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Ovarian cancer is a highly lethal malignancy specific to women. We have set up the infrastructure at Mayo for an Ovarian Cancer Research Program utilizing the rich resources of clinical material. This Program Project focuses specifically upon a genetic analysis of ovarian cancer using the transcriptional profiling strategies of cDNA microarrays and subtraction suppression hybridization. We have linked the work of cancer geneticists with cell biologists to begin to understand the functional role that some of the consistently aberrantly-regulated genes play in ovarian cancer development. There are three projects in this grant. The first project focuses on the identification of consistently down-regulated genes in ovarian cancer. The second project focuses on the role of gene amplification in familial versus sporadic ovarian cancer. The third project is to characterize genes that are consistently down-regulated in ovarian tumors that are derived from within common fragile site regions. This Program Project is centered within a larger institutional effort to better understand the biology of the development of ovarian cancer and to devise better strategies for the prevention, early detection, and treatment of this lethal disease.

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Introduction

Of the cancers unique to women, ovarian cancer has the highest mortality rate. Very little is known about the genetic alterations that result in the development of this lethal disease. However, it is clear that there may be many genetic changes that occur which lead to this disease. The purpose of this Program Project is to use the strategy of transcriptional profiling to identify large numbers of aberrantly regulated genes in primary ovarian tumors. We have been using microarrays containing 25,000 human genes to monitor the level of expression of these genes in ovarian tumors as compared to normal ovarian epithelial cells. This work will also be complemented with the construction of subtraction suppression hybridization cDNA libraries to identify additional aberrantly regulated genes, not on the microarrays. There are then three interconnected projects that will analyze some of the aberrantly regulated genes identified using transcriptional profiling. The first projects goal is to characterize the down-regulated genes and to test these as candidate tumor suppressor genes. The second project is to analyze over-expressed genes in both sporadic and hereditary ovarian cancer to characterize regions of amplification in sporadic versus hereditary ovarian tumors. The third project is to study the expression of genes that are localized to regions containing common fragile sites. The overall goal of this work is to better characterize the key genetic alterations that lead to the development of ovarian cancer. We have made significant progress on all these Projects in the last year of support. In addition, the support from the Department of Defense has enabled us to set-up a now recognized translational program in Ovarian Cancer as part of the Mayo Clinic Cancer Center.

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PROJECT #1: Down-regulated genes in ovarian cancer. Viji Shridhar, P.I.

The specific goal of this project is to identify genes that are down regulated during the development of ovarian cancer with an expression based strategy. The two main strategies are 1) In collaboration with Millennium Predictive Medicine (MPMx, Cambridge, MA), use their 25K cDNA gene expression arrays to screen for changes in expression of primary ovarian tumors and cell lines; and 2) Generate suppression subtraction cDNA libraries between ovarian tumors from patients with different stages of the disease and normal ovarian epithelial cells

Genes identified from this preliminary screening will be characterized in the following ways:

(A) The expression profiles of down regulated genes will be confirmed by Northern and semi-quantitative RT-PCR analysis in primary tumors and cell lines.

(B) The down-regulated genes from (A) will be analyzed on a corresponding Southern blot of DNA from primary ovarian tumors and cell lines to identify any altered bands at the genomic level.

(C) Identify corresponding BAC clones and map it to specific chromosomal; regions either by FISH or by radiation hybrid mapping panel.

(D) Test candidate genes for mutations using high throughput capabilities of denaturing high performance liquid chromatography.

(E) The final specific aim of this project is to correlate the expression of down regulated genes in a significant proportion of a large panel of primary ovarian tumors from patients for whom we have extensive outcome data. This will allow us to determine the clinical significance of alterations in these genes.

KEY RESEARCH ACCOMPLISHMENTS

(1) In an attempt to understand early events in ovarian carcinogenesis, and to explore steps in its progression, we have applied multiple molecular genetic techniques to the analysis of 21 early

stage (stage I/II) and 17 advanced stage (stage III/IV) ovarian tumors. These techniques included expression profiling seven each of early and late stage tumors with cDNA micro-arrays containing approximately 18,000 expressed sequences, and comparative genomic hybridization to address the chromosomal locations of copy number gains as well as losses. **Results from the analysis indicate that "early stage" ovarian cancers exhibit profound alterations in gene expression, many of which are similar to those identified in late stage tumors. However, differences observed at the genomic level suggest differences between the early and late stage tumors, and provide support for a progression model for ovarian cancer development.** The manuscript describing these results has been published in *Cancer Research* (2001, 61:5895-5904). A copy of the manuscript is included in the Appendix.

(2) In order to identify novel tumor suppressor genes involved in ovarian carcinogenesis, we generated four down regulated suppression subtraction cDNA libraries from two early stage (stage I/II) and two late stage (stage III) primary ovarian tumors each subtracted against cDNAs derived from normal ovarian epithelial cell brushings. Approximately 600-700 distinct clones were sequenced from each library. Comparison of down regulated clones obtained from early and late stage tumors revealed genes that were unique to each library suggesting tumor specific differences. We found 45 down regulated genes that were common in all four libraries. We also identified several genes whose role in tumor development has yet to be elucidated, in addition to several under expressed genes whose potential role in carcinogenesis has previously been described. The differential expression of a subset of these genes was confirmed by semi-quantitative RT-PCR using GAPDH as control in a panel of 15 stage I and 15 stage III tumors of mixed histological subtypes. Chromosomal sorting of library sequences revealed that several of the genes mapped to known regions of deletion in ovarian cancer. Loss of heterozygosity analysis revealed multiple genomic regions with a high frequency of loss in both early and late stage tumors. In order to determine if loss of expression of some of the genes corresponds to loss of an allele by LOH, we utilized a microsatellite marker for one of the novel genes on 8q and have shown that loss of expression of this novel gene correlates with loss of an allele by LOH. In conclusion, our analysis has identified down regulated genes, which map to known as well as novel regions of deletions and may represent potential candidate tumor suppressor genes involved in ovarian cancer. **While some of the genes identified from these libraries were also identified as down regulated genes by transcriptional profiling of the same tumor, we identified several known and unknown genes of very low abundance only in the SSH libraries. The data from the SSH library analysis also revealed that there were many genes which were differentially expressed in both early and late stage tumors.** The revised manuscript describing the results from this analysis has been submitted to *Cancer Research* (See attached manuscript in the Appendix).

