

AD_____

Award Number: W81XWH-08-1-0636

TITLE: Premalignant Genetic and Epigenetic Alterations in Tubal Epithelium from
Women with BRCA1 Mutations

PRINCIPAL INVESTIGATOR: Anton Krumm

CONTRACTING ORGANIZATION: University of Washington
Seattle WA 98195-9472

REPORT DATE: October 2009

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.				
1. REPORT DATE (DD-MM-YYYY) 01-10-2009		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 15 Sept 2008 - 14 Sept 2009
4. TITLE AND SUBTITLE Premalignant Genetic and Epigenetic Alterations in Tubal Epithelium from Women with BRCA1 Mutations		5a. CONTRACT NUMBER		
		5b. GRANT NUMBER W81XWH-08-1-0636		
		5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Anton Krumm Email: akrumm@u.washington.edu		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Washington Seattle WA 98195-9472		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Material Command Fort Detrick Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT Increasing evidence suggests that many types of ovarian cancers originate within the fallopian tube. The scope of this Translational Partnership project is to define a unique premalignant gene expression profile and to identify causal epigenetic relationships. As outlined in the statement of work, our analyses have identified a premalignant expression signature which potentially reflects early steps in ovarian carcinogenesis. While genes differentially expressed in BRCA1 normal Fallopian Tube epithelia and BRCA1 ovarian carcinoma were investigated in the Swisher lab, we have further established the proof-of-principle that demonstrates the role of DNA methylation of CTCF binding sites in cancer tissues. We gathered evidence that genes that are part of the premalignant signature are differentially methylated in ovarian cancer cell lines. We have begun to isolate and pool samples by laser-capture microscopy. In the coming year, we will perform extensive mapping of DNA methylation to precisely determine epigenetic changes that affect the normal expression of genes in ovarian cancer.				
15. SUBJECT TERMS Ovarian Cancer, chromatin, DNA methylation, transcription factor				
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-5
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	5
References.....	6
Appendices.....	7-9

Premalignant Genetic and Epigenetic Alterations in Tubal Epithelium from Women with BRCA1 Mutations

OC073389 Progress Report October 8, 2009

INTRODUCTION

The development of novel strategies and treatment methods for ovarian cancer, the most lethal of gynecological cancers, has been hindered by the lack of understanding of the biology of malignant transformation in the ovary. Previous evidence led to the hypothesis that many ovarian cancers originate within the fallopian tube. However, the precise mechanism of aberrant gene expression profiles remained unknown. In the first half of this funded 2-year project, Dr. Swisher's lab has identified candidate genes that may contribute to early carcinogenesis using fallopian tube tissue from women with BRCA1 mutations. To identify the underlying cause and mechanism for deregulated expression of this subset of genes, we initiated experiments to define the epigenetic status of this gene set. In the first year we established experimental approaches capable of identifying differential methylation patterns at specific gene loci using small cell/tissue samples similar to those that are derived from laser capture microscopy (LCM).

BODY

Comparison of gene expression in normal and cancerous tubal epithelia from both BRCA1 mutation carriers and control groups revealed a surprisingly low number of deregulated gene loci. In total, 41 concordant overlapping probe sets identified 29 genes which were upregulated and 12 which were downregulated. Previous work in several labs including ours has revealed that epigenetic aberrations including methylation of CTCF target sites are an early and key event in carcinogenesis [[1], reviewed in [2]]. CpG methylation of CTCF target motifs inhibits binding of CTCF and permits spreading of DNA methylation and subsequent silencing of genes such as tumor suppressor genes [3]. While gene profiling studies in laser captured cells were ongoing in year one, we tested this model at the HOX gene locus in cancer cell lines established from ovarian, breast and prostate tissue. The HOXA gene cluster is a family of homeotic genes that encode transcription factors which are frequently inactivated in cancer cell types.

The HOX gene domain contains several CTCF sites (hx1-hx5) previously identified in our ChIP-Chip in the breast epithelial cell line HBL100. Importantly, CTCF binding at hx1 is absent in the prostate epithelial cell line PC3 [4]. Genomic sequencing of the hx1 region revealed complete sequence identity at this site in HBL100 and PC3 cells, suggesting that epigenetic mechanisms account for the loss of CTCF binding in PC3 prostate cancer cells. Using a combination of methylation-sensitive restriction enzymes and PCR we analyzed the level of DNA methylation in the ovarian cancer cell line A2780 and compared it to the prostate cancer cell lines PC3 and C4-2. These experiments identified DNA methylation at the hx1 binding site in A2780 and PC3 cells but not in HBL100 cells and C4-2 cells. Most importantly, these preliminary studies reveal a complete correlation of DNA methylation and loss of CTCF binding; while hx1 in the prostate cell line C4-2 is both unmethylated and bound by CTCF, hx1 in the A2780 and PC3 cell lines is methylated and not bound by CTCF. These data further support our hypothesis that epigenetic mechanisms and loss of CTCF binding contribute to reprogramming of gene expression during disease progression.

