1 was consistently gained in CaP (100%), but within a region (8q21.11 – qter) that was commonly gained in >50% of

the CaP samples. A list of the genes present within these consensus or commonly gained regions is displayed in Table 2.

Discussion

In this study we have used a 2,400-element BAC microarray with a resolution of ~1 Mb to study CNAs in a set of 16 patient samples comprised of 8 HPIN cases and 8 CaP cases. The likely reason for increase in CNAs in this HPIN lesion is its close proximity to a region of cancer within the tissue. Although study of the tissue sections was inconclusive, it is possible that during post operative tissue processing, adjacent regions of HPIN and cancer may have been separated with the latter residing it in a separate portion of tissue that we did not have access to. This close proximity is important as previous work by our group has indicated that foci of HPIN and cancer situated immediately adjacent to one another demonstrate a higher degree of telomeres loss compared to HPIN located away from foci of CaP (Vukovic *et al.*, 2003). In the context of CNAs this similarity in loss of telomeres suggests a similar level of chromosomal instability in the HPIN and cancer foci, which would explain the increase in aberrations for CaP 8.

As with other cancers, CaP development and progression is likely to be the outcome of a series of stepwise genetic changes. The accumulation of CNAs, which occurs during this process, although likely due to increased genetic instability, is non-random and may indicate chromosomal regions important in tumourgenesis. Examination of our array results indicates that aberrations involving parts or all of 1p, 6q, 7p, 7q, 8p, 8q, 10q, 13q

16p and 16q are most common and thus may constitute a programmed cycle of events that promote tumour development, progression and survival.

A comparison of the CNAs present in the HPIN and CaP samples identified a significant increase in copy number for 7q ($p<0.01$, chi squared test) and a significantly increased frequency of loss for 10q ($p<0.01$, chi squared test) and 13q ($p<0.0001$, chi squared test). In genotype/phenotype correlations, gain of chromosome 7q (Paiss *et al.*, 2003) and loss of 13q (Dong *et al.*, 2001) have been associated with advancing tumour stage and aggressiveness. Which is in agreement with the results presented here. However, gain of 8q (Alers *et al.*, 2000) and loss of 16q (Matsuyama *et al.*, 2003) have also been linked to tumours progression but our data does not show any significant difference for these CNAs in our HPIN and CaP samples (8q, $p = 0.2655$; 16q, $p = 0.3375$). This would suggest that these are likely to be early events in the tumourigenic process. In addition, they may also identify HPIN and CaP samples that are likely to progress.

Additional alterations that include gains on 12q (HPIN) and loss of 4q (HPIN and CaP) have also been identified. Whether the identification of these regions will provide additional insight into CaP progression is not yet clear. However, what is apparent is that the combination of LCM, WGA and CGH used here has made such identifications possible. Further analysis using a platform such as tissue microarrays, which permit the screening of different disease stages from large patient cohorts, will help better identify their frequency and also their potential use in diagnosis.

Previous reports have demonstrated a relationship between alterations in chromosomal copy number and alterations in gene expression (Shayesteh *et al.*, 1999;

Collins *et al.*, 1998). The ability to distinguish these regions will point to genes, which may either directly (TSG loss or oncogene gain) or indirectly contribute to tumour development and progression. As a result CGH can be used as a surrogate for gene identification. Candidate genes (Table 2), which have previously been implicated in CaP, include CASP3 (4q), RAD21, MYC, PSCA, NBS1 (all 8q), PTEN, MXI1, ANXA7 (all 10q), MDM2 (12q), CDH1, DERPC, WWOX (all 16q) and MMP9, E2F1 (both 20q). The roles of MYC (Cassinelli *et al.*, 2004; Savinainen *et al.*, 2004; Bernard *et al.*, 2003), PSCA (Zhigang *et al.*, 2004; Fuessel *et al.*, 2004; Reiter *et al.*, 1998), PTEN (Dreher *et al.*, 2004; Fenic *et al.*, 2004; Koksal *et al.*, 2004) and MDM2 (Chang *et al.*, 2004; Mu *et al.*, 2004; Leite *et al.*, 2001) have all been well reported and alterations in gene dosage correlate well with their change in expression. However, other candidate genes have been less well studied. For example, EBAG9 whose increased expression in CaP is a negative prognostic indicator has a potential role in progression by enabling cancer cells to evade the immune response (Takahashi *et al.*, 2003). NBS1, which has been identified as a founder mutation causing an increased susceptibility to prostate cancer (Cybulski *et al.*, 2004), is involved in processing/repair of DNA double strand breaks and in cell cycle checkpoints, thus its dysregulation will further contribute to chromosomal instability. FKHR, which is a member of the FOXO forkhead transcription factor family, is thought to play a regulatory role in several cellular functions including cell proliferation and survival (Huang *et al.*, 2004). Loss of FKHR expression, as observed in CaP cell lines, is likely to abrogate this control leading to tumour cell growth. Though these genes have previously been implicated in CaP there are additional genes, including both oncogenes and tumour suppressor genes, which reside within all of the affected regions that may

play an important role in the aetiology of the disease. Further analysis of these other candidates, may identify their potential as molecular targets for diagnosis and treatment.

The process of telomere maintenance has previously been implicated in prostate cancer and involves a series of "telomere associated" proteins known as the telosome complex (Liu *et al.*, 2004). The data presented here indicates that regions of the genome that are commonly affected by alterations in gene dosage in CaP contain members of this complex, including TERF1 (8q13), TERF2 (16q22.1), TNKS (8p23.1), PTOP (16q22.1), POT1 (7q31.33), NBS1 (8q21) and PINX1 (8p23). This finding indicates that loss or gain of genetic material has a potentially crucial role in the dysregulation of telomere maintenance. The net result of these alterations in the telosome complex is the massive chromosomal instability characteristic of CaP.

In summary, the combination of techniques used in this study has allowed for the identification of consistent regions of copy number change, ranging from specific cytobands to whole chromosomes, starting with as little as 5-10ng of DNA. With the use of laser capture microdissection and whole genome amplification we have been to able obtain pure HPIN and CaP DNA and thus identify the particular chromosomal changes associated with the two disease stages. In addition, we have identified several lower frequency CNAs, which may not have been detected when using bulk extracted DNA due to the heterogeneous nature of prostate tissue. *In silico* analysis of regions of copy number change has identified several interesting candidate genes and although some have already been extensively studied others, which have not, may prove to be of clinical importance. Genetic screening strategies that combine FISH and immunohistochemistry

for the detection of various combinations of chromosomal gains and/or losses and altered gene expression are likely to be of great use in diagnosis and prognosis prediction.

Acknowledgments

This work was supported by award the United States Army Medical Research and Materiel Command [DAMD17-03-1-0154].

References

Alers JC, Rochat J, Krijtenburg PJ, Hop WC, Kranse R, Rosenberg C, Tanke HJ, Schröder FH, van Dekken H. 2000. Identification of genetic markers for prostatic cancer progression. Lab Invest 80:931-942.

Al-Maghrabi J, Vorobyova L, Toi A, Chapman W, Zielenska M, Squire JA. 2002. Identification of numerical chromosomal changes detected by interphase fluorescence in situ hybridization in high-grade prostate intraepithelial neoplasia as a predictor of carcinoma. Arch Pathol Lab Med 126:165-9.

Beheshti B, Braude I, Marrano P, Thorner P, Zielenska M, Squire JA. 2003. Chromosomal localization of DNA amplifications in neuroblastoma tumors using cDNA microarray comparative genomic hybridization. Neoplasia 5:53-62.

Bernard D, Pourtier-Manzanedo A, Gil J, Beach DH. 2003. Myc confers androgen-independent prostate cancer cell growth. J Clin Invest 112:1724-31.

Bostwick DG, Shan A, Qian J, Darson M, Maihle NJ, Jenkins RB, Cheng L. Independent origin of multiple foci of prostatic intraepithelial neoplasia: comparison with matched foci of prostate carcinoma. Cancer. 1998 Nov 1;83(9):1995-2002.

Cassinelli G, Supino R, Zuco V, Lanzi C, Scovassi AI, Semple SC, Zunino F. 2004. Role of c-myc protein in hormone refractory prostate carcinoma: cellular response to paclitaxel. Biochem Pharmacol. 68:923-31.

Chang CJ, Freeman DJ, Wu H. 2004. PTEN regulates Mdm2 expression through the P1 promoter. J Biol Chem 279:29841-8.

Clark J, Edwards S, Feber A, Flohr P, John M, Giddings I, Crossland S, Stratton MR, Wooster R, Campbell C, Cooper CS. 2003. Genome-wide screening for complete genetic loss in prostate cancer by comparative hybridization onto cDNA microarrays. Oncogene 22:1247-52.

Cleton-Jansen AM, Buerger H, Haar Nt N, Philippo K, Van De Vijver MJ, Boecker W, Smit VT, Cornelisse CJ. 2004. Different mechanisms of chromosome 16 loss of heterozygosity in well- versus poorly differentiated ductal breast cancer Genes Chromosomes Cancer 41:109-16.

Collins C, Rommens JM, Kowbel D, Godfrey T, Tanner M, Hwang SI, Polikoff D, Nonet G, Cochran J, Myambo K, Jay KE, Froula J, Cloutier T, Kuo WL, Yaswen P, Dairkee S, Giovanola J, Hutchinson GB, Isola J, Kallioniemi OP, Palazzolo M, Martin C, Ericsson C, Pinkel D, Albertson D, Li Wu-Bo, Gray J. 1998. Positional cloning of ZNF217 and NABC1: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proc Natl Acad Sci U S A 95:8703-8.

Cybulski C, Gorski B, Debniak T, Gliniewicz B, Mierzejewski M, Masojc B, Jakubowska A, Matyjasik J, Zlowocka E, Sikorski A, Narod SA, Lubinski J. 2004. NBS1 is a prostate cancer susceptibility gene. Cancer Res 64:1215-9.

Dong JT, Boyd JC, Frierson HF Jr. 2001. Loss of heterozygosity at 13q14 and 13q21 in high grade, high stage prostate cancer. Prostate 49:166-71.

Dreher T, Zentgraf H, Abel U, Kappeler A, Michel MS, Bleyl U, Grobholz R. 2004. Reduction of PTEN and p27kip1 expression correlates with tumor grade in prostate cancer. Analysis in radical prostatectomy specimens and needle biopsies. Virchows Arch 444:509-17.

Fenic I, Franke F, Failing K, Steger K, Woenckhaus J. 2004. Expression of PTEN in malignant and non-malignant human prostate tissues: comparison with p27 protein expression. J Pathol 203:559-66.

Fuessel S, Sickert D, Meye A, Klenk U, Schmidt U, Schmitz M, Rost AK, Weigle B, Kiessling A, Wirth MP. 2003. Multiple tumor marker analyses (PSA, hK2, PSCA, trp-p8) in primary prostate cancers using quantitative RT-PCR. Int J Oncol 23:221-8.

Huang H, Muddiman DC, Tindall DJ. 2004. Androgens negatively regulate forkhead transcription factor FKHR (FOXO1) through a proteolytic mechanism in prostate cancer cells. J Biol Chem 279:13866-77.

Hughes, S., Beheshti, B., Marrano, P., Lim, G., Squire, J. A. 2004a. Comparative Genomic Hybridization Analysis using Metaphase or Microarray Slides. Immunohistochemistry and In situ hybridization of human carcinomas, Volume 2. Elsiever/Academic Press.

Hughes S, Lim G, Beheshti B, Bayani J, Marrano P, Huang A, Squire JA. 2004b. Use of whole genome amplification and comparative genomic hybridisation to detect chromosomal copy number alterations in cell line material and tumour tissue. Cytogenet Genome Res 105:18-24.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. 1992. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818-21.

Kimura Y, Noguchi T, Kawahara K, Kashima K, Daa T, Yokoyama S. 2004. Genetic alterations in 102 primary gastric cancers by comparative genomic hybridization: gain of 20q and loss of 18q are associated with tumor progression. Mod Pathol [Epub ahead of print]

Koksal IT, Dirice E, Yasar D, Sanlioglu AD, Ciftcioglu A, Gulkesen KH, Ozes NO, Baykara M, Luleci G, Sanlioglu S. 2004. The assessment of PTEN tumor suppressor gene in combination with Gleason scoring and serum PSA to evaluate progression of prostate carcinoma. Urol Oncol 22:307-12.

Leite KR, Franco MF, Srougi M, Nesrallah LJ, Nesrallah A, Bevilacqua RG, Darini E, Carvalho CM, Meirelles MI, Santana I, Camara-Lopes LH. 2001. Abnormal expression of MDM2 in prostate carcinoma. Mod Pathol 14:428-36.

Liu D, Safari A, O'Connor MS, Chan DW, Laegeler A, Qin J, Songyang Z. 2004. PTOP interacts with POT1 and regulates its localization to telomeres. Nat Cell Biol 6:673-80.

Matsuyama H, Pan Y, Yoshihiro S, Kudren D, Naito K, Bergerheim US, Ekman P. 2003. Clinical significance of chromosome 8p, 10q, and 16q deletions in prostate cancer. Prostate 54:103-11.

Mu Z, Hachem P, Agrawal S, Pollack A. 2004. Antisense MDM2 oligonucleotides restore the apoptotic response of prostate cancer cells to androgen deprivation. Prostate 60:187-96.

Paiss T, Worner S, Kurtz F, Haeussler J, Hautmann RE, Gschwend JE, Herkommer K, Vogel W. 2003. Linkage of aggressive prostate cancer to chromosome 7q31-33 in German prostate cancer families. Eur J Hum Genet 11:17-22.

Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, Witte ON. 1998. Proc Natl Acad Sci U S A. 95:1735-40.

Savinainen KJ, Linja MJ, Saramaki OR, Tammela TL, Chang GT, Brinkmann AO, Visakorpi T. 2004. Expression and copy number analysis of TRPS1, EIF3S3 and MYC genes in breast and prostate cancer. Br J Cancer 90:1041-6.

Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, Gray JW. 1999. PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet :99-102.

Squire J, Marrano P, Kolomietz E: FISH: A Practical Approach. (Oxford University Press, Oxford 2002).

Takahashi S, Urano T, Tsuchiya F, Fujimura T, Kitamura T, Ouchi Y, Muramatsu M, Inoue S. 2003. EBAG9/RCAS1 expression and its prognostic significance in prostatic cancer. Int J Cancer 106:310-5.

van Dekken H, Alers JC, Damen IA, Vissers KJ, Krijtenburg PJ, Hoedemaeker RF, Wildhagen MF, Hop WC, van der Kwast TH, Tanke HJ, Schroder FH. 2003. Genetic evaluation of localized prostate cancer in a cohort of forty patients: gain of distal 8q discriminates between progressors and nonprogressors. Lab Invest 83:789-96.

van der Kwast TH, Labrie F, Tetu B. 1999. Persistence of high-grade prostatic intra-epithelial neoplasia under combined androgen blockade therapy. Hum Pathol 30:1503-7.

Vukovic B, Park PC, Al-Maghrabi J, Beheshti B, Sweet J, Evans A, Trachtenberg J, Squire J. 2003. Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. Oncogene 22:1978-87.

Wolf M, Mousses S, Hautaniemi S, Karhu R, Huusko P, Allinen M, Elkahloun A, Monni O, Chen Y, Kallioniemi A, Kallioniemi OP. 2004. High-resolution analysis of gene copy number alterations in human prostate cancer using CGH on cDNA microarrays: impact of copy number on gene expression. Neoplasia 6:240-7.

Zhigang Z, Wenlv S. 2004. Prostate stem cell antigen (PSCA) expression in human prostate cancer tissues and its potential role in prostate carcinogenesis and progression of prostate cancer. World J Surg Oncol 2:13.

Table 1. Chromosomal changes in microdissected HPIN and CaP DNA samples.