The expression of the down regulated genes were characterized in the following ways:

(A) By Northern and semi-quantitative RT-PCR analysis –See attached manuscripts for details.

(B) Southern blot analysis is still ongoing. We have not detected any altered bands at the genomic level with some of the down-regulated genes tested.

(C) Chromosomal mapping of corresponding BACS will not be performed due to the extensive chromosomal sequences available as a result of the human genome project. For most of the genes identified from these screens, chromosomal positions are known.

(D) Mutation screening using DHPLC for some of the genes is ongoing.

(E) The clinical significance of alterations in these genes is also ongoing.

PROJECT #2: Characterization of the role of amplified oncogenes in the development of familial and sporadic ovarian cancer. Fergus Couch, P.I.

Background: The specific goal of this project is to identify genes that are amplified and overexpressed during the development of ovarian cancer, and to use these genes to test the hypotheses 1) that gene amplification contributes significantly to the development and progression of ovarian cancer, and 2) that familial and sporadic ovarian tumors have different progression pathways. To achieve this goal we proposed the following four major aims. **Specific Aim #1**: Assembly of collections of frozen familial and sporadic ovarian tumors. **Specific Aim #2:** Assessment of the extent of gene amplification in familial and sporadic tumors. **Specific Aim #3:** Identification of novel amplicons and amplified genes in familial and sporadic ovarian tumors. **Specific Aim #4:** Characterization of the oncogenic activity of candidate oncogenes. Much of this work involved a close collaboration with Millennium Predictive Medicine, MPMx.

Results:
We feel that we have made excellent progress towards the completion of these goals.

Specific Aim #1/Task 1: Our goal in this section was to identify a series of ovarian tumors that contained mutations in the BRCA1 and BRCA2 genes in order to make direct comparisons between familial and sporadic ovarian tumors. We have now completed mutation screening of 75 ovarian tumors. BRCA1 mutations were found in 45% and BRCA2 mutations were found in only 10% of the tumors derived from patients with a family history of breast and or ovarian cancer. This numbers match the results from previously published studies and suggest. In addition BRCA1 mutations were found in 5% of a series of sporadic serous ovarian tumors, which is again in keeping with previous studies. We have now identified sufficient familial tumors to carry out Aims #2,3, and 4. Thus, this section of the proposal is complete.

Specific Aim #2/Task 2: Our goal in this section was to determine the extent of gene amplification in ovarian tumors using cytogenetic approaches. We have completed CGH analysis on a series of early and late stage ovarian tumors and have determined that amplification is generally restricted to late stage tumors. Amplification of known oncogenes such as Cyclin D1 on 11q13, c-Myc on 8q24, AIB-1 on 20q12, ZNF217 on 20q13.2, Cyclin E on 19q12, and PI3KCA on 3q26.3 was detected at a frequency of greater than 10%. A number of other amplified regions including 1p, 1q, 8p, 11p, 12p, 16p, and 19p were also identified. Oncogenes associated with ovarian cancer have not previously been identified in these regions. AmpliOnc array studies of the tumors will not be carried out because the array reader available at the Mayo Clinic cannot scan the new generation of AmpliOnc arrays produced by Vysis Inc. However, the success of the CGH technique and some of the other studies described in Task 3 suggest that the AmpliOnc Array analysis is no longer necessary.

Specific Aim #3/Task 3: Our goal in this section was to identify novel amplified and overexpressed genes in ovarian tumors. To achieve this goal we have now profiled a total of 78 ovarian tumors and 5 normal ovarian epithelial cell samples on a 30,000 gene expression array in collaboration with MPMx. We have used bioinformatics analysis of this dataset to generate moving median profiles of each tumor by plotting the median expression level of sequential 50 gene sets along each chromosome. Randomized datasets have been used to generate confidence intervals and to identify gene windows that are likely upregulated by more than chance alone. As these windows likely represent areas of amplification, by identifying peaks on the moving window plot we can select candidate regions of amplification. To date we have detected 15 novel regions. In Table 1 we identify candidate oncogenes that are located within these areas.

It is important to note that this type of analysis only detects aberrant regions and does not accurately define regional boundaries of amplified regions. Therefore, the regions of actual amplification could be larger or smaller than the regions defined here in Table 1. To verify that these candidate regions are actually amplified in the tumors we have initiated Fluorescent *In Situ* Hybridization (FISH) studies. Tissue Microarrays containing 48 of the 78 tumors have been generated and two representative Bacterial Artificial Chromosomes (BACs) probes from each of the candidate regions have been selected. These BAC probes will be hybridized to the Tissue Microarrays along with relevant centromeric probes (Vysis Inc.) and a ratio of BAC probe signals to centromeric signals in 60 nuclei from each sample will be calculated to determine if amplification is present. To date all probes have been ordered and initial FISH studies have begun for the 12p, 3q, 19q, and 20q11.2 regions. In addition, we have verified amplification of CCND1 and c-Myc in 20% of the tumors and have established that N-Myc is not amplified in these tumors.

Table 1.

Cytoband Location of Amplification	Amplified Region - Radiation Hybrid Map Location (GeneMap'99)*	Significant Genes with Respect to Cancer
Resistance		
1p34	110-118 cR3000	AK2 adenylate kinase 2 MYCL1 v-myc oncogene homolog 1
1q	740-777 cR3000	
2q	312-340 cR3000	
2q22	502-515 cR3000	ARHE ras homolog member E NMI N-myc (and STAT) interactor
3q	429-451 cR3000	RAB7 RAS oncogene homolog MCM2 minichromosome maintenance deficient (S. cerevisiae) 2 (mitotin)
3q	487-542 cR3000	
4q	332-419 cR3000	KIT v-kit GRO2 oncogene
12p	84-137 cR3000	KRAS2 v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
14q	231-269 cR3000	AKT1 v-akt oncogene homolog CCNK cyclin K
16p	23-59 cR3000	
19q13.1	218.315-238.63 cR3000	Hs.200816 AKT2 oncogene
19p	269-285 cR3000	
20q11.2	198.18-207.61 cR3000	E2F1 transcription factor Hs.112594 hepatocellular carcinoma-associated antigen 58
20q13.1	225-267 cR3000	AIB1 regulator of ER MYBL2 v-myb oncogene homolog-like 2 TOP1 topoisomerase (DNA) I CSEIL/CAS chromosome segregation 1
20q13.1-13.2	278-336 cR3000	STK15/BTAK serine/threonine kinase 15 BCAS1 breast carcinoma amplified sequence 1 ZABC1/ZNF217 transcription factor PTPN1 protein tyrosine phosphatase, non-receptor type 1