To begin to address the potential role of CTCF binding and DNA methylation in deregulation of those genes identified by the Swisher lab, we scanned the genomic regions harboring the candidate genes for known CTCF binding sites. Importantly, the majority of the candidate genes is associated with one or more CTCF sites in a sequence space of 100 kb surrounding candidate loci. For only six loci is the closest CTCF binding site located more than 100 kb away. The distribution of CTCF sites across the subset of premalignant signature genes is similar to the distribution found genome-wide: About one half of CTCF sites are located in intergenic regions, with an average distance of approximately 47 kb. About 20% CTCF sites are located at transcription start sites,

and 34% are located within introns and exons. Three examples of loci with CTCF binding sites in the vicinity of the genes under study are shown in Figure 3.

To obtain initial data on differential binding of CTCF at premalignant signature genes, we performed methylation-sensitive PCR on genomic DNA from several cancer cell lines including the ovarian cancer cell line OVCAR3. This analysis takes advantage of the methylation-sensitive restriction enzyme *AciI* that digests only unmethylated genomic regions, eliminating templates for subsequent PCR. Thus, while unmethylated regions yield no PCR product, methylated regions are protected from restriction digest and produce amplified DNA fragments. Using this approach, we investigated the methylation status at PAK3, JAG1, and LOC388798 gene loci. As shown in Figure 3, the CTCF binding region in PAK3 is methylated in the ovarian cancer cell line OVCAR3 but is un-methylated in the prostate cell line LnCaP. In contrast, our analyses at the LOC388798 on chromosome 20 revealed that this region is unmethylated in all cell lines tested.

KEY RESEARCH ACCOMPLISHMENTS

- Within the list of genes that constitute our premalignant signature for ovarian cancer, we identified genomic loci that contain potential target sites for CTCF binding and/or DNA methylation.
- We generated proof-of-principle data that demonstrate the influence of cancer-specific DNA methylation patterns with loss of CTCF binding at the HOX loci.
- We provided initial evidence for antagonistic action of DNA methylation and CTCF binding at several loci in our premalignant signature. These results suggest that an extensive survey of epigenetic changes at ovarian cancer related genes will yield important results.

CONCLUSIONS

Our analyses have identified a premalignant expression signature which potentially reflects early steps in ovarian carcinogenesis. While genes differentially expressed in BRCA1 normal fallopian tube epithelia and BRCA1 ovarian carcinoma were identified in the Swisher lab, we have further established the proof-of-principle that demonstrates the role of DNA methylation of CTCF binding sites in cancer tissues. Importantly, we already gathered evidence that at least some of the genes that are part of the premalignant signature are differentially methylated in ovarian cancer cell lines. These data strongly encourage us to pursue an extensive survey of DNA methylation and CTCF binding in laser-captured tissues, as was proposed in our application. Thus, we have begun to isolate and pool samples for laser-capture microscopy. In the coming year, we will perform extensive mapping of DNA methylation to determine epigenetic changes that affect the normal expression of signature genes in ovarian cancer.

REPORTABLE OUTCOMES

As epigenetic analyses of this Translational Partnership grant were set to begin at the end of year one, manuscripts or abstracts on the epigenetics of ovarian cancer have not been submitted.

REFERENCES

1. Witcher, M. and B.M. Emerson, *Epigenetic silencing of the p16(INK4a) tumor suppressor is associated with loss of CTCF binding and a chromatin boundary*. Mol Cell, 2009. **34**(3): p. 271-84.
2. Phillips, J.E. and V.G. Corces, *CTCF: master weaver of the genome*. Cell, 2009. **137**(7): p. 1194-211.
3. Gombert, W.M. and A. Krumm, *Targeted deletion of multiple CTCF-binding elements in the human C-MYC gene reveals a requirement for CTCF in C-MYC expression*. PLoS One, 2009. **4**(7): p. e6109.
4. Rubio, E.D., et al., *CTCF physically links cohesin to chromatin*. Proc Natl Acad Sci U S A, 2008. **105**(24): p. 8309-14.