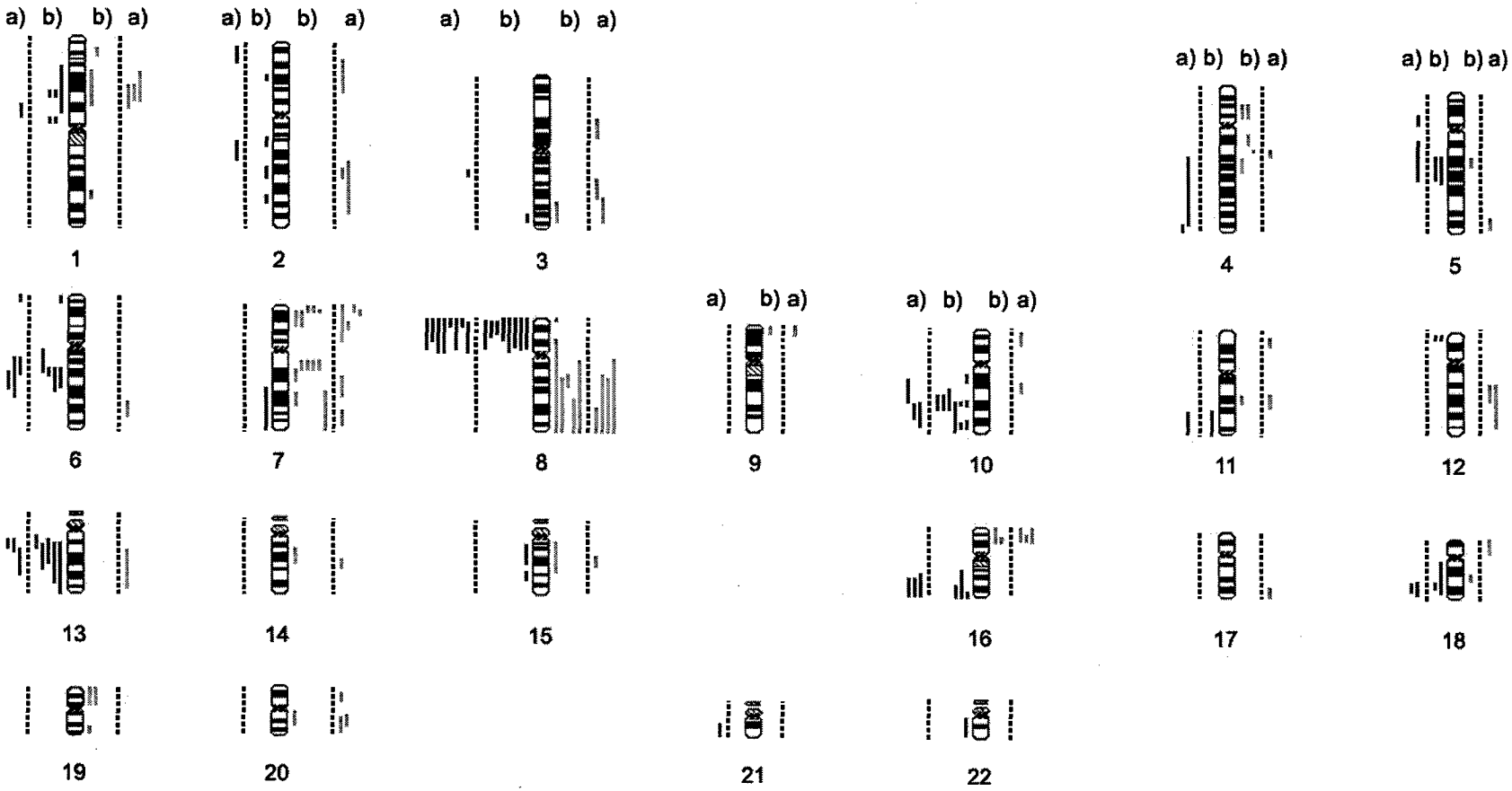
Tumor	Type	Loss	Gain
CaP 1	HPIN	3q13.33-q21.1; 6p24.1-pter; 8p11.1-p23.2; 13q14.3-q31.2	1p21.3-p31.1; 2q24.3-q31.1; 8q23.3-qter; 11p14.3-p15.1
CaP 2	HPIN	6q14.1-q16.3; 8p23.1-pter; 16q21-qter	1p22.3-p31.1; 7p15.1-p15.3; 8q13.3-qter
CaP 3	HPIN	2p23.1-p25.2; 4q22.1-q34.1; 8p11.23-pter	3q21.2-q24; 12q14.3-q21.33
CaP 4	HPIN	8p23.1-pter;	7p22.1-pter; 20q11.22-q13.11
CaP 5	HPIN	6q16.3-q22.1; 8p11.21-pter; 13q13.1-q14.3	7p21.3-p22.1; 8q21.11-qter; 16p12.3-pter; 20q11.23-q13.2
CaP 6	HPIN	8p21.1-pter; 10q21.3-q23.1; 18q21.32-q22.2; 21q21.3-qter	7q32.3-q36.1; 16p12.3-p13.13; 20p11.23-p12.1
CaP 7	HPIN	8p11.23-pter; 10q23.31-q25.1; 16q22.1-qter	16p12.2-pter; 17q24.2-qter
CaP 8	HPIN	1p13.1-p22.1; 2q14.2-q21.1; 2q21.3-q23.3; 3p12.3-q11.1; 4q32.3-q35.1; 5p14.3-q22.1; 6q14.2-q22.33; 8q11.1-pter; 9q34-qter; 10q11.1-q11.23; 10q23.2-q26.12; 11q22.2-q24.3; 13q13.3-q14.13; 16q12.2-qter; 18q11.1-qter	8q11.1-qter;
CaP 9	Cancer	2q14.2-q21.2; 5q14.1-q22.1; 6q16.1-q21; 8p21.3-p23.1; 10q22.1-q23.33; 13q14.11-q22.2	4q21.21-q21.23; 7p15.3-p21.3; 7q11.23
CaP 10	Cancer	1p21.1-p32.3; 2q33.1-q33.3; 5q14.1-q21.3; 6q16.1-q22.1; 8p11.23-pter; 10q23.31-qter; 13q12.13-q14.11	4p13-p15.1; 4q22.2-q24; 7q31.1-qter; 8q21.11-qter; 9p22.3-p24.1; 14q13.1-q21.3
CaP 11	Cancer	2q24.2-q31.1; 7q22.3-qter; 8p11.22-pter; 10q22.2-q23.33; 18q11.1-q22.3	
CaP 12	Cancer	8p11.21-pter; 10q22.2-q23.33; 12p13.2; 13q13.3-q32.2; 16q24.1-qter; 22q11.21-qter	1q32.1; 5q14.2-q14.3; 8q13.3-q21.2
CaP 13	Cancer	1p13.1-p13.2; 1p22.3; 2p16.3; 6q12-q16.1; 8p21.1-pter; 10q25.3-q26.11	7p22.1-pter; 7q11.23; 8q22.3-qter; 16p12.2-pter; 19p; 19q13.33-qter
CaP 14	Cancer	6p24.1-pter; 8p11.21-pter; 10q23.31-q23.32; 15q22.33-q24.1	1p21.3-p32.1; 3q25.33-q27.3; 4p13-p15.1; 4q13.1-q13.3; 7p15.3-p22.1; 7q21.11-q21.3; 7q31.1-q31.33; 8q11.22-qter; 11q14.1-14.3
CaP 15	Cancer	1p13.1-p13.2; 1p22.3; 6q16.1-q16.3; 8p21.2-p23.1; 8q12-qter; 10q21.1-q21.2; 10q23.1-q23.32; 10q25.2-q26.11; 11q22.3-qter; 12p13.2; 13q12.3-q21.33; 15q13.1-q21.3; 16q12.2-qter	7p22.1-pter; 7q11.23; 8p23.2-pter; 18q21.1; 20q11.21-q13.12
CaP 16	Cancer	3q26.33-q27.3; 8p11.22-p23.2; 13q13.3-qter; 16q23.1-qter; 18q21.33-q22.1	1p35.2-p36.11; 7p22.1-p22.2; 7q11.23; 15q11.2-q22.32; 16p12.2-13.11; 19p13.11-pter

Table 2. Consensus regions of copy number gain. Names in bold indicate those genes that have been implicated in prostate cancer. The *symbol indicates the same candidate genes from HPIN.

Chromosome	Region	Frequency	Candidate genes
HPIN Gain			
1p	1p22.3 – p31.1	37.5%	IGFBP10
2q	2q24.3 – q31.1	25%	TLK1, ITGA6
7p	7p22.1	37.5%	NUDT1, **PDGFA**
	7p15.1 – p15.3	25%	GPNMB, **AHR**
8q	8q21.11 – qter	>37.5%	PTK2, **RAD21**, MLZE, WISP1, **NOV**, ENPP2, **MYC**, **PSCA**, PTP4A3, KCNK9, **TPD52**, MMP16, **NBS1**, **FABP5**, E2F5, BAALC, **EBAG9**
12q	12q14.3 – q21.33	25%	**MDM2**
16p	16p12.3 – p13.13	37.5%	BFAR
20q	20q11.23 – q13.11	25%	**MMP9**, C20ORF1, **SRC**, **GHRH**, **E2F1**, DNLC2A, BASE, CDC91L1, WFDC2, SLPI, **CYP24**, **BMP7**, **CSE1L**
HPIN Loss			
4q	4q34.1	25%	FAT, MORF4, **CASP3**
6q	6q16.3	37.5%	
8p	8p11.23 – p23.3	>50%	SFRP1, **NKX3A**, TRIM35, REAM, RB1CC1, **PDGFRL**, FGL1, TNFRSF10B, **LZTS1**, DLC1, MTSG1, **TUSC3**, FLJ32642, MTSS1, **PINX1**, **DEFB1**, CSMD1, TNKS
10q	10q23.1	25%	
	10q23.2 – q25.1	25%	**PTEN**, **MXI1**, LGI1, PDCD4, **LAPSER1**, RNF27, SUFU, CASP7, LIMAB1, NEURL
13q	13q13.1 – q14.13		RFP2, TSC22, **DBM**, DDX26, **KCNRG**
	13q14.3	25%	DLEU2, DLEU1, **CHC1L**, **FAM10A4**, **FKHR**
16q	16q22.1 - qter	37.5%	PTOP, TERF2, **CDH1**, **DERPC**, **WWOX**, OKL38, CBFA2T3, **CDH13**, **WFDC1**, **MAF**, **FOXF1**, **MVD**
18q	18q21.32 – q22.2	25%	PMAIP1
CaP Gain			
4p	4p13 – p15.1	25%	UCHL1, CD38
7p	7p22.1	50%	*
7q	7q11.23	50%	**LIMK1**, CLDN4, **HSPB1**
	7q31.1 – q31.33	25%	NRCAM, PTPRZ1, POT1
8q	8q21.11 – qter	>50%	*
16p	16p12.2 – p13.13	25%	*
19p	19p13.11 - pter	25%	VAV1, RAB3D, ELAVL1, JUN-B, JUN-D, **EPOR**, DRIL1, BSG, ANGPTL4
CaP Loss			
1p	1p22.3	37.5%	
	1p13.1 – p13.2	25%	ST7L
5q	5q14.2 – q14.3	25%	
6q	6q16.1	50%	
8p	8p11.22 – p23.3	>50%	*
10q	10q22.2 - q23.33	>37.5%	UNC5B, BMPR1A, BLNK, **PTEN**
	10q25.3 – q26.11	37.5%	**DMBT1**, TACC2, WDR11, **FGFR2**, DEC, BCCIP
12p	12p13.2	25%	CD9
13q	13q13.3 – q26.11	>50%	BRCA3, **KLF5** *
16q	16q24.1 - qter	37.5%	*
18q	18q21.33 – q22.1	25%	*

* indicates the same candidate genes from HPIN. Names in bold indicate those genes that have been implicated in prostate cancer.

Figure 1. Chromosomal alterations observed for a) HPIN and b) CaP DNA samples. Green bars to the left of the chromosomes indicate regions of loss and red bars to the right of the chromosomes indicate regions of gain.



APPENDIX 2

Pathway-specific array analysis of DNA Damage Signaling in Prostate Cancer

Yoshimoto M[1], Albert M[2], Wong L[2], Hughes S[3], Macgregor P[2], Squire J.A.[1,4,5].
Evans A[5,6]

Ontario Cancer Institute, Princess Margaret Hospital[1], Department of Laboratory Medicine and Pathology[4], Department of Medical Biophysics[5], Division of Pathology, Faculty of Medicine[6], Microarray Centre, Toronto General Hospital, University Health Network[2], Institute of Cancer Research, UK[3]

Key Words: Prostate cancer, genomic instability, DNA damage, gene expression

Corresponding author:

Jeremy Squire, Ph.D.

Princess Margaret Hospital

Ontario Cancer Institute

University Health Network

610 University Avenue, Room 9-717

Toronto, Ontario

M5G 2M9, Canada

Email: jeremy.squire@utoronto.ca

Abstract

Prostate cancer (CaP) has become the most commonly diagnosed cancer in men in North America. Understanding of the molecular mechanisms behind the development of CaP will enable the identification of differential molecular biomarker(s), as well as, fundamental insights concerning the regulatory pathways of the integrity of genome that may lead to multistep field cancerization. Human DNA damage signaling pathway-specific array of 11 amplified aRNA from ten radical prostatectomy specimens and one normal control were used for gene expression assembly to ATR/ATM signaling network and transcriptional targets of DNA damage response. The expression profiling of 96 key genes was categorized into three functional groups: cell cycle arrest, apoptosis, and genomic stability and repair pathways. Overall, two tumors clusters were obtained using TMEV software version 3.1, whereas one of these groupings was divided in two more specific tumor clustering. In addtion, 36 genes were found to show differential expression levels in CaP. MRE11A gene was the most down regulated gene, followed by the members of apoptosis and cell cycle arrest pathways (TNFRSF4, P53AIP1, BAX, and CDC20, GSTE1, respectively). The highest increase is seen predictably in RAD51. In total of 22 genes showing increase in expression, 12 genes were associated to genomic stability/repair pathway. These data suggest that the genome of CaP cells display mutational mechanisms in DNA mismatch repair genes associated with genetic instability. Significantly, RAD51 dysfunction suggests a molecular mechanism to account for the most frequent chromosomal aberration in CaP: isochromosome 8q. It is suggested that failure in the fidelity of homologous recombination within the repetitive

sequences, that comprise the kinetochore complex, could lead to recuurent loss of 8p and gain of 8q by rearrangement of chromosome 8-specific alphoid centromeric sequences.

Introduction

Prostate cancer (CaP) is a major male health challenge throughout the world, although there is a large variation in its incidence (Schaid, 2004). Although the causes of this variation are likely to be differences in screening methods, diet and health-related behaviors, clinical practice patterns and environmental risk factors, the role of genetic differences is unknown (De Marzo *et al.*, 2004; Schaid, 2004). Because of patient-to-patient heterogeneity in the clinical behavior of this disease, prognostic markers that may help tailor therapeutic strategies to individual clinical situations are continuously re-assessed (Grasso *et al.*, 2004).

Regardless of the timing of the morphological events differs from cell to cell, recent studies of the molecular alteraions in CaP cells have begun to provide clues as to how prostate cancer may arise and progress (Shaid, 2004). Oxidative damage to DNA and other cellular components accompanying chronic or recurrent inflammation may connect prostate inflammation with cancer, since the genomic damage modulates cancer progression and the steps of CaP development are androgen independence, inhibition of apoptosis, and metastasis (Deutsch *et al.*, 2004). In addition, it has long been argued that the tumor microenvironment contributes to genetic instability and it is associated with increased DNA damage, enhanced mutagenesis and functional impairments in DNA repairs pathways (Reynolds *et al.*, 1996; Brinda & Glazer, 2005). Despite the recent advances in the understanding of the environmental, hormonal, and nutritional origins, gene mutations reported in CaP appear quite heterogeneous, from case to case, or even from lesion to lesion in a single case.

The objetive of this study was to better understanding the early development of CaP and to identify significant differential genes by use of pathway-specific array. Understanding of the molecular mechanisms behind the development of CaP will enable the identification of differential molecular biomarker(s), as well as, fundamental insights concerning the regulatory pathways of the integrity of genome that may lead to multistep field cancerization.

Material and Methods

Prostate tissue samples were collected through the Prostate Centre tissue bank (Princess Margaret Hospital, Ontario Cancer Institute) and the study protocol was approved by the University Health Network. Ten primary tumors from radical prostatectomy and commercially avaliable pooled RNA from 16 normal prostatic tissues (Clonetech Laboratories Inc., Palo Alto, CA, USA) were included in this preliminary study. The tumors were pathologically staged according to the pathological tumor node metastasis (pTNM) classification (Ayala *et al.*, 1989) and graded according to the Gleason grading system (Table 1). All tissue samples were snapped frozen in liquid nitrogen and embedded in optimal cutting temperature (OCT) solution and sectioned into six 10μm thick slides for RNA extraction, flanked by two 6μm thick slides for hematoxilin and eosin (H&E) staining and four 4μm thick slides for immunohistochemistry (IHC).

Macrodissection and RNA extraction

Macrodissection was performed only on samples that had relatively high tumor content (>75%) but lack the wide spread of tumor using adjacent H&E-stained sections as guidance. The portions of interest of each section were separated by a scalpel and sterilized blades, and placed into a 1.5ml tube with lysis buffer containing a strong protein denaturant, guanidine thiocyanate, which aids in effective cell lysis and prevents RNase degradation. The extraction process was carried out using the Absolutely RNA® Microprep Kit as described by manufacturer's instructions (Stratagene, La Jolla, CA, USA), and allowed quick purification of high quality RNA from samples that contains small number of cells (Dolter & Braman, 2001). RNA 6000 Nano LabChip® Kit (Agilent, Mississauga, ON, Canada) and RiboGreen (Molecular Probes, Eugene, OR, USA) application were employed to obtain accurate readings of quality and quantities of RNA, respectively.

T7-Amplification

The efficient reproducibility of high yield of aRNA from 200ng of mRNA was generated using the Amino Ally MessageAmp™ aRNA kit (Ambion Inc., Austin, TX, USA), following the procedures by Wang *et al.* (2000). The first-strand cDNA synthesis of total RNA was primed with 1μl of T7 oligo(dT) promoter sequence by reverse transcription. Previously to cDNA purification, the single-strand cDNA was cleaved into small fragments and converted to double-strand sDNA by addition of second-strand master mix containing 1μl of RNase H and 2μl of DNA polymerase, during 2 hours of incubation at 16°C. The amino allyl modified aRNA was produced by large scale *in vitro*

transcription reaction of purified cDNA. In this step, 3µl of amino allyl-UTP was added to the aRNA master mix synthesis. After a quick purification step, RNA 6000 Nano LabChip® Kit (Agilent, Mississauga, ON, Canada) and RiboGreen (Molecular Probes, Eugene, OR, USA) application were assessed to aRNA yield and quality, respectively.

Pathway-specific array

The amplified aRNA were hybridized in GEArray® Q Series pathway array containing 112 sites each (3 blanks, 3 negative reference spots, 10 household genes, and 96 pathway-specific human genes) (SuperArray Bioscience Corporation, Frederick, USA). Human cDNA DNA Damage Signaling Pathway Microarray (HS-029) was designed to assess the differential expression levels of 96 prostate cancer-related genes and 96 key genes involved in DNA damage signaling pathway. The protocol and data analysis of arrays were performed according to SuperArray technical specifications and the gene list and array position for expression profiling are avaliable at the web site http://www.superarray.com.

In vitro convertion of 2µg of amplifed aRNA to biotinylated-dUTP cDNA probe was generated by applying MMVL reverse transcriptase (Promega, Nepean, ON, Canada) and biotin-16-dUTP (Roche, Laval, Quebec, Canada), following the probe sysnthesis for chemiluminescent detection instructions from AmpoLabeling Linear Polymerase Reaction (LPR) Kit (SuperArray, Frederick, USA). The biotinylated cDNA probes were previously detectable at the 1000-fold dilution to probe denaturation and hybridization to nylon membranes spotted with gene-specific cDNA fragments. Chemiluminescent

analysis of membranes was subjected to quantification of binding-signals of alkaline phosphatase-conjugated streptavidin with CDP-Star substrate.

The array image was recorded using Kodak X-OMAT LS film (Amersham Biosciences, Piscataway, NJ, USA) and SprintScan 35 Plus (Polaroid Canada Inc., Mississauga, ON, Canada). Several exposure times for capturing the membrane image were archieved to produce the largest possible dynamic range in the individual spots. The adjustment of exposure setting was based on the minimal and maximal difference between the blank spots with no array signal and the brightest spots with maximal array signal, since the over/underexposure of a number of spots can affect the linearity of the relationship between RNA amount and signal intensity. The image was converted into grayscale and 16-bit tiff format, and the numerical data of expression level of each gene on the GEArrays was quantified using the GenePix software (Axon, Union City, CA, USA) (Figure 1). By allowing the GEArrayAnalyzer software analysis (SuperArray, Frederick, MD), the raw data was inter-normalized with the normal prostate control and intra-normalized with cyclophilin A values present on each of the GEArray. Output values were also log2 converted for easier interpretation and three criteria were used to determine differentially expressed genes (Table 2). (1) Number of values present among the 4 tumour samples for each gene greater than 75%. (2) Relative standard deviation was calculated for each gene across the tumour samples with a 100% cut-off. (3) Only genes with average fold larger than –1,5 and +1.5 were considered for increase/decrease in expression. The hierarchical clustering was performed using TIGR MultiExperiment Viewer (TMEV) software version 3.1 (Saeed *et al.*, 2003) (Figure 2).