Once amplification is verified for specific regions we will correlate the expression profiles with the amplified regions to select the candidate oncogenes. As the map location of all 30,000 genes on the arrays is now known due to the human genome project, there will be no need to pursue radiation hybrid mapping of candidates. In addition there will be no need to establish physical maps of candidate regions because this has already been accomplished by the Human Genome Project.

In an effort to improve upon this approach we have also established a collaboration aimed at using 1 Mbp resolution gCGH arrays to accurately define amplicons in the tumors. This involves hybridizing genomic DNA from the same set of tumors to an array of 4,500 BAC clones. Amplification can be detected as an enhanced signal relative to a normal specimen. DNA from 30 tumors has been extracted and these studies will commence shortly. By combining the expression and gCGH profiles we aim to identify specific candidate oncogenes from the amplicons that are detected in the tumors. Thus Task #3 will be completed in the near future.

Specific Aim #4/Task 4: Work on this area has not yet begun because we have not yet identified candidate amplified oncogenes.

PROJECT #3: Common fragile sites and ovarian cancer. David I Smith, P.I.

There were four specific aims to our original proposal. **Specific Aim #1:** The cloning and characterization of FRA6E (6q21) and FRA6F (6q26) as these two common fragile sites are derived from chromosomal regions that are frequently deleted during the development of ovarian cancer. **Specific Aim #2:** The isolation of genes from the FRA6E and FRA6F regions, followed by examining each of them as potential tumor suppressor genes or as sensors of genomic damage. **Specific Aim #3:** The third specific aim was to perform clinical correlative studies in ovarian cancer with our impressive resource of fresh frozen ovarian tumors with full clinical follow-up. **Specific Aim #4:** The final specific aim, and the one that linked this project to the other projects within this Program Project Grant, was to characterize aberrantly expressed genes derived from chromosomal bands containing common fragile sites, to determine if these genes actually do reside within common fragile site regions. We feel that we have continued to make excellent progress towards the completion of these goals.

Specific Aim #1: Our first goal was to characterize two common fragile site regions that were derived from regions on the long arm of chromosome 6 that were frequently deleted during the development of ovarian cancer. Although our plan or work called for us to characterize both common fragile sites regions, we have focused most of our efforts on the characterization of the FRA6E (6q26) region, and have collaborated with the group of Dr. Barbanti-Brodano (University of Ferrara, Italy) who have been analyzing the 6q21 region.

In our analysis of FRA6E we have now determined that aphidicolin-induced instability within the 6q26 region extends for a total of 2.2 megabases. The common fragile sites are characterized by hybridizing fluorescently labeled large-insert clones (usually cosmids or BACs) to metaphase spreads produced from aphidicolin-treated lymphocytes and then observing where the labeled clones hybridize relative to aphidicolin-induced decondensation/breakage within specific common fragile site regions. Since the regions of instability where decondensation/breakage occurs is considerably larger than the labeled clones an individual clone can hybridize proximal, distal or actually crossing the region with a decondensation/break. Clones which hybridize with approximately equal frequency proximal and distal to breaks in different metaphases are considered to reside within the "center" of the common fragile site region. Clones which hybridize more frequently proximal than distal to breaks are considered to reside

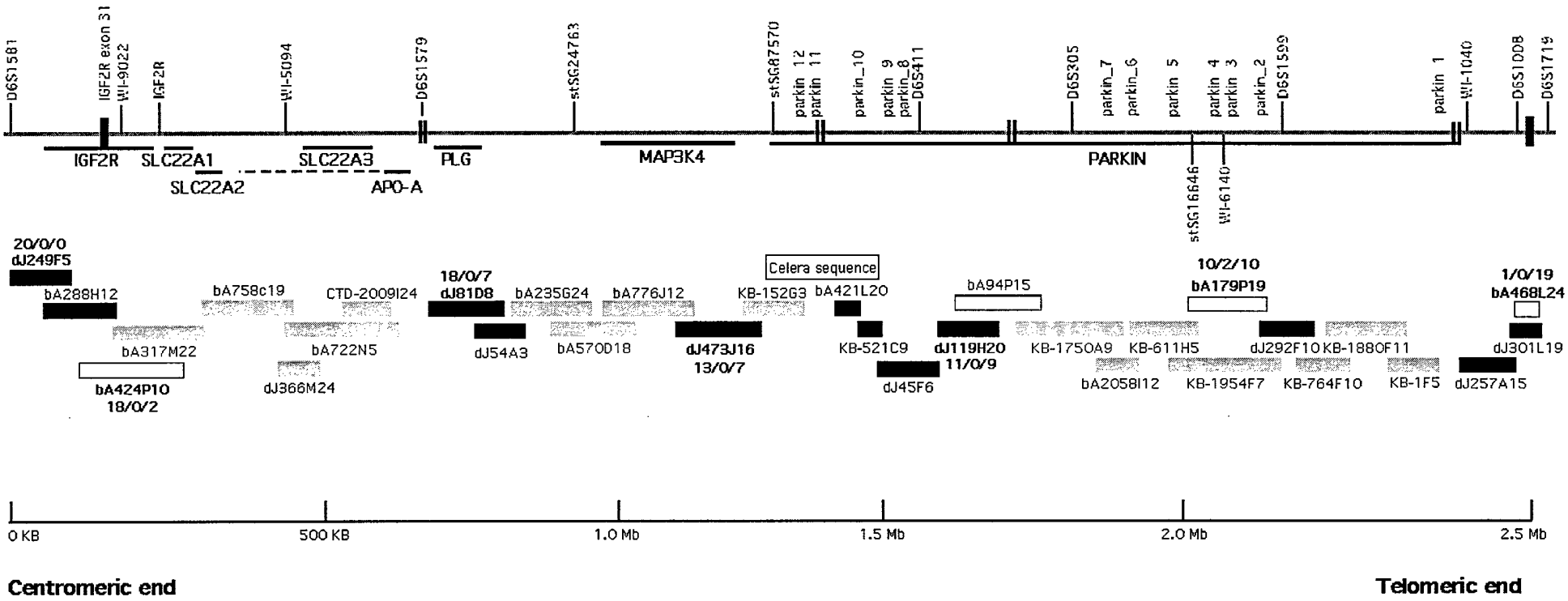
on the proximal side of the common fragile site region. Finally, if a clone hybridizes always on one side of a fragile site (in at least 20 metaphases with good discernible breakage within the common fragile site), that clone is outside of the common fragile site region. Utilizing this assay we were able to determine that the "center" of this common fragile site actually resides on the telomeric end of the entire region of instability. The size of the distal region is only 300 Kb while the proximal region of instability extends for over 1 megabase.