SUPPORTING DATA

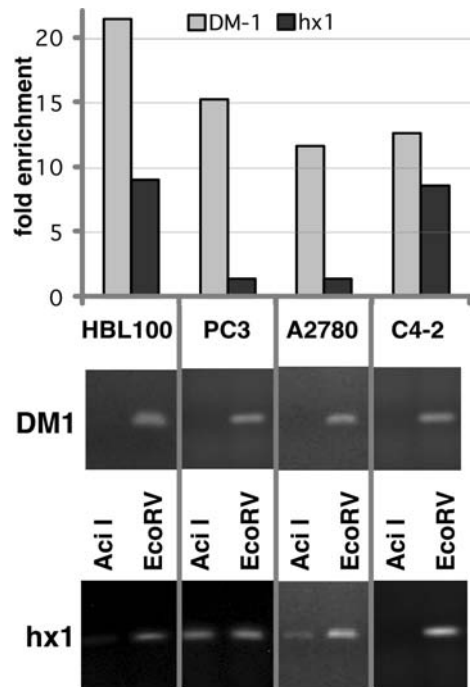


Figure 1. DNA methylation-sensitive binding of CTCF at the HoxA locus. Upper panel, enrichment of CTCF bound sequences at the DM1 locus (grey bars) and the hx1 binding site (black bars) in ChIP in breast epithelial cell type HBL100, ovarian cancer cell line A2780, and prostate cancer cell lines PC3 and C4-2. While CTCF is bound to the DM1 locus in all cell lines (grey bars), it fails to bind to the hx1 site at the HoxA gene domain in PC3 and A2780 (black bars). Lower panel, DNA methylation analysis by PCR of genomic DNA after restriction digest with methylation-sensitive AcI I or EcoRV (control digest; amplicons do not contain EcoRV sites) reveal no DNA methylation at DM1 (no PCR product due to digest of DNA). In contrast, hx1 in PC3 and A2780 is methylated, leading to inhibition of CTCF binding.