Results

Arrayed nylon membranes of human DNA damage signaling from 10 radical prostatectomy specimens (CaP 1 – CaP 10) and one normal prostatic tissue (11) were used for gene expression assembly to ATR/ATM signaling network and transcriptional targets od DNA damage response. The expression profiling of 96 key genes was categorized into three functional groups: cell cycle arrest, apoptosis, and genomic stability and repair pathways, as shown in Table 3 (modified version of functional gene grouping from SupeArray). Overall, two tumors clusters (CaP 5, 6, 7 and CaP 1, 2, 3, 4, 8, 9, 10) were obtained using TMEV software version 3.1, whereas one of these groupings was divided in tthree more specific tumor clustering (CaP 1, 3, 4; CaP 2, 8, 9 and CaP 10) (Figure 1).

Of genes identified as expressed by the GEArrayAnalyzer (>10% above background levels), 36 were found to show differential expression levels in CaP as defined by statistical normalization and ratio cutoffs of 1.5 and 0.5 for increase/decrease in expression. The meiotic recombination (*S. cerevisiae*) 11 homolog A (MRE11A) gene was the most down regulated gene (average fold -30.28) in CaP compared to normal prostate in DNA damage pathway, followed by the members of apoptosis and cell cycle arrest pathways (TNFRSF4, P53AIP1, BAX, and CDC20, GSTE1, respectively). The highest increase is seen predictably in RAD51 (*S. cerevisiae*) homolog (*E. coli* RecA homolog) (RAD 51), with a fold change of 18.21 (Table 2). In total of 22 genes showing increase in expression, 12 genes were associated to genomic stability/repair pathway.

Discussion

Prostate cancer is a multifatorial disease with genetic and environmental components involved in its etiology (Nwosu *et al.*, 2001; Visakorpi, 2003; Schaid, 2004). The knowledge of genetic basis of CaP has increased over the past years, mainly in the pathways that underlie tumorigenesis and growth and drug resistance for each clinical stage and specific histology (Karan *et al.*, 2002; Wolter *et al.*, 2002; Carles *et al.*, 2004). Considering the general trends that can be decipted from the clustering algorithms, at least 2 tumor-specific clustering were obtained using TMEV software version 3.1 based on similar gene expression profiles (Figure 1). Since a correlation was not observed between tumor hierarchical clustering, Gleason scoring, pTNM system and serum PSA level, future studies should include larger cohorts with long-term follow-up data.

It has been well established that tumor progression is correlated with genetic instability. Recent evidence suggests that the tumor microenvironment itself constitutes a significant source of such genetic instability by dysregulation of DNA repair pathways (Bindra & Glazer, 2005). The genome of cancer cells displays mutation mechanisms in DNA mismatch repair gene associated with microsatellite instability (MIN), in addition to numerical and complex structural changes to whole chromosomes recognized by chromosomal instability (CIN) (Caburet *et al.*, 2002; DeMarzo *et al.*, 2003; Storchova and Pellman, 2004). Although the mechanisms inducing CIN are complex and numerous, the most important are: defective mitotic checkpoints and abnormal DNA replication events leading to missegregation or aneuploidy (Duensing and Munger, 2001; Caburet *et al.*, 2002; Storchova and Pellman, 2004) telomere dysfunction (Artandi *et al.*, 2000; O'Hagan *et al.*, 2002; Charames and Bapat, 2003; Vukovic *et al.*, 2003), and breakage-fusion-bridge cycles (Caburet *et al.*, 2002). Following to complexity of genetic

instability, it seems that high fidelity process of homologous recombination is the major DNA repair pathway, which is indispensable for the maintenance of genetic stability (Thompson and Schild, 2001). Hyperactivity of the homologous recombination pathway could also contribute to inappropriate recombination between expansion repeat resulting in translocations, deletions, or duplications (Zhou *et al.*, 2001). Human RAD51 (s. cerevisiae) homolog (E. coli RecA homolog) (RAD51) gene is a relatively small protein, approximately 38 kDa that is likely to be a target for regulator factors such as p53 (Baumann & West, 1998; West, 2003). The result of a higly positive expression of RAD51 in this study implies that this gene can be involved in cancer development. The recent finding that linked increase homologous recombination to tumor susceptibility supports this hypothesis. Overexpression of the homologous recombination and DNA repair protein RAD51 has been reported in immortalized and tumor cells, providing a correlation between elevated RAD51 protein levels, genome instability, and tumor progression (Flygare *et al.*, 2001). RAD51 dysfunction suggests a molecular mechanism to account for the most frequent chromosomal aberration in CaP: isochromosome 8q (Fan *et al.*, 2004; Macoska *et al.*, 2004). It is suggested that failure in the fidelity of homologous recombination within the repetitive sequences, that comprise the kinetochore complex, could lead to recuurent loss of 8p and gain of 8q by rearrangement of chromosome 8-specific alphoid centromeric sequences.

Additionally, the result of a negative expression of GADD45a proposes the involvement of cancer development, increasing the transformation of carcinoma. The growth arrest and DNA damage-inducible protein 45alpha (GADD45a) gene is predominantly a nuclear protein and it has a important role in cellular response to DNA

damage because it is considered to partake in controlling the cell-cycle checkpoint, apoptosis, and DNA repair after DNA damage (Carrier *et al.*, 1994, Daino *et al.*, 2003). It was shown that cells derived from GADD45a-null mice exhibited genomic instability, proto-oncogene activation, increase cellular proliferation and reduced DNA repair (Hildesheim *et al.*, 2002).

The majority of neoplasias develop as a result of a complex accumulation of genetic alterations. The precise contribution played by each mutation remains unclear, although many associations have been made between gene mutation and disease stage. A key challenge has therefore been to link individual genetic changes with the cellular mechanisms underlying disease. DNA double strand breaks represent the most threatening lesion to the integrity of the genome in cells exposed to ionizing radiation and radiomimetic chemicals. Those breaks are recognized, signaled to cell cycle checkpoints and repaired by protein complexes. Several distinctive expressed genes involved in ATR/ATM signaling network were identified: MRE11A, CHEk1, RAD1, ATM and HUS1. Ataxia–telangiectasia-mutated (ATM) is a member of the phosphoinositide 3-kinase (P13-kinase) family that also includes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and A–T and Rad3-related protein (ATR) all of which are involved in the recognition of damage in DNA (Abraham, 2001). ATM is one of a growing number of proteins which when mutated compromise the stability of the genome and predispose to tumour development. The MRE11A gene is part of a complex, Mre11/Nbs1/Rad50 (MNR) complex, which acts as a sensor to DNA damage (Stewart et a., 1999). The MNR complex is associated with the DNA DSBs and remains at these sites

until the damage is repaired (Lavin *et al.*, 2005). It is suggested that pertubation of the MNR complex through mutated MRE11 predisposes cancers (Giannini *et al.*, 2004).

Conclusions

Postate cancer represents a fundamental research challenge: multiple foci and genetic heterogeneity between different primary foci in the same prostate. Without isolating and identifying common genetic features, it is difficult to propose realistic mechanistic models, and without such models it is impossible to propose changes to present treatment regimens. The pathway-specific array data suggest that the genome of CaP cells display mutation mechanisms in DNA mismatch repair gene associated with genetic instability and the high fidelity process of homologous recombination can be the major DNA repair pathway, which is indispensable for the maintenance of genetic stability. Thus, a detailed genomewide approach is required to answer questions concerning genetic variations in cancer.

Acknowledgments

This work was supported by award the United States Army Medical Research and Materiel Command [DAMD17-03-1-0154].

References

Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes

Dev. 2001; 15(17): 2177-96.

Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature. 2000; 406(6796): 641-5.

Ayala AG, Ro JY, Babaian R, Troncoso P, Grignon DJ. The prostatic capsule: does it exist? Its importance in the staging and treatment of prostatic carcinoma. Am J Surg Pathol. 1989; 13(1): 21-7.

Baumann P, West SC. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. Trends Biochem Sci. 1998; 23(7): 247-51.

Bindra RS, Glazer PM. Genetic instability and the tumor microenvironment: towards the concept of microenvironment-induced mutagenesis. Mutat Res. 2005; 569(1-2): 75-85.

Caburet S, Conti C, Bensimon A. Combing the genome for genomic instability. Trends Biotechnol. 2002; 20(8): 344-50.

Carles J, Lloreta J, Salido M, Font A, Suarez M, Baena V, Nogue M, Domenech M, Fabregat X. Her-2/neu expression in prostate cancer: a dynamic process? Clin Cancer Res. 2004; 10(14): 4742-5.

Carrier F, Smith ML, Bae I, Kilpatrick KE, Lansing TJ, Chen CY, Engelstein M, Friend SH, Henner WD, Gilmer TM. Characterization of human Gadd45, a p53-regulated protein. J Biol Chem. 1994; 269(51): 32672-7.

Charames GS, Bapat B. Genomic instability and cancer. Curr Mol Med. 2003; 3(7): 589-96.

Daino K, Ichimura S, Nenoi M. Comprehensive search for X-ray-responsive elements and binding factors in the regulatory region of the GADD45a gene. Radiat Res (Tokyo). 2003; 44(4): 311-8.

De Marzo AM, Meeker AK, Zha S, Luo J, Nakayama M, Platz EA, Isaacs WB, Nelson WG. Human prostate cancer precursors and pathobiology. Urology. 2003; 62(5 Suppl 1): 55-62.

De Marzo AM, DeWeese TL, Platz EA, Meeker AK, Nakayama M, Epstein JI, Isaacs WB, Nelson WG. Pathological and molecular mechanisms of prostate carcinogenesis: implications for diagnosis, detection, prevention, and treatment. J Cell Biochem. 2004; 91(3): 459-77.

Deutsch E, Maggiorella L, Eschwege P, Bourhis J, Soria JC, Abdulkarim B. Environmental, genetic, and molecular features of prostate cancer. Lancet Oncol. 2004; 5(5): 303-13.

Duensing S, Munger K. Centrosome abnormalities, genomic instability and carcinogenic progression. Biochim Biophys Acta. 2001; 1471(2): M81-8

Flygare J, Falt S, Ottervald J, Castro J, Dackland AL, Hellgren D, Wennborg A. Effects of HsRad51 overexpression on cell proliferation, cell cycle progression, and apoptosis. Exp Cell Res. 2001; 268(1): 61-9.

Giannini G, Rinaldi C, Ristori E, Ambrosini MI, Cerignoli F, Viel A, Bidoli E, Berni S, D'Amati G, Scambia G, Frati L, Screpanti I, Gulino A. Mutations of an intronic repeat induce impaired MRE11 expression in primary human cancer with microsatellite instability. Oncogene. 2004; 23(15): 2640-7.

Grasso M, Lania C, Blanco S, Baruffi M, Mocellin S. Reduction in PSA messenger-RNA expression and clinical recurrence in patients with prostatic cancer undergoing neoadjuvant therapy before radical prostatectomy. J Transl Med. 2004; 2(1): 13.

Fan R, Kumaravel TS, Jalali F, Marrano P, Squire JA, Bristow RG. Defective DNA strand break repair after DNA damage in prostate cancer cells: implications for genetic instability and prostate cancer progression. Cancer Res. 2004; 64(23): 8526-33.

Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, Fornace AJ Jr. Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. Cancer Res. 2002; 62(24):

7305-15.

Hu JJ, Hall MC, Grossman L, Hedayati M, McCullough DL, Lohman K, Case LD. Deficient nucleotide excision repair capacity enhances human prostate cancer risk. Cancer Res. 2004; 64(3): 1197-201.

Karan D, Kelly DL, Rizzino A, Lin MF, Batra SK. Expression profile of differentially-regulated genes during progression of androgen-independent growth in human prostate cancer cells. Carcinogenesis. 2002; 23(6): 967-75.

Lavin MF, Birrell G, Chen P, Kozlov S, Scott S, Gueven N. ATM signaling and genomic stability in response to DNA damage. Mutat Res. 2005; 569(1-2): 123-32.

Luo JH, Yu YP, Cieply K, Lin F, Deflavia P, Dhir R, Finkelstein S, Michalopoulos G, Becich M. Gene expression analysis of prostate cancers. Mol Carcinog. 2002; 33(1): 25-35.

Macoska JA, Paris P, Collins C, Andaya A, Beheshti B, Chaib H, Kant R, Begley L, MacDonald JW, Squire JA. Evolution of 8p loss in transformed human prostate epithelial cells. Cancer Genet Cytogenet. 2004; 154(1): 36-43.

Nwosu V, Carpten J, Trent JM, Sheridan R. Heterogeneity of genetic alterations in prostate cancer: evidence of the complex nature of the disease. Hum Mol Genet. 2001; 10(20): 2313-8.

O'Hagan RC, Chang S, Maser RS, Mohan R, Artandi SE, Chin L, DePinho RA. Telomere dysfunction provokes regional amplification and deletion in cancer genomes. Cancer Cell. 2002; 2(2): 149-55.

Reynolds TY, Rockwell S, Glazer PM. Genetic instability induced by the tumor microenvironment. Cancer Res. 1996; 56(24): 5754-7.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 2003; 34(2): 374-8.

Schaid DJ. The complex genetic epidemiology of prostate cancer. Hum Mol Genet. 2004; 13 Spec No 1:R103-21. Epub 2004 Jan 28.

Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI, Jaspers NG, Raams A, Byrd PJ, Petrini JH, Taylor AM. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. Cell. 1999; 99(6): 577-87.

Storchova Z, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol. 2004; 5(1): 45-54.

Thompson LH, Schild D. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mutat Res. 2001; 477(1-2): 131-53.

Visakorpi T. The molecular genetics of prostate cancer. Urology. 2003; 62(5 Suppl 1): 3-10.

Vukovic B, Park PC, Al-Maghrabi J, Beheshti B, Sweet J, Evans A, Trachtenberg J, Squire J. Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. Oncogene. 2003; 22(13): 1978-87.

Wang E, Miller LD, Ohnmacht GA, Liu ET, Marincola FM. High-fidelity mRNA amplification for gene profiling. Nat Biotechnol. 2000; 18(4): 457-9.

West SC. Molecular views of recombination proteins and their control. Nat Rev Mol Cell Biol. 2003; 4(6): 435-45.

Wolter H, Gottfried HW, Mattfeldt T. Genetic changes in stage pT2N0 prostate cancer studied by comparative genomic hybridization. BJU Int. 2002; 89(3): 310-6.

Zhou ZH, Akgun E, Jasin M. Repeat expansion by homologous recombination in the mouse germ line at palindromic sequences. Proc Natl Acad Sci U S A. 2001; 98(15): 8326-33.

Table 1. Clinicopathological information from ten radical prostatectomy specimens

Sample	Tumor Type	Age* (years)	PSA*	pTNM System	Gleason Score	Gleason Grade	PIN	Tumor content**
CaP 1	ACT	66	5.00	pT3aNxMx	7/10	3+4	+	>75%
CaP 2	ACT	66	11.00	pT2cNxMx	7/10	4+3	+	>75%
CaP 3	ACT	55	19.56	pT3bN1Mx	9/10	4+5	N/A	>75%
CaP 4	ACT	67	9.80	pT2cNxMx	9/10	5+4	-	>75%
CaP 5	ACT	60	7.00	pT2cNxMx	7/10	3+4	+	>90%
CaP 6	ACT	63	14.06	pT3aNxMx	7/10	4+3	+	>90%
CaP 7	ACT	64	53.00	pT3bN0Mx	9/10	5+4	-	>75%
CaP 8	ACT	N/A	N/A	N/A	N/A	N/A	N/A	>75%
CaP 9	ACT	48	5.70	pT2cNxMx	7/10	3+4	-	>75%
CaP10	ACT	59	4.90	pT2cNxMx	7/10	3+4	N/A	>75%

PSA: Prostate-specific antigen; pTNM: pathological tumor, nodes, metastases system; PIN: Prostatic intraepithelial neoplasia; ACT: Adenocarcinom, conventional type; N/A: not available; +: present; -: ausent.

* Age and PSA value at time of prostatectomy; ** Tumor percentage before macrodissection.

Table 2. Data exploration of genes differentially expressed between CaP and normal prostatic tissue obtained using MS Excel® Microsoft (version 2003)

Gene Symbol	Linear Standard Deviation	Relative Standard Deviation	Linear Average	Log 2 Average	Average Fold Change (Tumor vs Normal)
MRE11A	0.10	316.2%	0.03	-4.92	-30.28
TNFRSF4	0.11	316.2%	0.04	-4.80	-27.86
P53AIP1	0.13	316.2%	0.04	-4.55	-23.43
CDC20	0.16	212.7%	0.08	-3.72	-13.18
GTSE1	0.58	246.4%	0.23	-2.10	-4.29
BAX	0.57	220.9%	0.26	-1.96	-3.89
RRM2B	0.43	158.2%	0.27	-1.88	-3.68
BAK1	0.40	142.8%	0.28	-1.85	-3.61
LRDD	0.58	194.6%	0.30	-1.75	-3.37
TNF	0.69	212.1%	0.33	-1.62	-3.08
CHEK1	0.57	122.2%	0.47	-1.09	-2.13
RPA3	0.79	131.0%	0.60	-0.74	-1.67
GADD45A	0.74	113.7%	0.65	-0.62	-1.54
FEN1	0.82	124.3%	0.66	-0.60	-1.52
CDC25B	2.31	139.2%	1.66	0.73	1.66
RAD1	1.80	104.4%	1.73	0.79	1.73
ERCC5	2.11	118.9%	1.78	0.83	1.78
BTG2	1.72	94.4%	1.82	0.86	1.82
PURA	2.38	121.9%	1.95	0.96	1.95
ERCC3	2.86	125.4%	2.28	1.19	2.28
BBC3	3.20	135.7%	2.36	1.24	2.36
RPL13A	0.53	20.8%	2.53	1.34	2.53
ATM	2.47	92.3%	2.68	1.42	2.68
ERCC1	5.18	186.7%	2.78	1.47	2.78
H2AFL	3.32	118.7%	2.80	1.48	2.8
E2-EPF	4.68	160.0%	2.92	1.55	2.92
CKN1	4.31	131.2%	3.29	1.72	3.29
UNG2	6.01	178.8%	3.36	1.75	3.36
PIN1	6.99	191.3%	3.65	1.87	3.65
BIRC3	5.50	143.2%	3.84	1.94	3.84
DDB1	4.46	95.9%	4.65	2.22	4.65
PTPRCAP	10.76	207.2%	5.19	2.38	5.19
HUS1	9.38	169.9%	5.52	2.47	5.52
DDB2	17.46	301.8%	5.79	2.53	5.79
MSH2	29.28	226.8%	12.91	3.69	12.91
RAD51	28.65	157.4%	18.21	4.19	18.21

Table 3. Functional Gene Grouping: modified version from SupeArray Database

ATR/ATM signaling network	DNA damage response pathways		
	Cell cycle arrest pathway	Apoptosis pathway	Genomic stability/repair pathway
53BP1, ATM, ATR, CDS1, CHEK1, CHEK2 (Rad53), FOXO3A, HUS1, MRE11A, NBS1 (nibrin), RAD1, RAD9, RAD17, RAD50, TREX1 (ATRIP)	CDC20 (p55cdc), CDC25A, CDC25B, CDC25C, CDK4, CDKN1A (p21), CENPE, DDIT3 (GADD153), E2-EPF, EXT1, GADD45A, GADD45B, GADD45G, GTSE1 (B99), KNSL5 (MKLP-1), KNSL6 (MCAK), MDM2, PIN1, PPM1D (Wip1), PTPRCAP (LPAP), REPRIMO, SFN (14-3-3 sigma), TP53, WEE1	APAF1, BAK1, BAX, BBC3 (PUMA), BCL6, BIRC2 (MIHB), BIRC3 (MIHC), BNIP3 (NIP3), LGMN (Legumain), LRDD (PIDD), P53AIP1, TNF (TNF-a), TNFRSF4 (OX40), TNFRSF6 (Fas), TNFRSF10 (DR5)	APEX (REF-1), ATRX (RAD54), BRCA1, BRCA2, BTG2, CKN1 (CSA), DDB1, DDB2, ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, ERCC6, FEN1, GADD45A, GADD45B, GADD45G, H2AFL, MLH1, MPG, MSH2, MSH3, MSH6, PA26, PCNA, PMS1, PMS2, PRKDC (DNA-PK), PURA, RAD23A, RAD23B, RAD50, RAD51, RAD52, RPA3, RRM2B (p53R2), UNG, UNG2, XPA, XPC, XRCC1, XRCC2, XRCC3, XRCC4, XRCC5 (Ku-80).