Specific Aim #2: Our second specific aim was to identify genes that resided within the FRA6E common fragile site and to determine the level of expression of these genes in ovarian cancer cell lines and in primary ovarian tumors. We have identified a total of 8 genes that reside within this 2.2 megabase region of instability. The Figure below shows all our results on the physical characterization of the FRA6E region. Included on this Figure is the BAC contig which we've constructed across this region. This contig, which contains only two gaps, is completely sequenced, which will facilitate future analyses on this region of instability. Also included on this Figure are the FISH results with several of the BACs from the contig. The centromeric end of FRA6E contains a number of small genes, including IGF2R, a gene which has been demonstrated to be mutated in some forms of cancer, and has been called a putative tumor suppressor. The most striking aspect of the FRA6E region is that 50% of the entire common fragile site is spanned by the large Parkin gene. Mutations and homozygous deletions in this gene have been observed in patients with autosomal recessive juvenile Parkinsonism (AJRP). In addition, the center of this common fragile site occurs right in the middle of the Parkin gene around exons 3 and 4. These are the two exons that are frequently homozygously deleted in AJRP patients with large deletions in Parkin. Please see Figure 1 on the following page.

We then went on to examine the expression of each of the genes from FRA6E and found that half of them showed a loss of expression in ovarian cancer cell lines and primary tumors and that Parkin was down-regulated in 80% of the primary ovarian tumors analyzed. Additionally, loss of heterozygosity (LOH) analysis of primary ovarian tumors using polymorphic markers in the 6q26 region revealed that markers that mapped within the large Parkin gene had the highest LOH of any of the markers within the FRA6E region. All of the work summarizing our work on the characterization of the entire FRA6E region and the expression analysis of genes within that region in ovarian cancer cell lines and primary tumors was submitted for publication in *Cancer Research* (manuscript included in the appendix).

Specific Aim #3: The third specific aim was to perform clinical correlative studies in ovarian cancer with our impressive resource of fresh frozen ovarian tumors with full clinical follow-up. We have isolated RNA from over 100 primary ovarian tumors representing different histologies (serous, clear-cell, and mucinous ovarian tumors), and different stages and grades. Unfortunately, we do not have any low stage, low grade serous tumors, although, we do have low stage, low grade mucinous tumors. We have also chosen ovarian tumor samples from patients that appeared to have chemosensitive tumors (resulting in disease-free survival of greater than 3 years), as well as patients that appear to be chemoresistant (tumor progresses in less than 6 months). We have just initiated an analysis of gene expression of the various genes in the FRA6E region in this resource of primary ovarian tumors. However, one thing that has actually slowed down our work on this Specific Aim has been the exciting recent results that we've obtained analyzing Parkin, and on the characterization of genes that map within other common fragile sites (described under Specific Aim #4).

Figure 1


D6S1581
IGF2R exon 31
WI-9022
IGF2R
WI-5094
D6S1579
stSG24763
stSG87570
parkin 12
parkin 11
parkin_10
parkin 9
parkin_8
D6S411
D6S305
parkin_7
parkin_6
parkin 5
parkin 4
parkin 3
parkin_2
D6S1599
parkin 1
WI-1040
D6S1008
D6S1719
IGF2R
SLC22A1
SLC22A2
SLC22A3
APO-A
PLG
MAP3K4
PARKIN
stSG16646
WI-6140
20/0/0
dJ249F5
bA288H12
bA317M22
bA424P10
18/0/2
bA758c19
CTD-2009I24
bA722N5
dJ366M24
18/0/7
dJ81D8
dJ54A3
bA235G24
bA570D18
bA776J12
dJ473J16
13/0/7
Celera sequence
KB-152G3
bA421L20
KB-521C9
dJ45F6
bA94P15
dJ119H20
11/0/9
10/2/10
bA179P19
KB-1750A9
KB-611H5
bA2058I12
KB-1954F7
dJ292F10
KB-764F10
KB-1880F11
KB-1F5
1/0/19
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1.5 Mb
2.0 Mb
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Centromeric end
Telomeric end

One of the stated goals under Specific Aim #2 was to analyze genes that map within the common fragile sites to determine if they are mutational targets in ovarian cancer cell lines and primary tumors. We have used a combination of different techniques to look for mutations within these genes, including analyzing cell lines and primary tumors for homozygous deletions, as well as CSGE analysis for smaller alterations.

We did not detect any homozygous deletions in any of the FRA6E genes in our resource of ovarian cancer cell lines or primary tumors. However, as part of a collaboration with Dr. Lewis Roberts who works on hepatocellular carcinoma, we have detected a homozygous deletion in the Parkin gene in one HCC cell line. We are currently characterizing this homozygous deletion, which appears to be complex (thus far we have found two distinct homozygous deletions in the middle of Parkin gene in this one cell line). We are currently analyzing several of the key regions within Parkin for smaller mutations using CSGE analysis and we have some preliminary indications that several of the cell lines and primary ovarian tumors may have mutations within Parkin.

