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PRINCIPAL INVESTIGATOR: Wayne D. Lancaster

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI 48201

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14. ABSTRACT

The objectives of this study were to (1) analyze gene expression in a model of human ovarian cancer from abenign to a malignant phenotype in cells grown in organotypic culture and (20 confirm gene expression patterns by RNA analysis. Two human cell lines were used, 96.9.18 and 1.24.96, and RNA extracted from cells at various stages of malignant change were queried on Affymetrix HU133A and B chips in triplicate (96.9.18) or HU133A plus chip in triplicate (1.24.96). Data analysis has revealed a number of genes that are dysregulated in both 96.9.18 and 1.24.96. An analysis of the interection of these genes showed that 5 genes are downregulated between the two cell lines and 183 genes are upregulated during malignant progression. Downregulated genes are too few to analyze but the upregulated genes are involved in apotosis, cytokine-cytokine receptor interactions, toll-like receptor signaling pathway, and the MAPK signaling pathway.

15. SUBJECT TERMS
Organotypic (raft) culture of human ovarian surface epithelial cells; Microarray analysis of gene expression during malignant progression

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Introduction: Most epithelial cell cancers (cervix, colon, skin, prostate, breast, etc.) develop from precursor lesions resulting from an accumulation of mutations in growth regulatory genes. Such precursor lesions have not been identified for OVCA but it has been proposed that OVCAs arise by a multistep process through increasingly aggressive stages. We have shown that immortalized human ovarian surface epithelial (HOSE) cells undergo stepwise progression to the malignant phenotype in vitro. This process has been reproduced with a mouse ovarian cancer model we have developed. Our preliminary results with gene expression analysis of HOSE cells confirms our hypothesis that this phenotypic presentation reflects changes in the expression of genes from benign cells to malignantly transformed cells. The long-range goal of these studies is to identify aberrantly expressed genes in HOSE cells at various stages along the path to the malignant phenotype for the purpose of characterizing biochemical pathways whose expression is dysregulated.

Body: Our progress to date in analysis of gene expression of HOSE cells is provided in the Table 1.

Table 1. Progress in analysis of gene expression by 96.9.18 and 1.24.98 HOSE cells.

HOSE cell	Passage	RNA isolated	RNA labeled	Affymetrix array	Data analysis
96.9.18	Low	yes	yes	yes	yes
	Intermed.	yes	yes	yes	yes
	Late	yes	yes	yes	yes
1.24.96	Low	yes	yes	yes	yes
	Intermed.	yes	yes	yes	yes
	Late	yes	yes	yes	yes

Triplicate cultures of HOSE cells (96.9.18 and 1.24.96) from early, intermediate and late passages were cultivated on collagen rafts to confluency and then raised to the air medium interface and grown for three weeks. Extracted RNA was purified and labeled per the Affymetrix Eukaryotic Target Preparation guide and hybridized to either the Human Genome U133 (HG-U133A & B) Set (96.9.18) which contains almost 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well substantiated human genes or the Human Genome U133 Plus 2.0 array that is identical to U133 A & B but contains an additional 6,500 transcripts in a single chip. Chips were scanned with an Affymetrix GeneChip 3000 scanner. Data for 96.9.18 and 1.24.96 was normalized using dChip and ANOVA (GeneSpring). GeneSpring software was used to identify genes with >1.2 fold change and a p value $\leq$0.1. Analysis showed that the B chip data showed poor correlation and was not valid due to defects in some of the chips. This rendered the statistics for B chip data unreliable and was not included in our analysis.Conditional trees in which the linear data is presented as a heirarchial collection of nodes is shown in Figure 1. Striking is the discrete separation of gene expression levels at a given cell passage. Thus, gene expression clearly changes during malignant transformation. The data for 1.24.96 is not as discrete and is due to the level of compression necessary to include all genes in the tree. There were 231 genes differentially expressed between early and late passage cells for 96.9.18 and 676 genes differentially expressed between early and late passage 1.24.96 cells.