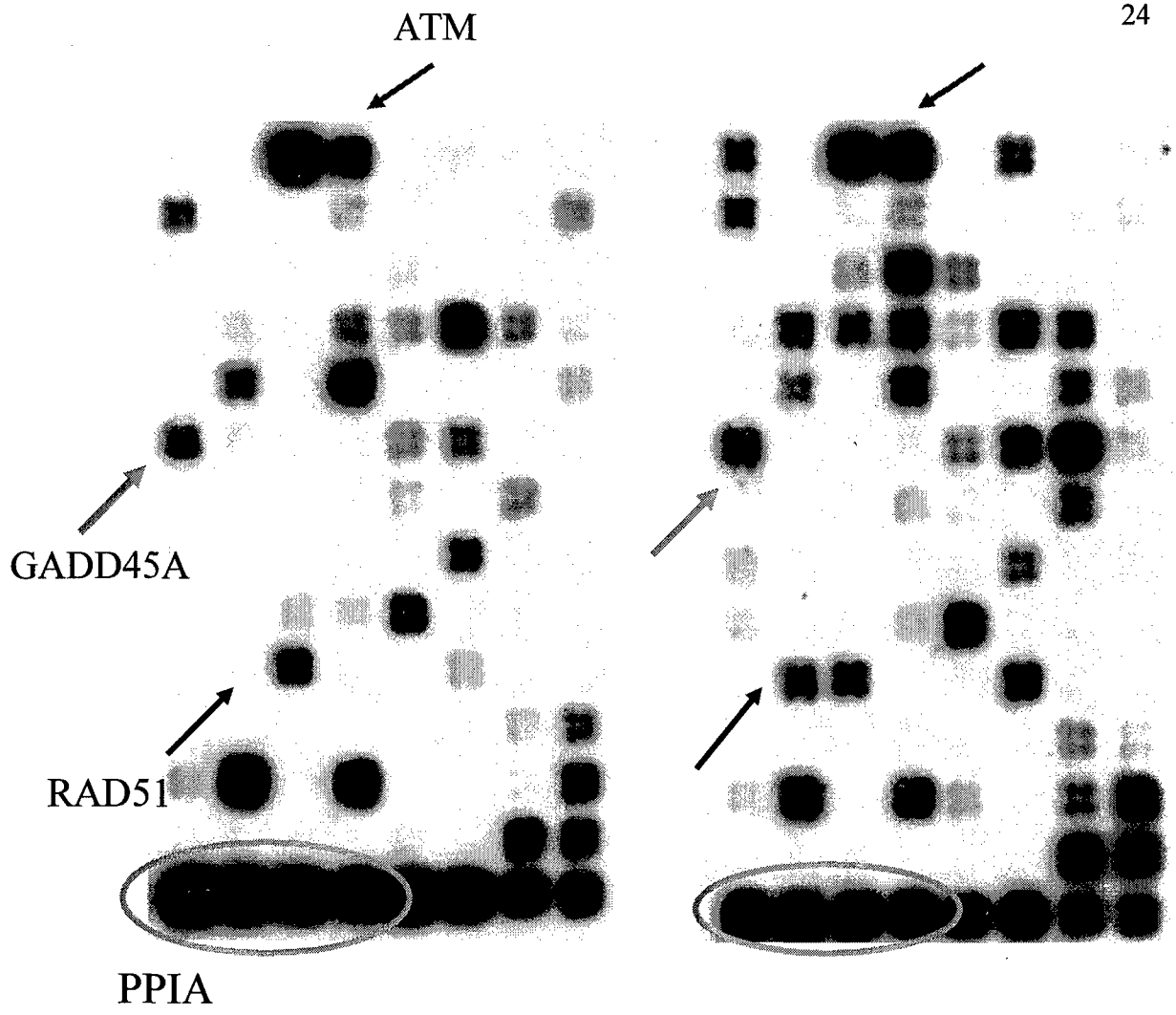


Figure 1. GEArray-Q Series Human DNA Damage Signaling Pathway Gene Array. DNA damage response profiles for CaP 1 and normal prostatic sample.

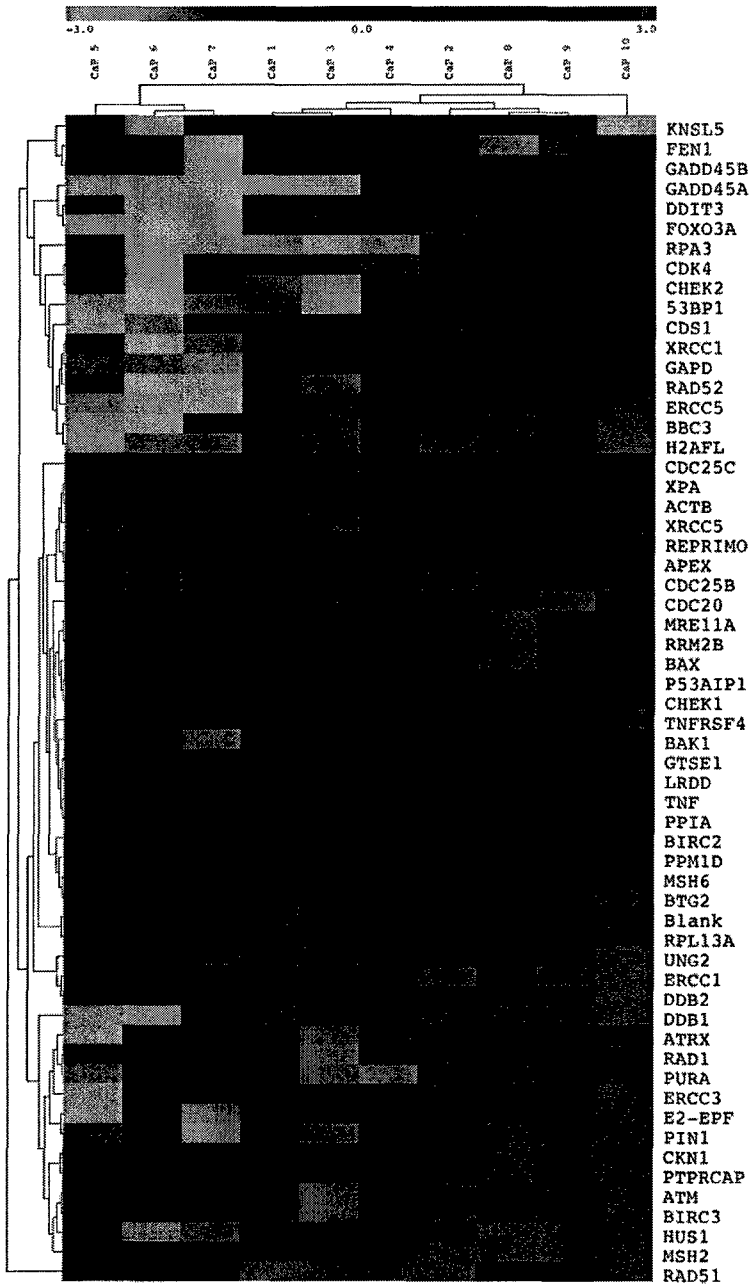


Figure 2. Illustration of the hierarchical clustering: 96 key genes and 10 household genes were identified through statistical significance and fold change criteria in 10 CaP samples. Levels of expression are represented on a scale from the lowest expression (bright green) to the highest expression (bright red). The color black represents a zero expression.

APPENDIX 3

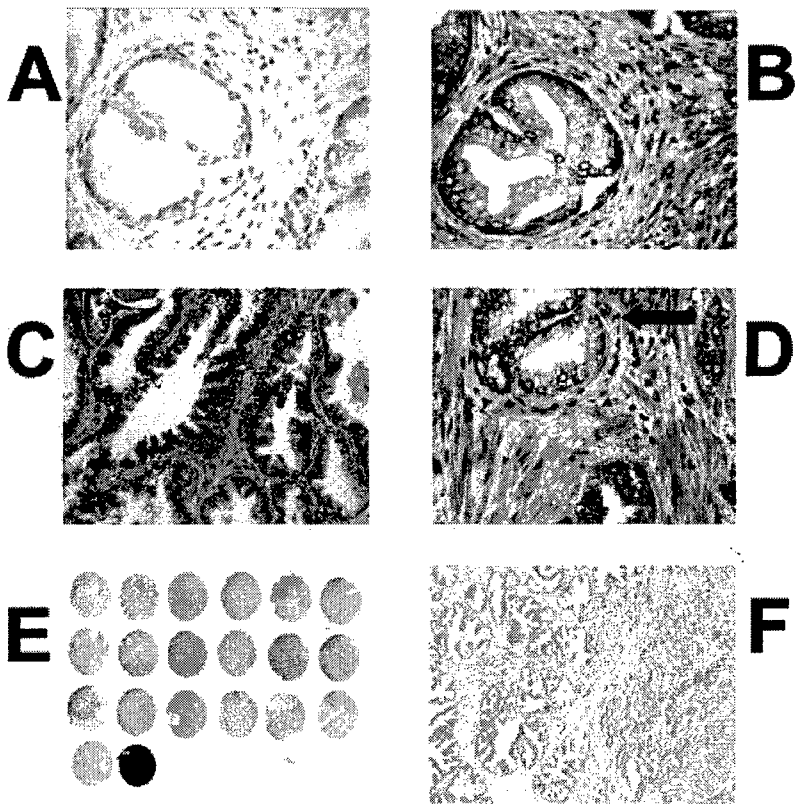


Figure 1. Immunohistochemical nuclear and cytoplasmatic patterns obtained with the commercially available PTEN mouse monoclonal antibody (1:100, clone 26H9) in Prostate cancer TMAs. **A-** The negative control treated identically but without incubation of the primary PTEN antibody. **B** and **C-** Strong and diffuse immunostaining positivity for PTEN in the same benign glandular epithelium and PIN, respectively. **D-** Focal low expression in areas of cancer in comparison with positivity in normal stromal cells. **E-** A low magnification of TMA and **F-** H&E section show foci of PIN and cancer.

APPENDIX 4

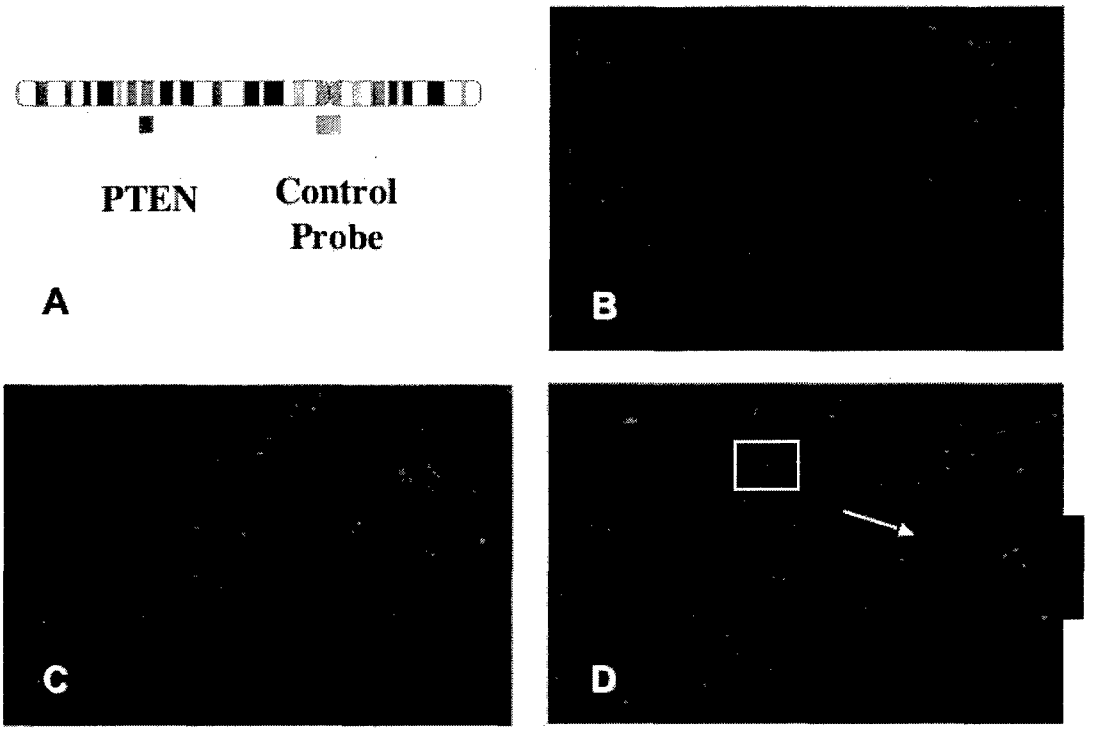


Figure 2. Illustration of nterphase FISH on prostate TMAs using dual color probes for PTEN locus (red signal) and centromere of chromosome 10 (green signal).

A- Probe configuration.
B- Overall, PTEN locus remained normal (2 red signals and 2 green signals) in some cancer cells.
C- Overall, PTEN locus remained normal (2 red signals and 2 green signals) in PIN.
D- PTEN locus deletion (1 red signal and 2 green signals) in cancer cells.

Appendix 5 Use of Whole Genome Amplification and Comparative Genomic Hybridisation to detect chromosomal copy number alterations in cell line material and tumour tissue.

S. Hughes[1,2], G. Lim[3,4], B. Beheshti[5], J. Bayani[1], P. Marrano[1], A. Huang[3], J.A. Squire[1,2,4].

Ontario Cancer Institute, Princess Margaret Hospital, University Health Network[1]. Department of Medical Biophysics[2], Hospital for Sick Children[3], Department of Laboratory Medicine and Pathology[4], Faculty of Medicine, University of Toronto[5],

Key Words: DOP-PCR, whole genome amplification, comparative genomic hybridization, genomic DNA arrays

Corresponding author: Jeremy Squire, Ph.D.
Ontario Cancer Institute
610 University Avenue
Toronto, Ontario, M5G 2M9
CANADA
e-mail: Jeremy.squire@utoronto.ca

Abstract

We have established that whole genome amplification (WGA) in conjunction with genomic DNA array comparative genomic hybridisation (gaCGH) allows for the identification of genome-wide copy number abnormalities (CNAs) in DNA extracted from both cell line and patient material. To determine the fidelity and reproducibility of WGA to detect copy number imbalances using gaCGH, well characterized cell line genomic DNA was analysed. The gaCGH data obtained from non-amplified DNA and amplified DNA for the neuroblastoma cell line NUB7 and a paediatric medulloblastoma patient was almost identical. In addition, laser capture microdissection (LCM) of prostate tumour cells and subsequent WGA allowed for the detection of a number of CNAs that may not have been identified if DNA had been extracted in bulk from heterogeneous tissue. The results presented here demonstrate the use of WGA for generating sufficient DNA for gaCGH analysis without the introduction of significant sequence representation bias. The combination of amplification and gaCGH using DNA extracted from archival patient material has the potential for permitting the studying of DNA from small cancerous or pre-cancerous foci, which may help to identify potential genomic markers for early diagnosis.

Introduction

Comparative genomic hybridisation (CGH) has provided a significant contribution to our understanding of chromosomal changes associated with tumour development and progression, making it possible to detect regions of chromosomal gain and loss in a single experiment (James et al., 1999). Conventional CGH uses metaphase chromosomes and provides a typical resolution of 10 Mb for a simple loss or gain (Kallioniemi et al., 1992) and approximately 2 Mb for a high copy number gain. Though this offers a suitable starting point for analysis of chromosomal changes, the limited resolution means that small focal changes may be missed. Recent advances that have substituted the metaphase chromosomes with well-defined arrayed sequences of DNA, have allowed for a increase in resolution from whole chromosomes, to considerably shorter cDNA, bacterial artificial chromosome (BAC) or P1 artificial chromosome (PAC) clones (Cai et al., 2002; Kraus et al. 2003). This innovation has allowed for a more accurate measure of chromosomal changes thus greatly refining the results produced by chromosome CGH (Hui et al., 2001; Wessendorf et al. 2003). Despite this progress, a major drawback with the current CGH methodology is its dependence on the availability of large quantities of genomic DNA extracted from bulk tissue. This requirement is prohibitive as for many cancers those key genetic changes that are of major interest are likely to occur at an early phase in the tumourigenetic process. These changes are likely to be restricted to a small portion of pre-malignant or malignant cells within the specimen under examination. As a result, such alterations may be masked, as

the data obtained from CGH for bulk extracted DNA represents an average value for all cells within the specimen. Even though CGH can detect chromosomal changes that are present in as little as 30-50% of starting tumour cells (du Manoir et al., 1995), it is likely that these changes will correspond to original high copy number changes, whereas low copy number changes may be obscured by the 50-70% of contaminating normal cells.

Recent work with prostate cancer, which is characterized by the presence of pre-malignant and malignant lesions interspersed within normal non-cancerous tissue, has demonstrated the utility of a microscope-guided hollow bore to obtain a relatively pure cell population that provides sufficient DNA for use in array CGH (Paris et al., 2003). However, in many instances, as the amount of patient tissue is a limiting factor and as lesions of interest may only consist of a few hundred cells, it is possible that boring will also collect a significant number of contaminating normal cells.