Specific Aim #4: The final specific aim was to characterize aberrantly expressed genes derived from chromosomal bands containing common fragile sites, to determine if these genes actually do reside within common fragile site regions. One of the stated goals of this Program Project Grant was to encourage interactions and collaborations between the three separate Projects. When we analyzed the transcriptional profiling results obtained by Dr. Shridhar, we found that many of the consistently down-regulated genes were derived from chromosomal bands that contain common fragile sites. We chose a sub-set of these genes to determine which of these genes were derived from a common fragile site region. The genes that were chosen were picked based upon a number of criteria including whether they were genes that were previously described as being important in the development of ovarian cancer (such as NOEY2), or whether the gene was derived from a chromosomal region that has been shown to be consistently deleted in ovarian cancer. The Table below lists the genes that were chosen for further study.

Gene	Image Clone	Accession	Band	CFS
NOEY 2	345680	W72033	1p31	FRA1C
RGP4	429349	AA007419	1q21	FRA1F
FHIT	-	XM043137	3p14.2	FRA3B
PDGFRA	52096	H23235	4q12	FRA4B
FST	434768	AA701860	5p14; 5q11	FRA5E; Hecht et al 1988
M6P/IGF2R[a]	67055	T70421	6q26	FRA6E
PLG[a]	875979	T73187	6q26	FRA6E
SLC22A3[a]	127120	R08121	6q26	FRA6E
CAV-1	-	XM057981	7q31.2	FRA7G
CAV-2	-	NM001233	7q31.2	FRA7G
TESTIN	-	?	7q31.2	FRA7G
PSAP	291255	N72215	10q21-22	FRA10C; FRA10D
TSG101	123087	R02529	11p15	FRA11C; FRA11D; FRA11E
TPM1	341328	W58092	15q22.1	FRA15A
WWOX	-	NM016373	16q23.2	FRA16D

In order to determine if any of these genes resided within a common fragile site region we first needed to identify BAC clones that spanned each of these genes. In some instances, BAC clones could be identified electronically. However, for the majority of the genes chosen we needed to screen the Research Genetics BAC pools to identify BAC clones that spanned these genes. Once the BACs were isolated, they were used as FISH-based probes to determine if they localized within a common fragile site region. If a BAC clone always hybridized proximal or distal to the region of aphidicolin-induced decondensation/breakage in 20/20 metaphases examined, that clone localizes outside of the CFS region of instability. Clones which hybridized with approximately equal frequencies proximal and distal to the region of decondensation/breakage were derived from the "center" of that CFS region. Clones which hybridized more frequently distal to the region of breakage were derived from the distal portion of the CFS, and thus we could localize BACs as either outside the respective CFS region, within the center of those regions, or at the "ends" of that CFS region. We analyzed the BAC clones that were identified to span each of the 10 genes chosen and found that 9 of the genes localized within a CFS region. Three of the genes (FST, IGF2R, and PDGFRA) localized to the ends of their respective CFS. The remaining six genes (NOEY2, PSAP, TPM1, SLC22A3, PLG, and TSG101) localized within the "centers" of their respective fragile sites. This work has enabled us to localize a number of CFS regions that were not previously characterized. We have recently completed a manuscript describing this work and have submitted it for publication to *Oncogene* (Manuscript included in the Appendix).

ADMINISTRATIVE CORE

Background: The premise of our entire program project grant is that ovarian cancer develops upon a background of significant genetic alterations and that we can combine several powerful strategies, based in a rich tissue repository, to clone many of the genes involved in ovarian cancer development.

Objective: The overall purpose of the Administration Core is to support and oversee the work done by all three projects and the three other cores.

Relevance to Ovarian Cancer: There are ample data that frequent genetic alterations underlie the development of epithelial ovarian cancer. This is the premise underlying our entire program project grant. The Administration Core integrates all our efforts to (1) determine which of these myriad genetic abnormalities are relevant and (2) identify the biologic function of the relevant alterations.

Support Provided to Research Projects by Administration Core: The Administration Core performs these functions:

- Continue to build our tissue repository
- Obtain clinical and follow-up data to match tissue specimens
- Develop system to provide ready access to tissue repository
- Link tissue and clinical data via a relational database
- Coordinate gyn specific pathology review for all specimens
- Provide statistical support to all projects and cores
- Oversee all projects and cores including budgets
- Manage all human subject issues including approvals through the Mayo Institutional Review Board and the Department of Defense Human Subjects Reviews Groups; address all patient/family questions about research participation
- Organize meetings of investigators and external advisors
- Maintain a liaison with education and outreach specialists within the Mayo Women's Cancer Program and Mayo Clinic Cancer Center (MCCC)
- Formalize a partnership with representatives of the advocacy community

Methodology and Design: Our methods for the repository/database and statistical support are described here and in the following Results section.

Tissue repository: The Ovarian Program has stored specimens of a variety of ovarian tumors since 1991. At the present time we have 1,250 ovarian specimens in a fresh, frozen state – 992 epithelial (including borderline) cancer specimens and 258 benign specimens. Over 90% of the epithelial samples have been re-read by our gyne pathologist Dr. Gary Keeney. The details regarding tissue acquisition and pathology review are provided in the following section on the Tissue Acquisition Core facility. The clinical information to match the tissue specimens is currently being abstracted from the medical records – this will include key risk factors such as family history and follow-up data. In the Results section below, we provide the current status of the tissue repository, clinical information, and relational database. Regarding statistical support, Dr. Steve Iturria in the Section of Biostatistics has provided statistical support for the genetic analyses. His duties have included maintaining all data files in a secure environment, performing diagnostics to ensure data quality, extracting data sets for analyses, and assisting investigators in the identification and use of appropriate statistical methods.

Results:

Tissue repository: At the present time, we have over 1,200 epithelial ovarian tumors in a fresh frozen state.

Chart abstraction for clinical follow-up: To date, we have abstracted 570 charts and this work is proceeding on schedule.