The differentially expressed gene lists for 96.9.18 and 1.24.96 were dissimilar. In order to identify potential target genes of interest we identified genes that were common between the two cell lines during the malignant progression. Only five genes in common were downregulated. Four of the five have known function and are related to the cytoskeleton. Upregulated genes numbered 193 with 180 mapped and these genes are associated with apoptosis, cytokine-cytokine receptor interactions, toll-like receptor signaling pathway and the MAPK signaling pathway and represents about 18% of the total number of genes in

common between the cell lines. There is cross-talk between the pathways mediated by cytokines and transcription factors. The genes in common between 96.9.18 and 1.24.96 involved in these 4 pathways and their cross-talk are shown in Table 2. Gene expression was validated by quantitative real-time polymerase chain reaction (QRT-PCR) using SYBR Green detection of product.

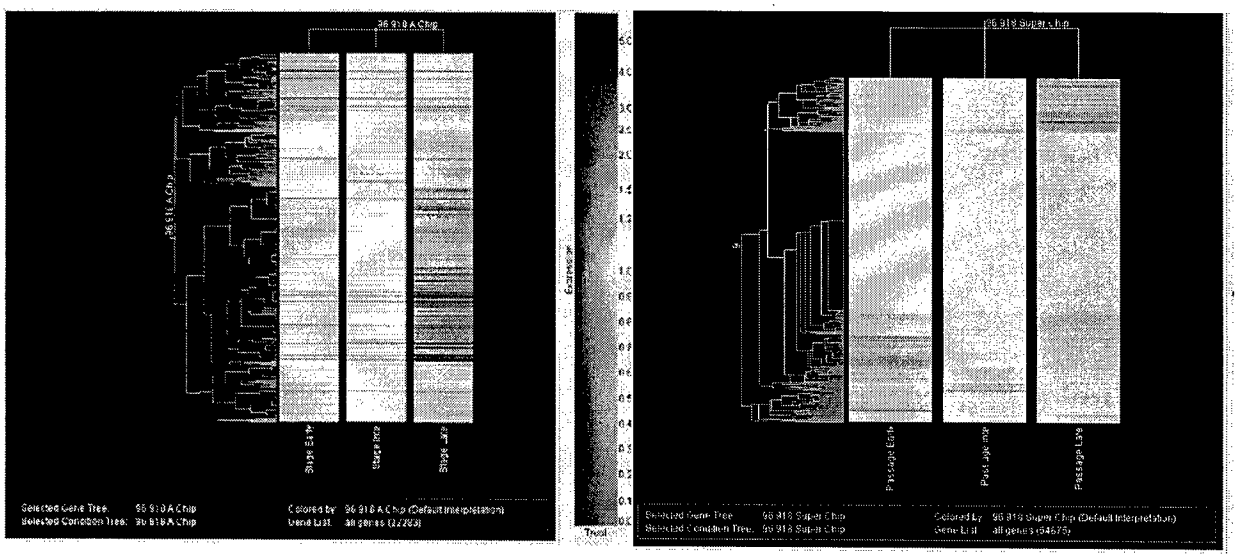
Table 2. Common upregulated genes in malignant transformation of 96.9.18 and 1.24.96. Genes that interact in more than one pathway are emboldened italics.

MAP kinase	Apoptosis	Cytokine	Toll receptor
IL1B	***IL1B***	***IL1B***	***IL1B***
IL1A	***IL1A***	***IL1A***	***IL8***
RAC2	NFKB1A	IL8	MKK3/6
MKNK2	***IRAK1***	MET	IKBA
GADD45A	CYCS	CXCL1	***IRAK1***
DUSP6	CFLAR	CXCL2	REC1
MAP2K1	***TNFRSF10B***	CXCL3	
MAP2K2		CSF2	
ATF4		LIF	
		TNFRSF10B	

These genes would be logical targets to investigate for potential therapeutic interventions or diagnostics.

Figure 1. Conditional trees for gene expression levels in 96.9.18 and 1.24.96 early, intermediate and late passage cells. Expression levels are red=high and green=low as indicated by center bar.

96.9.18 1.24.96



Key Research Accomplishments:

- Have demonstrated that our RNA isolation procedures are compatible with labeling protocol used for Affymetrix oligonucleotide arrays.