Previous studies have established the efficacy of WGA approaches to obtain sufficient amounts of DNA for array CGH (Lage et al., 2003). However, to date, analysis has been limited to use of whole genome cDNA arrays and genomic DNA arrays for individual chromosomes. In this report we show that WGA approaches, such as degenerate oligo-nucleotide primed (DOP)-PCR and strand displacement amplification (SDA) in conjunction with whole genome gaCGH, allow for identification of CNAs from cell line and patient DNA that show a close correlation to the results obtained for gaCGH from non-amplified DNA. In combination, these results demonstrate that WGA, specifically SDA, allows for

faithful amplification of genomic DNA. The fidelity of SDA when combined with laser capture microdissection (LCM) and gaCGH can allow for the detection of CNAs from a small number of starting cells.

Materials and Methods

Laser capture microdissection

Fresh-frozen prostate tissue embedded in Frozen Section Medium (Stephens Scientific, Riverdale NJ, USA) was cut onto microscope slides (5μm thickness) and stained using the HistoGene LCM frozen section staining kit (Arcturus, Carlsbad, USA). Regions of cancer, constituting in excess of 1000 cells, were identified and obtained by laser capture microdissection (LCM) using the Cell Robotics LaserScissors system (Cell Robotics, Inc., Albuquerque, NM, USA).

DNA Extraction

Reference DNA (from human placental tissue; 46, XY) and test DNA (from the NUB7 cell line, a paediatric medulloblastoma (90% tumour) and the captured prostate cancer cells) was prepared by proteinase K digestion and phenol-chloroform extraction (Molecular Cloning: A laboratory manual, Third Edition). DNA concentration was determined by measuring absorbance at A260, with the exception of the DNA obtained from captured cells where the concentration was determined using the PicoGreen dsDNA Quantitation kit (Molecular Probes, Inc., Eugene, OR, USA).

DOP-PCR amplification

DOP-PCR was carried out in two individual steps. The Step 1 reaction mixture contained 5-10ng template DNA (from diluted reference or test DNA), 1μl of 10X Thermosequenase Buffer (Amersham, Baie D'Urfe, Canada), 1μM of DOP-PCR primer (5'-CCGACTCGAGNNNNNNATGTGG-3'), 0.2mM dNTPs, 4 units of Thermosequenase (Amersham), and sterile water up to a final volume of 10μl. The PCR profile for Step 1 was: denaturation at 94°C for 60 seconds, annealing at 30°C for 60 seconds, ramping to 72°C over a 3 minute period (3.5°C/15 seconds) and extension at 72°C for 2 minutes. The reaction was carried out for 5 cycles with a final extension at 72°C for 10 minutes. The Step 2 reaction mixture contained 4μl of 10X PCR reaction buffer (Invitrogen, Burlington, Canada), 1.5mM magnesium chloride, 0.3μM of DOP-PCR primer, 0.4μM dNTPs, 1.25 units of *Thermus aquaticus* (*Taq*) polymerase (Invitrogen) and sterile water up to a final volume of 40μl. The step 1 and 2 reaction mixtures were then combined. The PCR profile for Step 2 was: denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 2 minutes. The reaction was carried out for 30 cycles with a final extension at 72°C for 10 minutes.

Strand displacement amplification

Strand displacement amplification was carried out in two individual steps. The step 1 reaction mixture contained 5-10ng of DNA (from diluted reference or test DNA), 1X ThermoPol buffer (New England Biolabs, Pickering, Canada), a

primer (modified random heptamer 5' [NITP][NITP]NNNnN 3') at 30μM (in final reaction volume of 20μl) and sterile water up to a volume of 6μl. This solution was first heated at 95 °C for 2 minutes, then left at room temperature for 10 minutes and finally chilled on ice. The step 2 reaction (amplification) mixture contained 1X ThermoPol buffer (New England Biolabs), dNTPs at 0.4mM (Invitrogen), 0.4% DMSO (Sigma-Aldrich, St. Louis, USA), 0.35 units of Bst DNA polymerase (New England Biolabs), 5.2ng of T4 gene 32 protein (Amersham) and sterile water up to a final volume of 14μl. The step 1 and 2 reaction mixtures were combined and then incubated at 50°C for 6 hours. The enzymes were inactivated by heating at 65 °C for 10 minutes, followed by cooling to 4 °C.

Assessment of amplification

Five microlitres of each amplification reaction was electrophoresed through a 1% agarose gel and stained with ethidium bromide in order to assess product yield and product length. All amplification products were purified by phenol-chloroform extraction and DNA concentration and purity were determined by measuring absorbance at A260 and A280.

Spectral Genomic BAC arrays

The genomic DNA arrays used in these experiments were obtained from Spectral Genomics Inc. (Houston, Texas, USA) and consist of 1403 unique BAC and PAC clones, which provide an average genomic resolution of 2-4 Mb.

DNA labeling

Array experiments were performed using both amplified and non-amplified DNA. Reference DNA and test DNA were first digested overnight at 37°C using 10 units of *RsaI* (Invitrogen) in a 10μl reaction. The digested DNA was labeled using the protocol optimized by Spectral Genomics, with separate labeling reactions for Cy3 and Cy5 being set up for both reference and test DNA. Briefly, labeling reaction were set up containing 1-2μg of DNA (genomic or amplified), 20μl of 2.5X random primer/reaction buffer mix (Invitrogen) and sterile water up to a final volume of 45μl. The reaction mix was boiled for 5 minutes prior to cooling on ice and the addition of 2.5μl of Spectral labeling buffer (Spectral Genomics, Houston, U.S.A), 1.5μl of either Cy3-dCTP (1mM, Applied Biosystems, Foster City, U.S.A) or Cy5-dCTP (1mM, Applied Biosystems) and 1μl of Klenow Fragment (BioPrime labelling kit, Invitrogen). The reaction was mixed gently and then incubated for 2 hours at 37°C. Following incubation the reaction was stopped by the addition of 5μl 0.5M EDTA (pH8) and heating at 72°C for 10 minutes.

Hybridisation

The Cy3 labeled test DNA was combined with the Cy5 labeled normal reference DNA and vice versa. Each combined probe was mixed with 45μl of Spectral Hybridisation Buffer (Spectral Genomics), 11.3μl of 5M NaCl and 110μl of room temperature isopropanol. The samples were incubated in the dark at room temperature for 10-15 minutes and centrifuged at 16,000 x g for 10 minutes

and the supernatant discarded. The pellets were then washed with 500μl of 70% ethanol. The supernatant was carefully removed and the pellets air-dried at room temperature in the dark. For hybridisation, the pellets were first resuspended in 10μl of sterile water prior to being mixed with 30μl of Spectral Hybridisation Buffer II (Spectral Genomics) by pipetting. The reconstituted probes were then incubated at 72°C for 10 minutes, placed on ice for 5 minutes and then incubate for 30 minutes at 37°C. The probes were hybridised to BAC arrays, covered with a 22x60mm coverslip and incubated for 12-16 hours at 37°C in a humified chamber.

Washes

The wash buffers, with the exception of Wash I, were pre-warmed to 40°C. The slides were gently dipped into and out of Wash I (2X SSC, 0.5% SDS) until the coverslip detached from the slide. The slides were then washed once in Wash II (2X SSC, 50% deionized Formamide, pH 7.5) for 20 minutes, followed by successive washes in Wash III (2X SSC, 0.1% NP-40, pH 7.5) for 20 minutes and Wash IV (0.2X SSC, pH 7.5) for 10 minutes. All washes were performed at 40°C, with the exception of Wash I. The slides were briefly submerged in distilled deionized water for 5-10 seconds and centrifuged for 3-5 minutes at 750 rpm to dry.

Data collection and Analysis

The slides were scanned using an Axon GenePix 4000A confocal scanner, each fluorescence signal was collected separately and quantified with the GenePix Pro 3.0 software (Axon Instruments, Union City, U.S.A). The data was normalised and analysed using Normalise Suite v1.63 (Beheshti et al., 2003). Software and complete data sets for all samples can be viewed at http://www.utoronto.ca/cancyto under the study data section.

Results

Whole genome amplification of DNA from tissue culture cells and tumour tissue

Using DOP-PCR and SDA we were able to consistently amplify in excess of 1μg of DNA from a starting concentration of 5-10ng. Though amplification was possible from lower concentrations of starting material (less than 1ng) the product quality, as assessed by the array results, was not consistent. The length of fragments obtained using the two WGA approaches differed considerably. DOP-PCR generated products ranging from 0.2kb to 3kb, whereas SDA consistently produced products in excess of 10kb. Variation in amount of starting material (in the range of 5-10ng), for either protocol, had little effect on the corresponding product size or smear intensity. No detectable background amplification was observed in the negative controls for either protocol.

Genomic DNA array CGH for the NUB7 cell line

For the NUB7 cell line studied in this work the presence or absence of CNAs, as observed by gaCGH using either non-amplified or amplified DNA, was consistent with previous findings. The gaCGH results depicted in Figure 1 for NUB7 (Dimitroulakos et. al., 1994) were obtained using non-amplified, SDA amplified and DOP-PCR DNA. The gray shading denotes chromosomal regions that display CNAs for all three types of DNA preparation. The arrows indicate CNAs introduced by DOP-PCR. gaCGH for NUB7 detected and further refined all of the CNAs that had previously been identified using chromosome CGH and cDNA microarrays (Squire et al., 2003), these being gains of 1p32.1 - qter, 7q32.2 - qter and 17q12 - qter and loss from 4q11 - q22.1 and 6q16.1 - qter (Figure 1). In addition, a loss of 11q13.3 - qter was also detected.

The gaCGH results obtained for NUB7 using DNA amplified by DOP-PCR identified the entire set of CNAs listed above, however additional CNAs were observed that were not corroborated by the results obtained using the non-amplified DNA (Figure 1). As a consequence, from this point onwards we decided to focus on the use of SDA, as the results obtained for the NUB7 cell line showed 100% of the previously identified CNAs, and importantly no additional alterations.

The control CGH experiments comparing differentially labeled non-amplified and/or amplified placental DNA did not display any chromosomal imbalances. In addition, those ideograms that do not display any CNAs in non-amplified DNA are very similar to those from the amplified DNA (Figure 1).

These results show that WGA introduces little or no amplification bias indicating that the chromosomal changes observed for these samples are not artifacts of the experimental procedures.

Genomic DNA array CGH from tissue

When gaCGH was performed using amplified and non-amplified DNA extracted from a paediatric medulloblastoma, alterations constituting loss of 17p and gain of 17q were observed, in addition to a focal amplification at 5p12 - p13 for both DNA types (Figure 2). The gaCGH results obtained for the two microdissected prostate DNA samples (CaP11-01 and CaP32-01) identified several CNAs (Table 1, Figure 3), which are in keeping with previous result observed for prostate cancer (Cunningham et al., 1996; Qian et al., 1999).

Discussion

Analysis of genomic alterations in tumour tissue is often restricted by the limited amount of sample material from which only small amounts of genomic DNA can be obtained. Whole genome amplification using DOP-PCR (Hirose et al. 2001; Grant et al. 2002) or SDA (Lage et al. 2003; Hosono et al. 2003) has permitted the use of advanced molecular analyses that would previously not have been possible due to the limited quantity of DNA available.

The merit of WGA is its ability to faithfully amplify the entire genome with little representational bias. DOP-PCR utilises a degenerate oligonucleotide primer in combination with an initial low annealing temperature to achieve non-

specific amplification from multiple sites (approximately 10^6 sites in humans) within the genome (Telinius et al., 1992). The appeal of this approach is that no *a priori* knowledge of the DNA under investigation is required. Though DOP-PCR has been used for many applications its shortcomings have been well reported. It is acknowledged that DOP-PCR does not provide either complete coverage of all loci (Paunio et al., 1996) or copy the target DNA in its entirety (Chueng and Nelson, 1996). In addition, PCR-based WGA methods have been reported to cause 4 to 6-fold amplification bias (Dean et al., 2002). In this context, the use of DOP-PCR is not an ideal approach for WGA, as the amplification bias could produce false results.

Recently, a rolling circle amplification method, originally developed for the amplification of large circular DNA templates (Dean et al., 2001), has been adapted for the amplification of genomic DNA, and allows for the synthesis of DNA strands in excess of 10 kb in length. Similarly to DOP-PCR no previous knowledge of the target template is required. Using Bst polymerase, T4 gene 32 protein and exonuclease resistant random heptamers, DNA is amplified in a 50°C reaction. The polymerase is capable of synthesizing microgram quantities of DNA from as little as 5ng of starting material, making is ideally suited for genomic analysis where starting DNA is limited. Additionally, in contrast to DOP-PCR SDA provides good genome coverage and does not introduce significant amplification bias (Lage et al., 2003).

The use of DOP-PCR in the analysis of NUB7 introduced a number of additional CNAs that were not corroborated by the results obtained using the

non-amplified DNA (Figure 1). If this bias was repeated in the amplification of patient samples, where no additional information was available, it might lead to incorrect assignment or failure to detect CNAs. In contrast to DOP-PCR, SDA introduced no detectable bias in the samples studied, when compared to non-amplified DNA, confirming the fidelity of this approach.

To date, a majority of the CGH array data has been restricted to cDNA arrays (Pollack et al., 1999; Beheshti et al., 2003), however there are now an increasing number of articles describing CGH using other array platforms (Cai et al., 2002; Lucito et al., 2003). Despite clear advantages over chromosome CGH, cDNA arrays exhibit a lack of consistency in hybridisation and poor specific signal-to-noise ratios. This attribute can cause an overall reduction in sensitivity and, as a result, a decreased potential for detecting single copy changes (Hughes et al., in press). In contrast, genomic DNA-based arrays provide a more accurate approach for detecting single copy number gains and losses. This characteristic has the potential to allow for the discovery of CNAs that would otherwise not have been detected using either cDNA array CGH or metaphase CGH. This is observed here with the detection of additional CNAs in the NUB7 cell line. Despite the advantages of genomic DNA-based arrays, they currently have uneven chromosome coverage, resulting in many genomic regions being under represented. This lack of uniformity was demonstrated by the failure to detect the amplification of MYCN at 2p24, which has previously been reported for NUB7 (Squire et al., 2003), as a clone covering this region is not present on the

array. The ongoing development of higher resolution arrays will likely address this problem.

The data presented here for the NUB7 cell line and the paediatric medulloblastoma demonstrates that SDA, in conjunction with gaCGH, produces results almost identical to non-amplified DNA and thus allows for the faithful identification of chromosomal alterations. In addition, the detection of CNAs in amplified DNA obtained from microdissected prostate cancer foci indicates that SDA and gaCGH may provide an approach for studying chromosomal changes in cancerous and pre-cancerous lesions present within the same tissue section.

The strategy outlined above should provide new insights into the biology of many tumours that contain multiple foci, potentially allowing for the identification of genomic regions of imbalance associated with various stages of tumour progression and metastasis. The combination of these approaches have the promise to aid in the identification of specific genetic markers, which may prove to be important for making prognostic and/or therapeutic decisions.

References

Beheshti B, Braude I, Marrano P, Thorner P, Zielenska M, Squire JA. Chromosomal localization of DNA amplifications in neuroblastoma tumors using cDNA microarray comparative genomic hybridization. Neoplasia 5: 53-62 (2003).

Cai WW, Mao JH, Chow CW, Damani S, Balmain A, Bradley A. Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays. Na. Biotechnol 20: 393-396 (2002).

Chueng VG, Nelson SF. Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. Proc Natl Acad Sci 93: 14676-14679 (1996).

Cunningham JM, Shan A, Wick MJ, McDonnell SK, Schaid DJ, Tester DJ, Qian J, Takahashi S, Jenkins RB, Bostwick DG, Thibodeau SN. Allelic imbalance and microsatellite instability in prostatic adenocarcinoma. Cancer Res 56: 4475-4482 (1996).

Dean FB, Nelson JR, Giesler TL, Lasken RS. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res 11: 1095-1099 (2001).

Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS. Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci 99: 5261-5266 (2002).

Dimitroulakos J, Squire J, Pawlin G, Yeger H. NUB-7: a stable I-type human neuroblastoma cell line inducible along N- and S-type cell lineages. Cell Growth Differ 5: 373-384 (1994).

du Manoir S, Schrock E, Bentz M, Speicher MR, Joos S, Ried T, Liichter P, Cremer T. Quantitative analysis of comparative genomic hybridization. Cytometry 19: 27-41 (1995).

Grant SFA, Steinlicht S, Nentwich U, Kern R, Burwinkel B, Tolle R. SNP genotyping on a genome-wide amplified DOP-PCR template. Nucleic Acids Res 30: e125 (2002).

Hirose Y, Aldape K, Takahashi M, Berger MS, Feuerstein BG. Tissue microdissection and Degenerate Oligonucleotide Primed-Polymerase Chain Reaction (DOP-PCR) is an effective method to analyze genetic aberrations in invasive tumors. Journal of Mol Diag 3: 62-67 (2001).

Hosono S, Faruqi AF, Dean FB, Du Y, Sun Z, Wu X, Du J, Kingsmore SF, Egholm M, Lasken RS. Unbiased whole-genome amplification directly from clinical samples. Genome Res 13: 954-964 (2003).

Hughes S, Beheshti B, Marrano P, Lim G, Squire JA. Comparative Genomic Hybridisation Analysis using Metaphase or Microarray Slides. Immunohistochemistry and In situ hybridization of human carcinomas, Volume 2. Elsevier Academic Press (2004).

Hui AB, Lo KW, Yin XL, Poon WS, Ng HK. Detection of multiple gene amplifications in glioblastoma multiforme using array-based comparative genomic hybridization. Lab. Invest 81: 717-723 (2001).

James LA. Comparative genomic hybridization as a tool in tumour cytogenetics. J Pathol 187: 385-395 (1999).

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258: 818-821 (1992).

Kraus J, Pantel K, Pinkel D, Albertson DG, Speicher MR. High-resolution genomic profiling of occult micrometastatic tumor cells. Genes Chromos Cancer 36: 159-166 (2003).

Lage JM, Leamon JH, Pejovic T, Hamann S, Lacey M, Dillon D, Segraves R, Vossbrinck B, Gonzalez A, Pinkel D, Albertson DG, Costa J, Lizardi PM. Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. Genome Res 13: 294-307 (2003).

Lucito R, Healy J, Alexander J, Reiner A, Esposito D, Chi M, Rodgers L, Brady A, Sebat J, Troge J, West JA, Rostan S, Nguyen KC, Powers S, Ye KQ, Olshen A, Venkatraman E, Norton L, Wigler M. Representational Oligonucleotide Microarray Analysis: A High-Resolution Method to Detect Genome Copy Number Variation. Genome Res: [Epub ahead of print] (2003).

Paris PL, Albertson DG, Alers JC, Andaya A, Carroll P, Fridlyand J, Jain AN, Kamkar S, Kowbel D, Krijtenburg PJ, Pinkel D, Schroder FH, Vissers KJ, Watson VJ, Wildhagen MF, Collins C, Van Dekken H. High-resolution analysis of paraffin-embedded and formalin-fixed prostate tumors using

comparative genomic hybridization to genomic microarrays. Am J Pathol 162: 763-770 (2003).