Relational database: At the present time, the details of our ovarian tissue collection are housed in an Access database, created to store information by tumor code numbers (patient identification is not distributed to individual investigators or cores). The clinical data and follow-up information are housed in a clinical database. We now wish to create a relational database joining the laboratory elements with the clinical data. The requirements of the clinical component of the relational database have been communicated to the programmers, who have developed web-based screens needed to enter and retrieve clinical information. The programmers are designing methods to download the data from SAS to load the web-based system. We anticipate testing and troubleshooting to be completed during the first quarter of 2002. The Mayo Clinic Cancer Center has agreed to have Dr. Piet de Groen, an expert in establishing databases using relevant clinical information resources, to take the lead on this effort

Statistical support: The statistical support that Dr. Iturria has provided thus far to the specific projects includes:

(Project #3) Examined the down-regulation of genes near known fragile sites in ovarian cancer. Tumor vs. normal expression ratios for genes lying in known fragile sites were contrasted with those of genes not lying in known fragile sites. Potentially unknown fragile sites were identified by constructing moving average plots for the tumor vs. normal expression ratios on each chromosome.
(Project #2) Identified over-expressed genes in ovarian cancer. Cluster analyses were performed to identify sets of genes that had common expression profiles across multiple experiments. Clusters, which exhibited a pattern consistent with over-expression in tumors, were identified. Various other gene-ranking methods were used to identify genes demonstrating significant increased expression in ovarian tumors. Moving average analyses were carried out to identify entire regions of gene amplification.

(Project #1) Identified under-expressed genes using methods similar to those used above.

Oversight: Drs. Smith and Hartmann communicate multiple times per week to ensure the fluid progress of all the projects and cores. They each interact independently with project and core leaders to address issues as they arise.

Human subjects issues; patient/family contact: We have developed a protocol for direct patient contact as we continue to build our tissue/clinical data repository. The steps for this protocol are as follows:

A. Tumor sent from Surgical Pathology to Tumor Acquisition Core Facility.
B. Tumor Acquisition Core sends e-mail notification of tumor received to Study Coordinator.
C. Surgical pathology results reviewed to determine appropriate participation level.
D. Patient contacted by Study Coordinator regarding participation in Ovarian Tumor Study.
E. Consent form signed, blood request ordered, and questionnaire completed.
F. Materials filed in master file along with Patient Enrollment Master Sheet.

All patient/family contact materials have been approved by the Mayo IRB and the Department of Defense Human Subjects Review Group (although it took considerably longer to obtain approval from the DOD to initiate this work).

Coordination of meetings: Our Ovarian Cancer Research group meets the third Monday of every month from 10:30 a.m.–12:00 noon. Fifteen to 20 individuals attend, including the project leaders, core leaders, technicians, and trainees involved in this ovarian cancer effort. At these meetings investigators present their scientific data for discussion, and new questions and collaborations are generated. Moreover, our Executive Committee meets monthly to assure smooth operations among the projects and within the MCCC. Dr. Hartmann meets weekly with the study abstracter and the laboratory personnel involved in sample acquisition.

Maintain liaison with education and outreach specialists within the Mayo Women's Cancer Program and MCCC. Ms. Julie (Quam) Ponto is the nurse coordinator for the Women's Cancer Program and routinely attends our scientific sessions as does Ms. Lisa Copeland, MCCC Communications. The issues of gynecologic cancer patients are routinely addressed at our MCCC public education events which occur in the fall and spring each year. The brochures from our 2001 event are included as appendices.

Formalize a partnership with representatives of the advocacy community. In the past year, we have developed a closer working relationship with the Minnesota Ovarian Cancer Alliance (MOCA). They hosted a special showing of the play Wit in Minneapolis in the spring of 2001 and Mayo purchased a block of 35 tickets, and most of our research group attended this very memorable performance. At the present time, 11 Mayo staff members serve on the MOCA Medical Advisory Board. Dr. Hartmann has been asked to serve on their grant review group. We are committed to incorporating direction and guidance from the patient advocacy community as we conduct our research and map out strategies for future studies.

Conclusions:

We have built upon our prior infrastructure in women's cancers within the MCCC to upgrade our database, mobilize needed resources to investigators in a timely manner, and provide a smooth organizational structure for the conduct of our ovarian cancer genetics research.

TISSUE ACQUISITION CORE FACILITY

The Tissue Acquisition Core provides a coordinated, centralized, and dedicated program for procurement and processing of biospecimens obtained from ovarian cancer patients and from

populations of women at risk of developing ovarian cancer. Clinically annotated human biospecimens have historically been one of the most valuable and unique resources available for translational research at Mayo Clinic Rochester and the GOAL of the Core is to procure a tissue and/or a blood specimen from every ovarian cancer patient seen at Mayo Clinic. The Core will coordinate acquisition of both normal and neoplastic ovarian tissues, will process blood samples, will provide investigators with DNA and RNA, and will continue to bank biospecimens for future translational research. The Core will also serve as a resource of expertise, collaborative support, and service for pathology, immunohistochemistry, *in situ* hybridization, laser capture microdissection, RT-PCR, and digital image analysis. The Core will interface and be electronically integrated with the Ovarian Cancer Patient Registry and the Biostatistics Core to provide investigators clinically annotated biospecimens. The collection, banking, and use of biospecimens will be performed with appropriate patient consent and institutional approval. The Core will interact and collaborate with other federally-funded Ovarian Cancer Programs to promote resource sharing and integrate scientific projects.

Dr. Gary Keeney, has re-read over 90% of all the epithelial cancers in our repository. His review has significantly decreased ambiguity about tumor morphologies and grades, and has allowed identification and exclusion of "ovarian tumors" deriving from a non-ovarian primary cancer.

Tumors that are to be used in assays are first sectioned and stained with hematoxylin and eosin. Dr. Pat Roche, Laboratory Medicine and Pathology and Head of the Tissue Acquisition and Processing Core Facility, looks at each slide and determines the percentage of the frozen section that is comprised of malignant cells. Only samples containing >70% tumor are used for analysis. Because of a consolidation of tumor acquisition and processing protocols within the last year, new tumors are screened prospectively for the percentage of the block comprised of malignant tissue. This will make decisions about use of specific tumor blocks more efficient in the future.