- Have successfully queried the U133A chip with 96.9.18 RNA and the U133 Plus 2.0 chip with 1.24.96 RNA for early, intermediate and late passage cells.
- Analysis of the 96.9.18 data indicates 231 genes and for 1.24.96 data 676 genes are differentially expressed between early and late passage cells many of which have previously been shown to be associated with either ovarian cancer or cancer at other sites.
- Dysregulated genes could be verified by QRT-PCR.
- There are common dysregulated genes between 96.9.18 and 1.24.96. Eighteen percent of the genes are in 4 pathways that exhibit cross talk.
- Potential gene targets for therapeutics or diagnostics for ovarian cancer have been identified for overexpressed genes.

Reportable Outcomes: Abstract: Gregoire L, Thota A, Lancaster WD. Changes in gene expression during progression to te malignant phenotype in a human ovarian surface epithelial cell model. American Association for Cancer Research, Advances in Cancer Research, Waikoloa, Hawaii, January 2004.

Conclusions: We requested and received a 12 month no cost extension because of difficulties with tissue culture that were beyond our control that made completion of the project in the 36 month time period impossible. We have completed all queries of the Affymetrix oligonucleotide arrays with two cell lines. Analysis of the results of these hybridizations indicated a number of genes that are differentially expressed in early, intermediate and late passage cells and that these differences can be validated by QRT-PCR. These results indicate that new avenues of investigation can be initiated to dissect development of ovarian cancer as well as development of therapeutics and early biomarkers of early ovarian cancer. The lack of description of a "premalignant" ovarian surface epithelial cell phenotype in situ has hindered progress in early diagnosis of epithelial ovarian cancer. The cell culture system we have developed mimics a premalignant condition in that there is local invasion of matrix by early passage cells. These cultures will be of value in identifying early changes in biochemical pathways that become dysregulated early during malignant progression. Manuscripts describing our results are in preparation.

References: None

Appendices: One abstract

Personnel: Wayne D. Lancaster, Ph.D., Lucie Gregoire Ph.D., Helen Peng, M.S.

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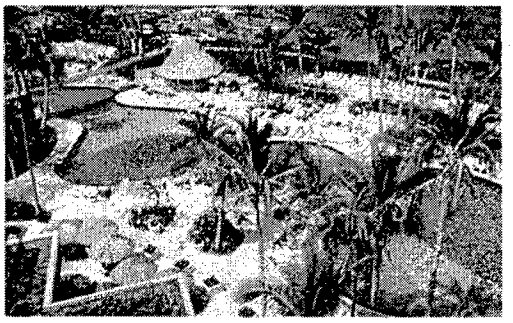

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Changes in Gene Expression during Progression to the Malignant Phenotype in a Human Ovarian Surface Epithelial Cell Model

Lucie Grégoire, Aditya Thota, Wayne Lancaster, Wayne State Univ. Medical School, Detroit, MI.

We have developed an in vitro human epithelial ovarian cancer model by growth of HPV-16 E6/E7 immortalized human ovarian surface epithelial (HOSE) cells in an organotypic (collagen raft) culture system (Gregoire et al. 2001. Clin. Cancer Res. 7:4280). At early passage, cells form a monolayer on collagen rafts. At intermediate passage there is focal stratification and single cell invasion of the collagen. At late passage, cells form colonies in soft agar and form mounds of invading cells on collagen. These later stage cells form tumors in immunodeficient mice. To understand what gene expression changes occur during this phenotypic progression we have queried Affymetrix U133B chips with low and intermediate passage HOSE cell RNA. Three independent cultures of the same passage were grown in parallel on collagen rafts and the RNA isolated and labeled in parallel. Parameters of the chip data that are used to evaluate the hybridization were all within limits indicating no degradation of RNA, sufficient level of label and low background. Comparison of a baseline data set with other 5 data sets showed a correlation of 0.8 indicating that only a small percentage of genes would be differentially expressed. The raw data was subjected to two different statistical analyses: t-test with an adjusted p value of 0.05 and a confidence analysis. The t-test showed that between the low and intermediate passage cells 180 genes were differentially expressed. The confidence analysis showed 210 genes were differentially expressed. The intersection of these two data sets showed that 20 genes were common to both methods of data manipulation. Of the 20 genes, 9 are known and 11 are either expressed sequence tags or hypothetical proteins. Of the 9 known genes, most are involved in cell growth, proliferation, differentiation, longevity, and transformation. This preliminary analysis is being expanded to include U133A chips and comparison of all three phases of phenotypic change.