Paunio T, Reima I, Syvanen AC. Preimplantation diagnosis by whole genome amplification, PCR amplification and solid-phase minisequencing of blastomere DNA. Clin Chem 42: 1382-1390 (1996).

Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 23: 41-46 (1999).

Qian J, Jenkins RB, Bostwick DG. Genetic and chromosomal alterations in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization. Eur Urol 35: 479-483 (1999).

Molecular Cloning: A Laboratory Manual, Third Edition. Sambrook J, Russell DW. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Squire JA, Pei J, Marrano P, Beheshti B, Bayani J, Lim G, Moldovan L, Zielenska M. High-resolution mapping of amplification and deletions in pediatric osteosarcoma using CGH analysis of cDNA microarrays. Genes Chromosomes Cancer 38: 215-225 (2003).

Telenius H, Carter NP, Bebb CE, Nordenskjöld M, Ponder BA, Tunnacliffe A. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 13: 718-725 (1992).

Wessendorf S, Schwaenen C, Kohlhammer H, Kienle D, Wrobel G, Barth TF, Nessling M, Moller P, Dohner H, Lichter P, Bentz M. Hidden gene

amplifications in aggressive B-cell non-Hodgkin lymphomas detected by microarray-based comparative genomic hybridization. Oncogene 22: 1425-1429 (2003).

Figure and Table legends

Figure 1. The use of (i) non-amplified, (ii) SDA amplified and (iii) DOP-PCR amplified NUB7 DNA in conjunction with gaCGH, allows for the detection of a gain of genetic material on chromosomes 1, 7 and 17 and a loss from chromosomes 4, 6 and 11. The gray shading denotes chromosomal regions that display CNAs for all three types of DNA preparation. The arrows indicate CNAs introduced by DOP-PCR.

Figure 2. Chromosomal alterations observed for a paediatric medullooblastoma, as determined by gaCGH from (i) non-amplified and (ii) SDA-amplified DNA. A focal gain was observed on 5p, in addition to loss of 17p and gain of 17q. Chromosomes 1 and 10, which do not possess any CNAs, are displayed to demonstrate that amplification using SDA does not introduce any aberrant CNAs. The gray shading denotes chromosomal regions that display CNAs for both DNA preparations.

Figure 3. The chromosomal alterations observed for the two prostate cancer samples, a) CaP11-01 and b) CaP32-01. The DNA used for CGH was obtained by laser capture microdissection of tumour cells and subsequent SDA.

Table 1. Chromosomal changes in microdissected prostate cancer DNA.

Table 1

Prostate sample	Gain	Loss
CaP11-01	8q22.1 - qter	2p16.3 – 22.1 8pter – p12
CaP32-01	14p11.2 – q11.2, 15pter – q23	3q26.2 – qter, 8p 13q12.2 – qter, 16q22 - qter

ELSEVIER

Progress in Biophysics & Molecular Biology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Progress in Biophysics & Molecular Biology

www.elsevier.com/locate/pbiomolbio

Appendix 6 The use of whole genome amplification in the study of human disease

Simon Hughes[a,b,*], Nona Arneson[a], Susan Done[a,b,c], Jeremy Squire[a,b,c]

[a] *Ontario Cancer Institute, Princess Margaret Hospital, University Health Network, 610 University Avenue, Toronto, Ont., Canada M5G 2M9*

[b] *Department of Medical Biophysics, University of Toronto, Toronto, Ont., Canada M5G 2M9*

[c] *Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ont., Canada M5G 2M9*

Abstract

The availability of large amounts of genomic DNA is of critical importance for many of the molecular biology assays used in the analysis of human disease. However, since the amount of patient tissue available is often limited and as particular foci of interest may consist of only a few hundred cells, the yield of DNA is often insufficient for extensive analysis. To address this problem, several whole genome amplification (WGA) methodologies have been developed. Initial WGA approaches were based on the polymerase chain reaction (PCR). However, recent reports have described the use of non-PCR based linear amplification protocols for WGA. Using these methods, it is possible to generate microgram quantities of DNA starting with as little as one nanogram of genomic DNA. This review will provide an overview of WGA approaches and summarise some of the uses for amplified DNA in various high-throughput genetic applications.

Keywords: Whole genome amplification (WGA); PEP; DOP-PCR; SCOMP; SDA; TLAD

1. Introduction

Recent advances in the study and understanding of human disease have largely been made possible by advances in molecular biology techniques. The sequencing of the human genome has provided the first human genetic blue print for all 23 chromosome pairs. Though the tools and the technologies for the analysis of this information are still in their infancy, the information they will eventually provide will allow scientists and clinicians to identify and treat those individuals at the risk of cardiovascular disease, neuro-psychiatric disorders, cancer and a myriad of other genetic

*Corresponding author.
E-mail address: sih3@yahoo.com (S. Hughes).

0079-6107/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pbiomolbio.2004.01.002

disorders, prior to development of disease. The ability to better diagnose, monitor and manage these disease processes is the basis of much of the research performed around the world.

The starting point for a large proportion of human disease research is DNA. However, acquisition of this most basic of requirements is often the stumbling block for many researchers. Human disease research often suffers from an inherent lack of sufficient representative genomic DNA from patient material. This can result from the mode of sample collection (Zheng et al., 2001), or the requirement for pure tumour cell populations obtained by macrodissection or (Chung et al., 2000) laser capture microdissection (LCM) (Wang et al., 2001; Hughes et al., in press). The net result of such factors is a restriction on the type and number of high-throughput genetic assays that can be performed. As a result, researchers are often required to implement a candidate gene or region specific methodology, in contrast to a more holistic whole genome approach, due to the limited quantities of patient DNA. Despite the success of candidate type approaches (Thakker et al., 1993; Bates et al., 1997), it is likely that many key genetic alterations that could prove to be important for making prognostic and/or therapeutic decisions may go undetected (Simpson et al., 2003). It is therefore clear that some form of whole genome amplification (WGA) is required in order to provide an ample supply of DNA for large-scale genetic studies. For molecular analyses there are currently three techniques that can be used: PCR-based approaches, strand displacement amplification and T7 based linear amplification. The ideal situation for any such approach is the generation of results from the amplified DNA that are indistinguishable from the results obtained from the original genomic DNA. (Information for each technique described in this review is listed in Table 1a and b).

Many of the papers reporting the use of WGA have applied it to the analysis of human cancers and though WGA has utility in other fields such as pre-implantation genetic diagnosis (Jiao et al., 2003; Ao et al., 1998; Sermon et al., 1996; Kristjansson et al., 1994) and prenatal diagnosis (Martel-Petit et al., 2001; Sekizawa et al., 1996), this review will concentrate on the application of WGA to the study of human malignancies. Below we will summarise the evolution of WGA methods (both PCR and non-PCR based) as well as discuss the limitations of each method as it pertains to human genetic research.

2. PCR-based approaches

The first PCR-based genome amplification approach, interspersed repetitive sequence (IRS) PCR (Nelson et al., 1989), used primers designed to anneal to repetitive sequences (Alu repeats) within the genome. The disadvantages of this approach were that it relied on; (1) the repeats being evenly distributed throughout the genome and (2) the repeats being suitably positioned to allow for the generation of a PCR product. Even though Alu repeats are present throughout the human genome, they have a tendency to be clustered at intrinsically unstable regions of the genome (Kolomietz et al., 2002) and in the G-light chromosome bands (Korenberg and Rykowski, 1988). Due to the location of the repeats there is a bias towards amplification of these regions. An alternative approach to overcome these limitations was introduced by Lüdecke et al. (1989). Their method, termed the linker adapter technique (LA) PCR, involved restriction digestion of the DNA and ligation of adaptors to serve as priming sites for subsequent PCR. These genome amplification approaches were initially applied to the cloning of microdissected chromosomes

Table 1
A summary of the currently available WGA techniques and a summary of approximate costs

Method	Reagents	Cost	Technical difficulty	Template requirements	Applications
(a) Currently available WGA techniques					
DOP-PCR	Roche has a DOP-PCR Kit; alternative method uses Thermosequenase and Taq for the two steps required	Expensive with kit. Cheaper with alternative protocol	Easy	Low concentration of starting material and poor quality DNA	Metaphase CGH (Ottesen et al., 2003), array CGH (Peng et al., 2003) SNP genotyping (Grant et al., 2002), microsatellite genotyping (Kittler et al., 2002) SSCP (Barbaux et al., 2001).
I-PEP	Uses Roche Expand High Fidelity PCR System	Medium price range	Easy	Low concentration of starting material and poor quality DNA	Sequencing (Dietmaier et al., 1999), microsatellite analysis (Zheng et al., 2001), LOH (Simpson et al., 2003).
LMP	Uses Roche Expand Long Template PCR System	Medium price range	Several steps required but can be optimised into one tube eliminating need for repeated purification steps	Low concentration of starting material, poor quality DNA is acceptable but not ideal	Metaphase CGH, LOH and sequence analysis (Klein et al., 1999), microsatellite genotyping (Tanabe et al., 2003).

Table 1 *(continued)*

Method	Reagents	Cost	Technical difficulty	Template requirements	Applications
SDA	GenomiPhi (Amersham) or alternate protocol using Bst Polymerase (NEB) and T4 Gene 32 protein (Amersham)	Medium price range. Cheaper when not using kit	Easy	Requires 5–10 ng of high-quality genomic DNA	Array CGH (Lage et al., 2003), SNP analysis (Tranah et al., 2003), quantitative PCR (Dean et al., 2002), microsatellite analysis (Hosono et al., 2003)
TLAD	No kit available	Expensive	Several purification steps required, technically difficult	Low concentration of starting material and poor quality DNA	

Item	Rxns	Price*	Price/Rxn*
(b) Approximate costs			
Genomic amplification methods			
DOP PCR Master	30	292.00	*9.73*
DOP PCR (Thermosequenase)			*4.45*
Thermosequenase (Amersham, 1000 U)	250	871.00	3.48
Primers	200	18.20	0.09
Taq polymerase (Invitrogen, 500 U)	200	175.00	0.88
I-PEP			*3.52*
Primers	200	10.20	0.05
Expand high-fidelity PCR system (2 × 360 U)	144	499.00	3.47

Scomp			*7.83*
Mse I (2500 U)	250	352.00	1.41
Expand long template PCR System (2 × 360 U)	144	499.00	3.47
T4 DNA ligase (Invitrogen, 500 U)	100	120.00	1.20
Primers	100	175.00	1.75
PRSG			*4.94*
Adaptor and primer	200	54.60	0.27
T4 DNA ligase (Invitrogen, 500 U)	100	120.30	1.20
Expand high-fidelity PCR system (2 × 360 U)	144	499.00	3.47
SDA			
GenomiPhi Kit (100 Rxns)	100	664.00	*6.64*
Alternate protocol			*5.79*
Bst polymerase (8000 U)	500	286.00	0.57
T4 Gene 32 protein (500 μg)	160	689.00	4.30
Primer	250	230.00	0.92
TLAD			*10.55*
AluI (2500 units)	250	352.00	1.41
Alkaline phosphatase (NEB, 5000 U)	2000	286.00	0.14
Terminal transferase (Roche, 24,000 U)	1200	265.00	0.22
Klenow (NEB, 1000 U)	500	260.00	0.52
Primer	100	175.00	1.75
T7 Megascript kit (Ambion,	40	215.00	5.37
SuperScript II Reverse Transcriptase-(Invitrogen, 200 U/μl	200	227.70	1.14

*All prices are in Canadian dollars.

(Saunders et al., 1989) and for the detection of regions of DNA involved in DNA/protein interaction (Kinzler and Vogelstein, 1989), not to the process of WGA.

In 1992, the first two WGA methods of degenerate oligonucleotide-primed (DOP) PCR (Telenius et al., 1992) and primer extension preamplification (PEP) (Zhang et al., 1992) were published. These techniques have allowed for the amplification of an entire genome-worth of DNA starting from as little as a single cell, which has also been shown to be possible for RNA (Eberwine et al., 1992). Modifications of DOP-PCR and PEP (long products from low DNA quantities (LL) DOP-PCR, Kittler et al., 2002; improved (I) PEP, Dietmaier et al. 1999) have allowed for increases in both PCR efficiency and DNA yield. In addition, the LA-PCR approach (Lüdecke et al., 1989) has since been adapted for WGA (Klein et al., 1999; Tanabe et al., 2003). These advances have increased the capacity for a more comprehensive genetic analysis of human diseases.

2.1. Degenerate oligonucleotide primed PCR (DOP-PCR)

Degenerate oligonucleotide primed (DOP) PCR is a well-established, technically straightforward method that is widely accepted as a mode of WGA. DOP-PCR-generated DNA has been used for many applications including metaphase CGH (Ottesen et al., 2003; Beheshti et al., 2002; Harada et al., 2002; Hirose et al., 2001; Larsen et al., 2001; Huang et al., 2000; Verhagen et al., 2000), array CGH (Peng et al., 2003; Daigo et al., 2001) single nucleotide polymorphism (SNP) genotyping (Grant et al., 2002; Barbaux et al., 2001), microsatellite genotyping (Kittler et al. 2002; Kim et al., 1999; Cheung and Nelson, 1996) mutation detection by single stranded conformational polymorphism (SSCP) and/or heteroduplex analysis (Barbaux et al., 2001). DOP-PCR was first described by Telenius et al. (1992) as a method for genome mapping studies that did not suffer from the limitations of IRS-PCR (Ledbetter et al., 1990) and was less technically challenging than LA-PCR (Lüdecke et al., 1989; Saunders et al., 1989). Since its conception, DOP-PCR has been adapted by several groups in order to lower the required amount of starting template (Hirose et al., 2001), to increase yield (Huang et al., 2000), fidelity and fragment length (Kittler et al., 2002), and to provide better genome coverage (Larsen et al., 2001; Huang et al., 2000; Kuukasjarvi et al., 1997). DOP-PCR uses a partially degenerate oligonucletoide primer that binds during several low-temperature annealing cycles at many sites in the genome (approximately 10^6 sites in humans). This pre-amplification step is followed by PCR amplification at a higher temperature to allow only those initial fragments that are tagged with the specific part of the primer sequence to be amplified.

There is considerable debate within reported studies regarding the possible limitations of DOP-PCR and its use in clinical applications. A comparison of the results obtained from genetic analysis of DOP-PCR amplified DNA with those from non-amplified DNA from the same specimens demonstrates that amplification provides good genome representation (Kim et al., 1999). In addition, Peng et al. state that "the sensitivity and specificity of CGH were scarcely influenced by preceding DOP-PCR amplification", but do acknowledge that there were differences in the quality of CGH microarray results. These have been attributed to different labelling methods as DOP-PCR labelling tended to give false positive results, while nick translation labelling of DOP-PCR products had limitations in detecting losses (Peng et al., 2003). Though its utility in SNP analysis has been proven (Jordan et al., 2002), Grant et al. note that

data clusters in SNP genotyping are not as tight, resulting in an increase in ambiguous calls for DOP-PCR amplified templates (error rate 0.7%). However, they believe that these errors (including unequal amplification and generation of extra alleles) could be attributed to the original quality of the DNA sample (Grant et al., 2002).

The amount and quality of the starting material used for DOP-PCR is an issue that has been addressed by several groups using this technique. In general it is agreed that starting with less than 1 ng of genomic DNA severely decreases the fidelity of the resulting experiment and increases the likelihood of allele dropout (Kittler et al., 2002) although some groups have successfully used less starting material (Huang et al., 2000). DNA extracted from fixed tissue has also been successfully DOP-amplified and used for subsequent analysis. Unfortunately issues regarding preferential amplification of alleles in DNA from fixed tissues have not yet been adequately addressed.

2.2. *Primer extension preamplification (PEP)*

PEP was first described by Zhang et al. (1992) who utilised PEP to amplify DNA from single sperm nuclei prior to analysis of specific genes. The original PEP protocol involves a 50 cycle PCR program using Taq polymerase and a 15 base random oligonucleotide primer. However, in contrast to a standard PCR, following denaturation the primers are allowed to anneal at a low stringency temperature (37°C), which is then gradually ramped (10 s/°C) up to 55°C prior to a 4 min elongation step (55°C). The choice to use random primers was based on the assumption that one primer extension product is just as likely to undergo primer extension during successive cycles as any other product. Through this work it was determined that via PEP approximately 78% of the genome from a single haploid cell can be copied at least 30 times (Zhang et al., 1992). It has been proposed that not all DNA sequences are capable of being amplified by PCR, possibly due to their repetitive nature or their sequence composition. Therefore the 22% of the genome that is not amplified by PEP could correspond to such regions. Over time, the PEP protocol has been optimised by utilisation of proof reading enzymes and altered PCR cycling conditions (improved (I) PEP, Dietmaier et al., 1999). These adaptations have allowed for greater WGA efficiency (Dietmaier et al., 1999; Heinmöller et al., 2002) from single cells and small cell clusters.

Since its conception, PEP has been used for many of the same applications as DOP-PCR and has allowed for multiple genetic analyses. However, compared to DOP-PCR, the results obtained from PEP have been reported to be more reliable in certain circumstances. The use of restriction fragment length polymorphism (RFLP) PCR to compare DOP-PCR, PEP and I-PEP, demonstrated that I-PEP has an increased efficiency for amplification of DNA from both individual cells (40% efficiency vs. 15% for standard PEP and 3% for DOP-PCR) as well as up to five cells (100% efficiency vs. 33% for standard PEP and 20% for DOP-PCR). In addition, WGA by I-PEP has allowed for 100% sequencing accuracy of greater than 4000 sequenced base pairs from Ki-ras (Dietmaier et al., 1999). A number of reports have highlighted the success of PEP using source DNA from both ethanol fixed paraffin embedded (EFPE) tissue (Heinmöller et al., 2002) and formalin fixed paraffin embedded (FFPE) tissue (Bataille et al., 2003; Heinmöller et al., 2003; Wang et al., 2001; Heinmöller et al., 2000; Dietmaier et al., 1999; Duddy et al., 1998). This is of particular interest to those researchers who are limited to the use of archival tissues fixed in this manner. Though microsatellite analysis can be performed on the DNA amplified from a single cell

from fixed tissue (Heinmöller et al., 2002), more reliable results are obtained from multiple cells (Dietmaier et al., 1999), or fresh tissue (Zheng et al., 2001).

One of the previously mentioned problems in the study of human cancers is the limitation on the number of tests that can be carried out on restricted quantities of DNA or individual cells. Advances in PEP-PCR have allowed for the most comprehensive allelotype analysis in pituitary tumours so far performed (Simpson et al., 2003). Analysis of 50 FFPE sporadic non-functioning pituitary tumours (25 non-invasive, 25 invasive) and 50 FFPE somatotrophinomas (25 non-invasive, 25 invasive) determined that LOH is more frequent in invasive tumours than in their non-invasive counterparts (Simpson et al., 2003). Not only is PEP suitable for the analysis of malignant cells, it can also be used for the study of microdissected premalignant cells, such as pancreatic intraductal lesions, which can possess many of the important genetic changes associated with pancreatic tumourigenesis (Heinmöller et al., 2003).