BIOLOGICAL FUNCTION CORE

The purpose of the Biological Function Core is to provide normal ovarian epithelial specimens for the studies outlined in Projects 1-3 and assess the functional consequences of the genetic alterations detected during completion of Projects 1-3. To perform this analysis, we proposed to: (1) generate new cell lines from primary ovarian cancers and normal ovarian epithelium; (2) transect these cell lines with appropriate plasmids to recapitulate the genetic alterations identified in Projects 1-3; (3) assess the effects of this transfection on proliferation rate, clonogenicity, and ability to form tumors in nude mice; (4) determine the effects of the transfected constructs on sensitivity of cell lines *in vitro* to agents commonly used to treat ovarian cancer; and (5) examine the effects of the genetic alterations on the therapeutic modalities when control or transfected cells are grown as xenografts in nude mice.

Hypothesis: The functional studies are being undertaken to determine whether the genetic alterations detected in ovarian cancer cells alter the proliferative rate, apoptotic threshold, and/or drug sensitivity of the tumor cells *in vitro* and *in vivo*.

Relevance to ovarian cancer: These activities are designed to: i) provide additional samples and models that can be used to study the biology of ovarian cancer vs. normal ovarian surface epithelium, ii) demonstrate how individual genetic alterations contribute to the cancer phenotype, and iii) potentially identify new gene products that can be investigated as possible therapeutic targets.

Core Facility Support Provided to Research Projects: As indicated below, the Biological Function Core has provided normal ovarian surface epithelial cells as normal controls in support of Projects 1-3.

In addition, the Core has taken genes identified in Project 3 and demonstrated their effects on drug sensitivity. Finally, the Core has begun to develop cell lines and animal models required for assessment of additional genes that are in the process of being identified in Projects 1-3.

Results:

Task 1: Provision of normal ovarian surface epithelial cells for study controls. Uncultured brushings of normal ovaries (documented to contain sheets of normal ovarian surface epithelial cells) and cultures of ovarian surface epithelium have been provided to the Smith, Shridhar and Couch laboratories to provide normal controls for Projects 1-3. In addition, normal ovarian surface epithelial cells bearing a temperature-sensitive SV40 large T antigen (described in the 2000 progress report) have been provided to the three labs to provide additional controls for experiments emanating from Projects 1-3.

Task 2: Optimization of current cell lines and development of new cell lines. As indicated below, many functional studies will involve expression of cDNA encoding a particular transcript into cells that have low levels or lack that transcript. To facilitate these studies, we optimized the transfection efficiency and geneticin-induced killing in each of six currently available low-passage ovarian cancer cell lines. We have also inserted a doxycycline sensitive transcriptional regulator into these six lines so that potentially toxic genes can be expressed in these cell lines in a conditional fashion. Because these genes might have different effects in a p53 wildtype background than a p53 mutant or p53 null background, we have over the past year also characterized the p53 status of all six of the cell lines. In addition, in an attempt to generate additional cell lines, ~30 fresh ovarian cancers were put into tissue culture under standard conditions (in addition to the 66 reported last year). Of these 30, two (one that turned out to be from a patient with a mixed Mullerian tumor and one from a patient with relapsed ovarian cancer) spontaneously gave rise to cell lines.

Over the past three months, we have explored an additional strategy for developing ovarian cancer cell lines. William Cliby, who is a member of the Biological Function Core, has been injected fresh ovarian cancer samples directly into nu/nu mice. Any that take will then be passaged in nude mice (see Task 5) as well as transferred to tissue culture.

Task 3: Assess the effect of genetic alterations on proliferation rate and cloning efficiency in vitro. In the 2000 progress report we described work on a gene call MC-J, which is silenced by methylation in ~70% of epithelial ovarian cancers. That work is now completed (Shridhar, V., *et al.* Loss of Expression of a New Member of the DNAJ Protein Family Confers Resistance to Chemotherapeutic Agents Used in the Treatment of Ovarian Cancer. *Cancer Res.* 61: 4258-4265, 2001). Over the past year the Biological Function Core has been assisting in the analysis of two additional genes, HTRA and PAPX.

HTRA is a serine protease that contains an insulin-like growth factor binding domain at its amino terminus (Zumbrunn, J., and Trueb, B. Primary structure of a putative serine protease specific for IGF-binding proteins FEBS Lett. 398, 187-192, 1996). Early studies demonstrated that HTRA expression in fibroblasts is suppressed by SV-40-transformation. Work of Dr. Shridhar has resulted in the demonstration that HTRA mRNA is diminished in >50% of ovarian cancer specimens relative to normal ovarian surface epithelium. To assess the functional consequences of HTRA down-regulation, the OV167 ovarian cancer cell line (which lacks HTRA expression) was transfected with HTRA under the control of the constitutive cytomegalovirus promoter or with empty vector. The growth rates of parental cells, empty vector controls, and HTRA transfectants were comparable, as were colony-forming efficiencies.

PAPX is an X-linked gene with no strong homology to any other gene in the database. Dr. Shridhar's data demonstrated that PAPX mRNA is diminished in all of the ovarian cancer cell lines tested and in >70% of ovarian cancer specimens relative to normal ovarian surface epithelium. The Biological Function Core has worked with Dr. Shridhar to try to create stable transfectants expressing PAPX cDNA behind the constitutive cytomegalovirus promoter. When stable transfectants were not obtained, transient transfection experiments were performed, which showed that all transfected successfully cells appeared to be undergoing apoptosis. Efforts are currently under way to develop cell lines expressing PAPX under the inducible control of the tetracycline-regulated tet-on system. This cell line will then be utilized to examine the role of PAPX in cell survival and proliferation. In the meantime, the Biological Function Core has also developed a polyclonal serum that recognizes the PAPX protein and has utilized this serum to confirm at the protein level that PAPX is downregulated in six different ovarian cancer cell lines relative to normal ovarian surface epithelium. This antiserum is being utilized to characterized the colonies resulting from PAPX transfection.

Task 4: Assess the effect of genetic alterations on sensitivity to various treatments in vitro. OV167 cells transfected with **HTRA** or empty vector were examined for sensitivity to cisplatin, paclitaxel, and topotecan *in vitro*. HTRA transfection slightly increased the sensitivity to topotecan and paclitaxel. Larger effects (a 3- to 5-fold change in IC_{90}) were observed with cisplatin. This enhanced sensitivity was also reflected in a more rapid induction of apoptosis after addition of cisplatin to the HTRA-transfected cells. Ongoing studies suggest that the alteration in cisplatin sensitivity does not reflect any change in cisplatin accumulation.