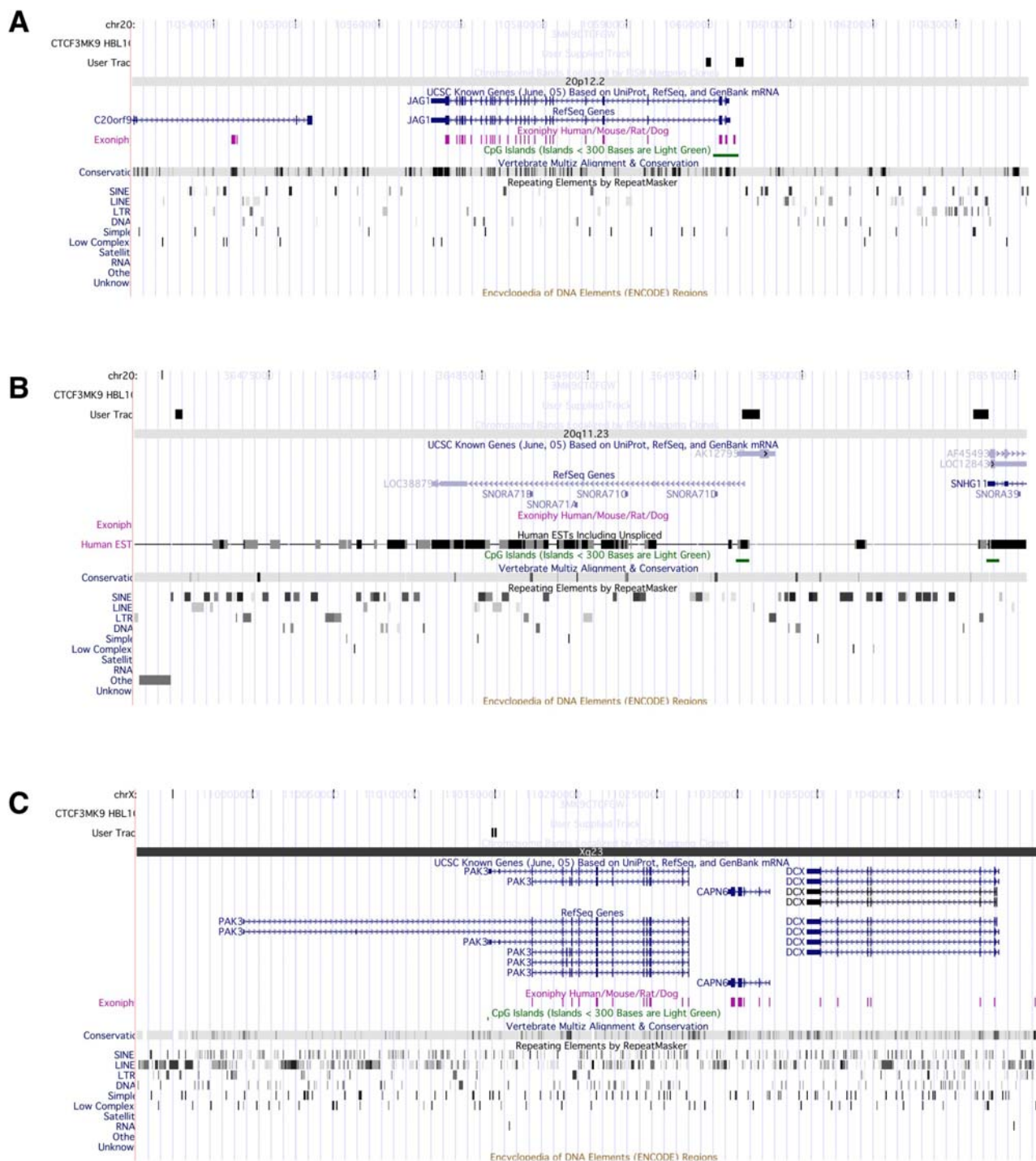


Figure 2. Distribution of CTCF sites at genomic loci that are differentially expressed and part of the premalignant gene signature. Custom tracks of the UCSC genome browser of the JAG1 (A), LOC38879 (B), and PAK3 (C) genes are shown as examples. CTCF binding regions are indicated by black boxes within “User track”. Chromosome and position are shown at the top.

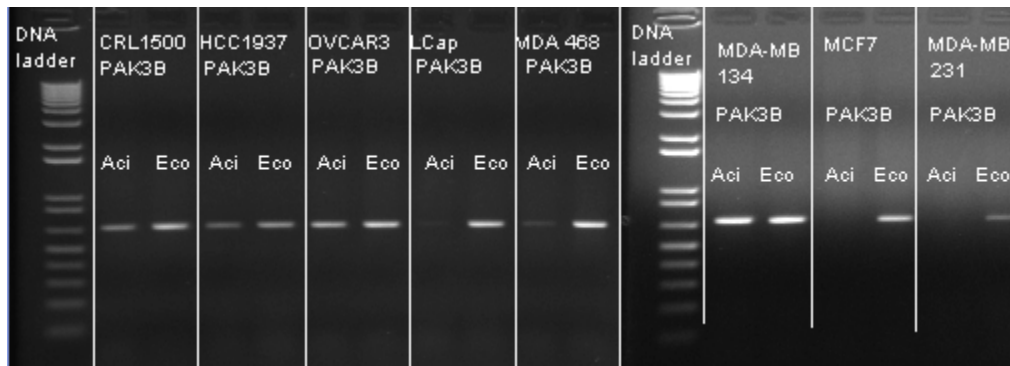


Figure 3. Differential methylation of CTCF binding sites at the PAK3 gene locus in several cancer cell lines. Genomic DNA isolated from indicated cell types was digested with AcI (CpG methylation sensitive) or EcoRV (control). Genomic region of PAK3A gene was subsequently amplified by PCR. CpG methylation at CTCF binding region blocks digest by AcI, and permits amplification of PAK3B region (e.g. ovarian cancer cell line OVCAR3 and breast cancer cell line MDA-MB134). In contrast, non-methylated PAK3 regions are digested in the presence of AcI, and PCR amplification does not yield any product (e.g. prostate cancer cell line LNCaP).



Figure 4. CTCF binding region at LOC388796 is invariably unmethylated in different tissues/cell lines (CRL1500, HCC1937, OVCAR3, LNCaP, MDA468, MDA-MB134, MCF7, and MDA-MB231). Genomic DNA digested with either AcI or EcoRV was amplified with primers specific for a CTCF binding region at LOC388796 (chr20). Absence of PCR product using genomic DNA digested by AcI indicates absence of methylation in all cell lines.