Although microsatellite analysis of PEP amplified DNA has permitted a refinement of LOH mapping in several tumours (ovarian cancer, Wang et al., 2001; cervical intraepithelial neoplasia, Chung et al., 2000; bladder cancer Simoneau et al., 1999; head and neck squamous lesions, Coombes et al., 1998) and allowed for the identification of chromosomal regions harbouring tumour suppressor genes (TSG), there are still specific limitations (see, Section 3). Like DOP-PCR, however, PEP is a relatively straightforward protocol that is easily implemented in most laboratory settings.

2.3. Ligation-mediated PCR (LMP)

Genetic analysis of small DNA samples using genomic representations (Lucito et al., 1998) is a method of WGA that has not been widely reported in the literature, likely due to the technical difficulties involved. However, this method, which involves ligating an adapter sequence on to a "representation" of DNA to facilitate subsequent PCR amplification, is showing new promise in the analysis of DNA samples.

Single Cell Comparative Genomic Hybridization (SCOMP) is a PCR-based strategy for global amplification of genomic DNA from a single cell (Klein et al., 1999). In principle it is very similar to the LA-PCR method described by Lüdecke et al. (1989) and Saunders et al. (1989). For SCOMP, genomic DNA (instead of individual chromosome bands) is digested with the restriction endonuclease, MseI, which transfers it to a high complexity representation (Lucito et al., 1998) with a fragment size distribution between 100 and 1500 base pairs. Two oligonucleotides are used in a pre-annealing step to form an adaptor complex that is then ligated to the overhangs on the genomic DNA fragments. By using a single primer for subsequent PCR amplification, the sequence complexities of multiple primer binding sites that are required by DOP-PCR and PEP techniques are avoided. The elegance of this protocol is that all steps including DNA isolation, restriction enzyme digestion, primer ligation and PCR amplification are performed in the same tube, which avoids template loss and excessive sample handling. Validation was achieved through successful metaphase CGH, LOH and sequence analysis to detect known DNA copy number aberrations and TP53 mutations (Klein et al., 1999). In addition, the breast cancer cell line MCF7 was analysed using interphase FISH and the results confirmed the heterogeneity detected in this cell line by CGH analysis. Comparison of DOP-PCR and SCOMP has determined that SCOMP is superior to DOP-PCR in eliminating PCR-based introduction of bias when using FFPE

samples combined with laser capture microdissection (Stoecklein et al., 2002). The use of SCOMP to generate sufficient DNA for CGH analysis of single disseminated cancer cells from the bone marrow of breast cancer patients has put forth compelling evidence that challenges the widely held view that the precursors of metastasis are derived from the most advanced clone within the primary tumour (Schmidt-Kittler et al., 2003). These studies highlight the importance of studying the genomic profiles of multiple single cells within a tumour to determine the genetic changes associated with progression and metastasis.

A further adaptation of this type of WGA has been to ligate primer sequences on to randomly sheared genomic DNA in a procedure termed adaptor-ligation-mediated PCR of randomly sheared genomic DNA (PRSG) (Tanabe et al., 2003). Tanabe and co-workers have used exon amplification and genotyping of 307 microsatellites, in addition to array CGH, to validate this method of WGA. The random shearing of the DNA may successfully mimic DNA that has been fixed prior to WGA.

As LMP has not yet been widely used for whole genome analysis, many of the problems associated with the technique, if they exist, will not yet have been reported. Though initial results from our lab and others are promising, a more thorough investigation of different tissue types and fixation conditions is required before it will be possible to determine relative advantages of LMP over other PCR based approaches.

Our group and others have been dissatisfied with the results produced using DOP-PCR and PEP (Sanchez-Cespedes et al., 1998; Hughes et al., in press). Imbalanced amplification of microsatellites (Cheung and Nelson, 1996) and SNPs (Grant et al., 2002; Paunio et al., 1996) as well as incomplete genome coverage (Dean et al., 2002) and failure to produce reliable CGH data (Wells et al., 1999) have all been observed. However, in contrast other groups have obtained satisfactory results (Kuukasjarvi et al., 1997; Umayahara et al., 2002). This discrepancy is likely a result of tissue handling and processing as well as the down stream application in which the amplified DNA is being used.

3. Non-PCR-based approaches

Though PCR-based WGA has been used for many applications, its shortcomings have been well reported. Protocols such as DOP-PCR and PEP amplify DNA in an exponential fashion and exponential amplification protocols are highly susceptible to bias and have a tendency to alter the information content of the DNA. In the worst case, this could cause regions of the genome to be over amplified or even lost. By the use of quantitative real time PCR Dean et al. (2002) have identified an amplification bias of 10^3–10^6 between genomic loci in PEP amplified products. In addition, PCR-based methods typically generate small products, usually 200 bp to 2 kb, which can limit some down stream applications. Furthermore, it has been acknowledged that DOP-PCR does not provide complete coverage of all loci (Paunio et al., 1996), or copy the target DNA in its entirety (Cheung and Nelson, 1996) and can preferentially amplify shorter alleles, Alu sequences and microsatellites (Wells et al., 1999). All of these factors can introduce experimental error with respect to LOH studies and SNP analysis. For these reasons, many researchers have switched to the use of non-PCR based linear amplification protocols. These protocols have a distinct advantage over PCR methods as they preserve sequence representation, do not introduce

sequence content bias and with reference to strand displacement amplification, provide good genome coverage (Dean et al., 2002).

3.1. Strand displacement amplification (SDA)

The process of SDA, or multiple displacement amplification (MDA, Lizardi, 2000) is based on the rolling circle amplification (RCA) type mechanism by which circular DNA molecules such as plasmids or viruses frequently replicate (Kornberg and Baker, 1992). This rolling circle amplification method was initially adapted for the amplification of large circular DNA templates (Dean et al., 2001) and more recently for the amplification of genomic DNA (Dean et al., 2002). Similar to other WGA approaches, no previous knowledge of the target template is required, however, in contrast to PCR-based methods the lengths of the DNA products following SDA are usually in excess of 10 kb.

SDA uses either phi29 DNA polymerase or a combination of Bst polymerase and T4 gene 32 protein in conjunction with modified random primers to amplify the entire genome (Dean et al., 2002; Lage et al., 2003). In contrast to PCR-based methods no repeated cycling is required. Instead a short initial (2–3 min) 94°C denaturation is followed by an enzyme specific incubation (phi29 30°C, Bst 50°C) step of 6–18 h and a final 65°C step of 15 min to inactivate the enzyme (Dean et al., 2002; Lage et al., 2003). The random primers first anneal to the denatured DNA and are then extended by thousands of bases using the single stranded DNA as template. The 5′ end of any extending strand is displaced by another upstream strand growing in the same direction (Fig. 1). The strand displacement reactions generate a hyperbranching mechanism that can produce hundreds or thousands of copies of the genome in only a few hours. As a result microgram quantities of DNA can be generated from as little as 5 ng of starting material, making it ideally suited for genomic analysis where starting DNA is limited.

It is important to note that phi29 polymerase has a greater SDA activity than the Bst polymerase/T4 gene 32 protein cocktail, and has been reported to introduce a significant level of under and over representation of sequences when compared to the enzyme cocktail (Lage et al., 2003). However, several groups that have used phi29 have not reported any inconsistencies in their results (Hosono et al., 2003; Lovmar et al., 2003; Tranah et al., 2003). In addition, recent work in our laboratory that has compared the two different approaches has generated near identical results.

To date, less than a dozen papers reporting the use of SDA for the study of human disease have been published. This is in stark contrast to the several dozen reporting the use of DOP-PCR and PEP. Despite this disparity, due largely to the recent introduction of this approach, there is data covering the use of SDA amplified DNA in array CGH (Hughes et al., in press; Lage et al., 2003), SNP analysis (Tranah et al., 2003), quantitative PCR (Hosono et al., 2003; Dean et al., 2002) and microsatellite analysis (Hosono et al., 2003).

A study of 47 loci in 44 patients by Hosono et al. (2003) identified an amplification bias ranging from 0.5 to 3 fold, which is in agreement with previous findings (Dean et al., 2002). It was also determined from their study that amplified DNA produced results indistinguishable from unamplified genomic DNA. Unfortunately they only assayed 5 SNPs and 10 microsatellites, which is hardly a comprehensive evaluation. Nevertheless, the results are promising. In addition, they were able to achieve successful SDA directly from clinical specimens, such as whole blood,

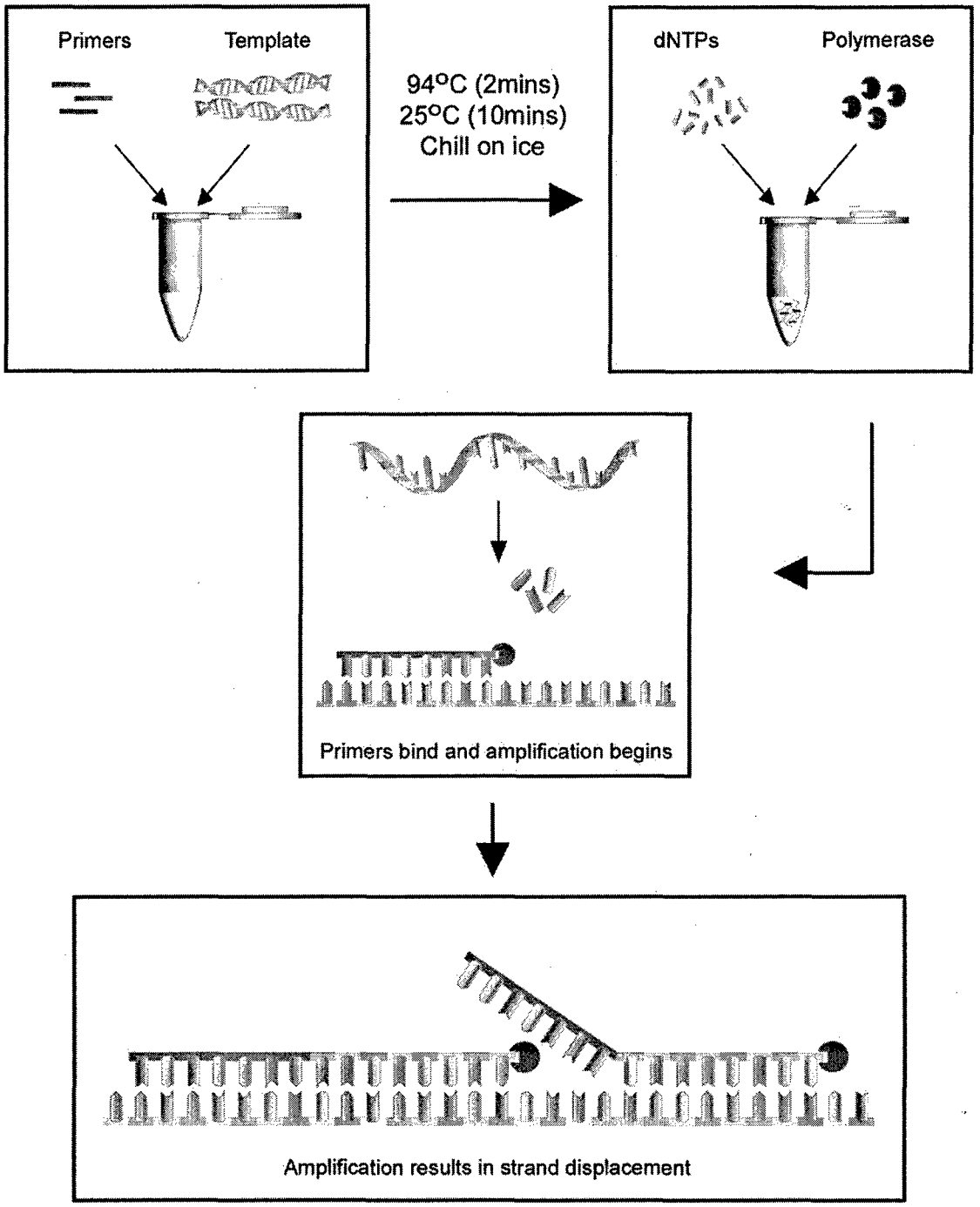


Fig. 1. Strand displacement amplification. Primers anneal at multiple sites on the template DNA. The polymerase initiates primer extension and new strand synthesis. As synthesis progresses, the 5′ end of the extending strand is displaced by another upstream strand extending in the same direction.

buccal cells and buffy coats, which eliminates the requirement for time-consuming DNA extraction processes.

So far, only three studies have reported a direct comparison between PCR-based methods and SDA. Dean et al. (2002) using TaqMan analysis, determined an amplification bias for tested loci of approximately 3-fold for SDA and up to 10^4 for PEP and 10^6 for DOP-PCR. In addition, an assessment of PEP amplified, SDA amplified and unamplified DNA using 45 SNPs established that PEP only showed 88.7% concordance with unamplified DNA, while SDA demonstrated 99.7% concordance (Lovmar et al., 2003). Finally, using array CGH we have determined that the results obtained with DOP-PCR amplified DNA are less reliable than those generated using SDA (Hughes et al., in press).

Unfortunately, papers reporting the use of SDA have so far concentrated more on an evaluation of the technique rather than a more extensive patient study. Current work in our laboratory suggests that the fidelity of SDA when combined with LCM and array CGH can allow for the detection of copy number abnormalities from a small number of starting cells.

3.2. T7-based linear amplification of DNA (TLAD)

The use of linear amplification strategies has been demonstrated by mRNA expression analysis to substantially reduce amplification bias (Iscove et al., 2002; Zhao et al., 2002; Wang et al., 2000; Phillips and Eberwine, 1996). However, until recently this type of approach had not been applied to the study of genomic DNA, due to the absence of an appropriate tag such as the polyA tail present on mRNA molecules. Liu et al. (2003) has devised a linear amplification protocol for genomic DNA, which will be applicable to the amplification of DNA of varying fragment size and quality. This will be of particular use in the study of the DNA extracted from FFPE tissue, as the quality of the DNA is often poor.

T7-based linear amplification is capable of generating microgram quantities of genomic DNA from as little as 2.5 ng of input DNA, while maintaining the variation in fragment size present in the starting material. TLAD is based on a protocol devised by Phillips and Eberwine (1996) for the amplification of mRNA for use on cDNA microarrays. For TLAD, the DNA is first digested with the restriction endonuclease, AluI. Terminal transferase is then used to add polyT tails to the 3′ ends of the digested DNA strands. An oligonucleotide primer, containing a 5′ T7 promoter and a 3′ polyA tract, is annealed to the genomic fragments, and Klenow is used for synthesis of the second strand. The reaction products of this synthesis are then used as templates for the in vitro transcription reaction. One of the major advantages of TLAD is that it does not introduce the sequence and length-dependent biases observed in PCR-based amplification. One of the major disadvantages is the requirement for sample purification following each step. This makes the protocol cumbersome and time-consuming and can result in sample loss.

To date TLAD has only been validated by the analysis of yeast genomic DNA obtained following chromatin immunoprecipitation (ChIP) (Liu et al., 2003), so it is of interest as to whether this approach will be appropriate for the study of human diseases. In our laboratory we have been able to successfully use TLAD for the generation of DNA for array CGH (unpublished data). The CGH profiles generated from amplified and non-amplified genomic DNA extracted from a well-characterized cell line show the same patterns of genomic alterations.

As was mentioned previously for LMP, more extensive investigation into the reliability of TLAD will be required before it will be generally accepted as an approach for faithful WGA.

4. Conclusions

Genetic alterations in cancer often involve loss or gain of chromosomal regions. Substantial research effort has been directed towards identifying the changes in a cell genome that occur during the development and progression of numerous tumour types. These analyses have focused upon different tumour stages in an attempt to determine which genomic changes occur early in the tumour development, which changes are accumulated during progression and which occur that may facilitate metastasis. In order to do this, it has been necessary to analyse DNA from cells in all the different stages. This has led to two problems, firstly if the DNA is limited, as is often the case when studying premalignant lesions (Chung et al., 2000; Heinmöller et al., 2003), or patient samples (Zheng et al., 2001), only a restricted number of assays can be performed. Secondly when studying heterogenous tissue, if larger quantities of "bulk extracted" genomic DNA are used, key genetic alterations that could be of major interest, may be masked by the contaminating normal cells present within the sample. These problems have led researchers to use WGA, often in conjunction with microdissection, to obtain sufficient amounts of DNA, extracted from pure cell populations, for genome analysis. Microdissection can be used to obtain a single cell or several thousands cells, however at best the yield is only nanograms of DNA. The limitations imposed by insufficient DNA can be overcome by use of WGA techniques that permit apparent unbiased amplification across the genome, thus opening up the possibility for new scientific approaches.

Despite their differences, WGA techniques all work on the premise of being able to generate microgram quantities of DNA, depending upon the method used, from as little as one cell's worth of DNA (Hirose et al., 2001). Though satisfactory results have been obtained from one cell using PEP, unequal allelic amplification has been observed (Faulkner and Leigh, 1998; Barrett et al., 1995). A possible explanation for this is the fact that between 10% and 20% of the genetic material from a single nucleus can be lost during tissue sectioning (Heinmöller et al., 2002) and subsequent LCM. In addition, fixation of tissues can introduce sequence variations and reduce overall DNA quality (Williams et al., 1999). As a result most researchers use a minimum of 10–1000 cells, depending on tissue type (Heinmöller et al., 2002), fixation method (Dietmaier et al., 1999) and mode of WGA (Lage et al., 2003; Lovmar et al., 2003; Dean et al., 2002; Zheng et al., 2001).

When considering the use of WGA it is important to take into account the entire experimental process, i.e. tissue accrual, fixation, sectioning, staining and dissection. All of these factors can effect DNA quality and thus have some bearing on the choice of WGA methodology (Ottesen et al., 2003; Heinmöller et al., 2002; Dietmaier et al., 1999). For instance, SDA, which in comparison to other methods outlined in this review provides good genome coverage and does not introduce significant amplification bias (Dean et al., 2002), has a requirement for good quality high molecular weight DNA usually obtained from fresh tissue. As a result, the poor quality DNA often extracted from fixed tissue will not be suitable for this approach (Lage et al., 2003). In contrast PCR-based methods and TLAD are less affected by DNA quality and are more applicable to all DNA sources. However, the use of PCR-based methods has its own inherent

problems. When studying tissue sections, prior examination of the quality of DNA in the unrequired regions of the section may indicate the appropriate WGA approach to use.

While many researchers recognize that WGA in all its various forms has a range of limitations, very few address them in terms of their own results, likely due to the difficulty in assessing the error rate that may be associated with WGA methods. In this context it is important to remember that WGA is sometimes the only means to significantly increase the amount of data that can be derived from limited clinical samples. If researchers are aware of the possible problems and correctly deal with the potential for missing data, WGA has the prospect of significantly expanding our knowledge of human diseases where the quantity of DNA is a limiting factor.

Acknowledgements

We would like to thank Dr. Rod Bremner, Dr. Johanne Weberpals, Mr. Ilan Braude and Miss Catherine Jones for their critical review of this manuscript. Financial support for this work is provided by the US Army Medical Research and Materiel Command Prostate Cancer Research Program (Research fellowship PC020888).

References

Ao, A., Wells, D., Handyside, A.H., Winston, R.M., Delhanty, J.D., 1998. Preimplantation genetic diagnosis of inherited cancer: familial adenomatous polyposis coli. J. Assist. Reprod. Genet. 15, 140–144.

Barbaux, S., Poirier, O., Cambien, F., 2001. Use of degenerate oligonucleotide primed PCR (DOP-PCR) for the genotyping of low-concentration DNA samples. J. Mol. Med. 79, 329–332.

Barrett, M.T., Reid, B.J., Joslyn, G., 1995. Genotypic analysis of multiple loci in somatic cells by whole genome amplification. Nucl. Acids Res. 23, 3488–3492.

Bataille, F., Rummele, P., Dietmaier, W., Gaag, D., Klebl, F., Reichle, A., Wild, P., Hofstadter, F., Hartmann, A., 2003. Alterations in p53 predict response to preoperative high dose chemotherapy in patients with gastric cancer. Mol. Pathol. 56, 286–292.

Bates, A.S., Farrell, W.E., Bicknell, E.J., McNicol, A.M., Talbot, A.J., Broome, J.C., Perrett, C.W., Thakker, R.V., Clayton, R.N., 1997. Allelic deletion in pituitary adenomas reflects aggressive biological activity and has potential value as a prognostic marker. J. Clin. Endocrinol. Metab. 82, 818–824.

Beheshti, B., Vukovic, B., Marrano, P., Squire, J.A., Park, P.C., 2002. Resolution of genotypic heterogeneity in prostate tumors using polymerase chain reaction and comparative genomic hybridization on microdissected carcinoma and prostatic intraepithelial neoplasia foci. Cancer Genet. Cytogenet. 137, 15–22.

Cheung, V.G., Nelson, S.F., 1996. Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. Proc. Natl. Acad Sci. USA 93, 14676–14679.

Chung, T.K., Cheung, T.H., Lo, W.K., Yu, M.Y., Hampton, G.M., Wong, H.K., Wong, Y.F., 2000. Loss of heterozygosity at the short arm of chromosome 3 in microdissected cervical intraepithelial neoplasia. Cancer Lett. 154, 189–194.

Coombes, M.M., Mao, L., Steck, K.D., Luna, M A., El-Naggar, A.K., 1998. Genotypic analysis of flow-sorted and microdissected head and neck squamous lesions by whole-genome amplification. Diagn. Mol. Pathol. 7, 197–201.

Daigo, Y., Chin, S.F., Gorringe, K.L., Bobrow, L.G., Ponder, B.A., Pharoah, P.D., Caldas, C., 2001. Degenerate oligonucleotide primed-polymerase chain reaction-based array comparative genomic hybridization for extensive amplicon profiling of breast cancers: a new approach for the molecular analysis of paraffin-embedded cancer tissue. Am. J. Pathol. 158, 1623–1631.

Dean, F.B., Nelson, J.R., Giesler, T.L., Lasken, R.S., 2001. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply primed rolling circle amplification. Genome Res. 11, 1095–1099.

Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M., Lasken, R.S., 2002. Comprehensive human genome amplification using multiple displacement amplification. Proc. Natl. Acad. Sci. USA 99, 5261–5266.

Dietmaier, W., Hartmann, A., Wallinger, S., Heinmoller, E., Kerner, T., Endl, E., Jauch, K.W., Hofstadter, F., Ruschoff, J., 1999. Multiple mutation analyses in single tumor cells with improved whole genome amplification. Am. J. Pathol. 154, 83–95.

Duddy, S.K., Gorospe, S., Bleavins, M.R., 1998. Genetic analysis of multiple loci in microsamples of fixed paraffin-embedded tissue. Toxicol. Sci. 46, 317–323.

Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettel, M., Coleman, P., 1992. Analysis of gene expression in single live neurons. Proc. Natl. Acad. Sci. USA 89, 3010–3014.

Faulkner, S.W., Leigh, D.A., 1998. Universal amplification of DNA isolated from small regions of paraffin-embedded, formalin-fixed tissue. Biotechniques 24, 47–50.

Grant, S.F., Steinlicht, S., Nentwich, U., Kern, R., Burwinkel, B., Tolle, R., 2002. SNP genotyping on a genome-wide amplified DOP-PCR template. Nucl. Acids Res. 30, e125.

Harada, T., Okita, K., Shiraishi, K., Kusano, N., Furuya, T., Oga, A., Kawauchi, S., Kondoh, S., Sasaki, K., 2002. Detection of genetic alterations in pancreatic cancers by comparative genomic hybridization coupled with tissue microdissection and degenerate oligonucleotide primed polymerase chain reaction. Oncology 62, 251–258.

Heinmöller, E., Dietmaier, W., Zirngibl, H., Heinmoller, P., Scaringe, W., Jauch, K.W., Hofstadter, F., Ruschoff, J., 2000. Molecular analysis of microdissected tumors and preneoplastic intraductal lesions in pancreatic carcinoma. Am. J. Pathol. 157, 83–92.

Heinmöller, E., Liu, Q., Sun, Y., Schlake, G., Hill, K.A., Weiss, L.M., Sommer, S.S., 2002. Toward efficient analysis of mutations in single cells from ethanol-fixed, paraffin-embedded, and immunohistochemically stained tissues. Lab. Invest. 82, 443–453.

Heinmöller, E., Bockholt, A., Werther, M., Ziemer, M., Muller, A., Ghadimi, B.M., Ruschoff, J., 2003. Laser microdissection of small tissue samples—application to chronic pancreatitis tissues. Pathol. Res. Pract. 199, 363–371.

Hirose, Y., Aldape, K., Takahashi, M., Berger, M.S., Feuerstein, B.G., 2001. Tissue microdissection and degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) is an effective method to analyze genetic aberrations in invasive tumors. J. Mol. Diagn. 3, 62–67.

Hosono, S., Faruqi, A.F., Dean, F.B., Du, Y., Sun, Z., Wu, X., Du, J., Kingsmore, S.F., Egholm, M., Lasken, R.S., 2003. Unbiased whole-genome amplification directly from clinical samples. Genome Res. 13, 954–964.

Huang, Q., Schantz, S.P., Rao, P.H., Mo, J., McCormick, S.A., Chaganti, R.S., 2000. Improving degenerate oligonucleotide primed PCR-comparative genomic hybridization for analysis of DNA copy number changes in tumors. Genes Chromosomes Cancer 28, 395–403.

Hughes, S., Lim, G., Beheshti, B., Bayani, J., Marrano, P., Huang, A., Squire, J.A., Use of Whole Genome Amplification and Comparative Genomic Hybridisation to detect chromosomal copy number alterations in cell line material and tumour tissue. Cytogenet. Genome Res., in press.

Iscove, N.N., Barbara, M., Gu, M., Gibson, M., Modi, C., Winegarden, N., 2002. Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. Nat. Biotechnol. 20, 940–943.

Jiao, Z., Zhou, C., Li, J., Shu, Y., Liang, X., Zhang, M., Zhuang, G., 2003. Birth of healthy children after preimplantation diagnosis of beta-thalassemia by whole-genome amplification. Prenat. Diagn. 23, 646–651.

Jordan, B., Charest, A., Dowd, J.F., Blumenstiel, J.P., Yeh Rf, R.F., Osman, A., Housman, D.E., Landers, J.E., 2002. Genome complexity reduction for SNP genotyping analysis. Proc. Natl. Acad. Sci. USA 99, 2942–2947.

Kim, S.H., Godfrey, T., Jensen, R.H., 1999. Whole genome amplification and molecular genetic analysis of DNA from paraffin-embedded prostate adenocarcinoma tumor tissue. J. Urol. 162, 1512–1518.

Kinzler, K.W., Vogelstein, B., 1989. Whole genome PCR: application to the identification of sequences bound by gene regulatory proteins. Nucl. Acids Res. 17, 3645–3653.

Kittler, R., Stoneking, M., Kayser, M., 2002. A whole genome amplification method to generate long fragments from low quantities of genomic DNA. Anal. Biochem. 300, 237–244.

Klein, C.A., Schmidt-Kittler, O., Schardt, J.A., Pantel, K., Speicher, M.R., Riethmuller, G., 1999. Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. Proc. Natl. Acad. Sci. USA 96, 4494–4499.

Kolomietz, E., Meyn, M.S., Pandita, A., Squire, J.A., 2002. The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors. Genes Chromosomes Cancer 35, 97–112.

Korenberg, J.R., Rykowski, M.C., 1988. Human genome organization: Alu, lines, and the molecular structure of metaphase chromosome bands. Cell 53, 391–400.

Kornberg, A., Baker, T.A., 1992. DNA replication. W.H. Freeman and Company, San Francisco.

Kristjansson, K., Chong, S.S., Van den Veyver, I.B., Subramanian, S., Snabes, M.C., Hughes, M.R., 1994. Preimplantation single cell analyses of dystrophin gene deletions using whole genome amplification. Nat. Genet. 6, 19–23.

Kuukasjarvi, T., Tanner, M., Pennanen, S., Karhu, R., Visakorpi, T., Isola, J., 1997. Optimizing DOP-PCR for universal amplification of small DNA samples in comparative genomic hybridization. Genes Chromosomes Cancer 18, 94–101.

Lage, J.M., Leamon, J.H., Pejovic, T., Hamann, S., Lacey, M., Dillon, D., Segraves, R., Vossbrinck, B., Gonzalez, A., Pinkel, D., Albertson, D.G., Costa, J., Lizardi, P.M., 2003. Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. Genome Res. 13, 294–307.

Larsen, J., Ottesen, A.M., Lundsteen, C., Leffers, H., Larsen, J.K., 2001. Optimization of DOP-PCR amplification of DNA for high-resolution comparative genomic hybridization analysis. Cytometry 44, 317–325.

Ledbetter, S.A., Nelson, D.L., Warren, S.T., Ledbetter, D.H., 1990. Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence polymerase chain reaction. Genomics 6, 475–481.

Liu, C.L., Schreiber, S.L., Bernstein, B.E., 2003. Development and validation of a T7 based linear amplification for genomic DNA. BMC Genomics 4, 19.

Lizardi, P.M., 2000. Multiple displacement amplification. United States Patent 6, 123,120.

Lovmar, L., Fredriksson, M., Liljedahl, U., Sigurdsson, S., Syvanen, A.C., 2003. Quantitative evaluation by minisequencing and microarrays reveals accurate multiplexed SNP genotyping of whole genome amplified DNA. Nucl. Acids Res. 31, e129.

Lucito, R., Nakimura, M., West, J.A., Han, Y., Chin, K., Jensen, K., McCombie, R., Gray, J.W., Wigler, M., 1998. Genetic analysis using genomic representations. Proc. Natl. Acad. Sci. USA 95, 4487–4492.

Lüdecke, H.J., Senger, G., Claussen, U., Horsthemke, B., 1989. Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification. Nature 338, 348–350.

Martel-Petit, V., Petit, C., Marchand, M., Fleurentin, A., Fontaine, B., Miton, A., Lemarie, P., Philippe, C., Jonveaux, P., 2001. Use of the Kleihauer test to detect fetal erythroblasts in the maternal circulation. Prenat. Diagn. 21, 106–111.

Nelson, D.L., Ledbetter, S.A., Corbo, L., Victoria, M.F., Ramirez-Solis, R., Webster, T.D., Ledbetter, D.H., Caskey, C.T., 1989. Alu polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. Proc. Natl. Acad. Sci. USA 86, 6686–6690.

Ottesen, A.M., Skakkebaek, N.E., Lundsteen, C., Leffers, H., Larsen, J., Rajpert-De Meyts, E., 2003. High-resolution comparative genomic hybridization detects extra chromosome arm 12p material in most cases of carcinoma in situ adjacent to overt germ cell tumors, but not before the invasive tumor development. Genes Chromosomes Cancer 38, 117–125.

Paunio, T., Reima, I., Syvanen, A.C., 1996. Preimplantation diagnosis by whole-genome amplification, PCR amplification, and solid-phase minisequencing of blastomere DNA. Clin. Chem. 42, 1382–1390.

Peng, D.F., Sugihara, H., Mukaisho, K., Tsubosa, Y., Hattori, T., 2003. Alterations of chromosomal copy number during progression of diffuse-type gastric carcinomas: metaphase- and array-based comparative genomic hybridization analyses of multiple samples from individual tumours. J. Pathol. 201, 439–450.

Phillips, J., Eberwine, J.H., 1996. Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells. Methods 10, 283–288.

Sanchez-Cespedes, M., Cairns, P., Jen, J., Sidransky, D., 1998. Degenerate oligonucleotide-primed PCR (DOP-PCR): evaluation of its reliability for screening of genetic alterations in neoplasia. Biotechniques 25, 1036–1038.

Saunders, R.D., Glover, D.M., Ashburner, M., Siden-Kiamos, I., Louis, C., Monastirioti, M., Savakis, C., Kafatos, F., 1989. PCR amplification of DNA microdissected from a single polytene chromosome band: a comparison with conventional microcloning. Nucl. Acids Res. 17, 9027–9037.

Schmidt-Kittler, O., Ragg, T., Daskalakis, A., Granzow, M., Ahr, A., Blankenstein, T.J., Kaufmann, M., Diebold, J., Arnholdt, H., Muller, P., Bischoff, J., Harich, D., Schlimok, G., Riethmuller, G., Eils, R., Klein, C.A., 2003. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. Proc. Natl. Acad. Sci. USA 100, 7737–7742.

Sekizawa, A., Watanabe, A., Kimura, T., Saito, H., Yanaihara, T., Sato, T., 1996. Prenatal diagnosis of the fetal RhD blood type using a single fetal nucleated erythrocyte from maternal blood. Obstet. Gynecol. 87, 501–505.

Sermon, K., Lissens, W., Joris, H., Van Steirteghem, A., Liebaers, I., 1996. Adaptation of the primer extension preamplification (PEP) reaction for preimplantation diagnosis: single blastomere analysis using short PEP protocols. Mol. Hum. Reprod. 2, 209–212.

Simoneau, M., Aboulkassim, T.O., LaRue, H., Rousseau, F., Fradet, Y., 1999. Four tumor suppressor loci on chromosome 9q in bladder cancer: evidence for two novel candidate regions at 9q22.3 and 9q31. Oncogene 18, 157–163.

Simpson, D.J., Bicknell, E.J., Buch, H.N., Cutty, S.J., Clayton, R.N., Farrell, W.E., 2003. Genome-wide amplification and allelotyping of sporadic pituitary adenomas identify novel regions of genetic loss. Genes Chromosomes Cancer 37, 225–236.

Stoecklein, N.H., Erbersdobler, A., Schmidt-Kittler, O., Diebold, J., Schardt, J.A., Izbicki, J.R., Klein, C.A., 2002. SCOMP is superior to degenerated oligonucleotide primed-polymerase chain reaction for global amplification of minute amounts of DNA from microdissected archival tissue samples. Am. J. Pathol. 161, 43–51.

Tanabe, C., Aoyagi, K., Sakiyama, T., Kohno, T., Yanagitani, N., Akimoto, S., Sakamoto, M., Sakamoto, H., Yokota, J., Ohki, M., Terada, M., Yoshida, T., Sasaki, H., 2003. Evaluation of a whole-genome amplification method based on adaptor-ligation PCR of randomly sheared genomic DNA. Genes Chromosomes Cancer 38, 168–176.

Telenius, H., Carter, N.P., Bebb, C.E., Nordenskjold, M., Ponder, B.A., Tunnacliffe, A., 1992. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 13, 718–725.

Thakker, R.V., Pook, M.A., Wooding, C., Boscaro, M., Scanarini, M., Clayton, R.N., 1993. Association of somatotrophinomas with loss of alleles on chromosome 11 and with GSP mutations. J. Clin. Invest. 91, 2815–2821.

Tranah, G.J., Lescault, P.J., Hunter, D.J., De Vivo, I., 2003. Multiple displacement amplification prior to single nucleotide polymorphism genotyping in epidemiologic studies. Biotechnol. Lett. 25, 1031–1036.

Umayahara, K., Numa, F., Suehiro, Y., Sakata, A., Nawata, S., Ogata, H., Suminami, Y., Sakamoto, M., Sasaki, K., Kato, H., 2002. Comparative genomic hybridization detects genetic alterations during early stages of cervical cancer progression. Genes Chromosomes Cancer 33, 98–102.

Verhagen, P.C., Zhu, X.L., Rohr, L.R., Cannon-Albright, L.A., Tavtigian, S.V., Skolnick, M.H., Brothman, A.R., 2000. Microdissection, DOP-PCR, and comparative genomic hybridization of paraffin-embedded familial prostate cancers. Cancer Genet. Cytogenet. 122, 43–48.

Wang, E., Miller, L.D., Ohnmacht, G.A., Liu, E.T., Marincola, F.M., 2000. High-fidelity mRNA amplification for gene profiling. Nat. Biotechnol. 18, 457–459.

Wang, V.W., Bell, D.A., Berkowitz, R.S., Mok, S.C., 2001. Whole genome amplification and high-throughput allelotyping identified five distinct deletion regions on chromosomes 5 and 6 in microdissected early stage ovarian tumors. Cancer Res. 61, 4169–4174.

Wells, D., Sherlock, J.K., Handyside, A.H., Delhanty, J.D., 1999. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. Nucl. Acids Res. 27, 1214–1218.

Williams, C., Ponten, F., Moberg, C., Soderkvist, P., Uhlen, M., Ponten, J., Sitbon, G., Lundeberg, J., 1999. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am. J. Pathol. 155, 1467–1471.

Zhang, L., Cui, X., Schmitt, K., Hubert, R., Navidi, W., Arnheim, N., 1992. Whole genome amplification from a single cell: implications for genetic analysis. Proc. Natl. Acad. Sci. USA 89, 5847–5851.

Zhao, H., Hastie, T., Whitfield, M.L., Borresen-Dale, A.L., Jeffrey, S.S., 2002. Optimization and evaluation of T7 based RNA linear amplification protocols for cDNA microarray analysis. BMC Genomics 3, 31.

Zheng, S., Ma, X., Buffler, P.A., Smith, M.T., Wiencke, J.K., 2001. Whole genome amplification increases the efficiency and validity of buccal cell genotyping in pediatric populations. Cancer Epidemiol. Biomarkers Prev. 10, 697–700.