This same approach will be applied to the **PAPX** transfectants once they become available.

Task 5: Assess the effect of genetic alterations on tumorigenicity and drug sensitivity in vivo. All six of the low passage ovarian cancer cell lines were injected into the flanks of nu/nu mice. Interestingly, none of these cell lines formed tumors. At present we are injecting minced fresh tumor specimens into nu/nu mice in an attempt to establish xenografts that can be utilized to a) serve as another potential source of cell lines (see Task 1) and b) further assess the effect of genes identified in Projects 1-3 on tumorigenicity and *in vivo* drug sensitivity are planned.

MOLECULAR CYTOGENETICS CORE FACILITY

We are extremely fortunate to have an outstanding molecular cytogeneticist working with our ovarian Program. Dr. Robert Jenkins is responsible with starting our Ovarian Program (with Dr. Hartmann) and he has done much work over the years on the characterization of many of the ovarian tumor specimens already collected. We do not request any funding from the Department of Defense to support this Core, as it is supported entirely by the Mayo Clinic Cancer Center. Nevertheless, the core continues to provide support for routine cytogenetic and molecular cytogenetic services to members of the Ovarian Cancer Program. Investigators on the grant are welcome to use the course facilities and have ready access to Dr. Jenkins' expertise. The Core's help in providing comparative genomic hyperitization to characterize 25 of our specimens was highlighted in Dr. Shridhar's August cancer research paper comparing the genomic differences between early and late stage ovarian cancer (see manuscripts published). This Core has provided excellent service to the Program Project as we have been able to use comparative genomic hybridization to characterize 25 of the ovarian tumor specimens. We will also be transcriptionally profiling the same ovarian tumors, and thus will have both expression and cytogenetic information on the same set of tumors.

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Key Research Accomplishments:

- Completed the transcriptional profiling using DNA microarrays containing 25,000 genes against 20 primary ovarian tumors.
- Constructed SSH cDNA libraries from several of the same tumors that were transcriptionally profiled. Several thousand clones from each of these libraries have been sequenced.
- Confirmed that 60% of the genes which appear to be down-regulated are indeed down-regulated in a panel of low-stage and high-stage ovarian tumors.
- Identified a number of ovarian tumors from patients with a family history of ovarian cancer. We have already identified three tumors with known BRCA1 or BRCA2 mutations.
- Completed comparative genomic hybridization analysis of 25 ovarian tumors, including the 20 tumors that were transcriptionally profiled.
- Cloned and characterized the FRA6E common fragile site. We have also localized at least eight different genes within this 800 Kb fragile site region. Several of these genes show a loss of expression in primary ovarian tumors.
- Found that many of the consistently down-regulated genes in the ovarian tumors are derived from chromosomal bands containing common fragile sites. We have used these genes to clone seven new common fragile sites.
- Integration of the work of our Ovarian Program with the outstanding Ovarian Group working at M.D. Anderson (Drs. Gordon Mills and Robert Bast).

REPORTABLE OUTCOMES:

Manuscripts Published:

- Muthusamy T, **Kaufmann SH, Couch FJ**. BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines. *J. Biol. Chem.* 275:33487-33496, 2000.
- **Shridhar V, Bible KC,** Staub J, Avule R, Lee YK, **Kalli K**, Huang H, **Hartmann LC, Kaufmann SH, Smith DI.** Loss of expressison of a new member of the DNAJ protein family confers resistance to chemotherapeutic agents used in the treatment of ovarian cancer. *Cancer Res.* 2001; 61:5895-5904.
- **Shridhar V,** Lee J, **Pandita A, Iturria S,** Avula R, Staub J, Morrissey M, Calhoun E, Sen A, **Kalli K, Keeeney G, Roche P, Cliby W,** Lu K, Schmandt R, Mills GN, Bast RC Jr., James CD, **Couch F, Hartmann LC,** Lillie J, **Smith DI.** Genetic analysis of early-versus late-stage ovarian tumors. *Cancer Res.* 2001; 61:4258-4265.

Manuscripts Submitted:

- **Denison SR, Callahan G,** Phillips LA, and **Smith DI.** Evidence for a role of a common fragile site (FRA6E) in autosomal recessive juvenile Parkinsonism and Ovarian Cancer. Submitted to *Cancer Res.* 9/13/01.
- **Shridhar V,** Sen A, Chien J, Staub J, Avula R, Kovarts S, Lee J, Lille J, and **Smith DI.** Identification of under-expressed genes in early and late stage primary ovarian tumors by suppression subtraction hybridization. Submitted to *Cancer Res.* 9/17/01.
- Bast RC, Urban N, **Shridhar V, Smith DI,** Zheng Z, Skates S, and Mills G. Early detection of ovarian cancer: Promise and reality. Submitted to

Abstracts:

- Muthusamy R, Kaufmann, SH, **Couch FJ**. Fas ligand and caspase-8 mediate BRCA1 induced apoptosis in breast and ovarian cancer cell lines. April 2000, American Association for Cancer Research, San Francisco, CA.
- **Couch, FJ.** Familial Breast and Ovarian Cancer Predisposition Genes. Oct. 2000, Womens Cancer Program Annual Education Symposium, Mayo Clinic, Rochester, MN.

- **Shridhar V**, Pandita A, Lee J, Iturria S, Staub J, Avula R, Sen A, Calhoun R, **Couch F**, James CD, **Hartmann L**, Lillie J, **Smith DI**. Comprehensive analysis of genetic alterations in ovarian cancer. Oncogenomics meeting in Tucson, Arizona, January 25-27, 2001.
- Becker N, Phillips LA, **Smith DI**. FRA3B is larger than published data and completely contains two genes. Amer. Assoc. for Cancer Research 42:A333, 2001.
- Callahan G, **Denison S, Shridhar V, Smith DI**. Cloning and characterization of FRA9E. Amer. Assoc. for Cancer Research 42:A338, 2001.
- **Denison SR, Phillips LA, Shridhar V, Smith DI**. FRA6E (6q26), a 1 MB gene-rich common fragile site. Amer. Assoc. for Cancer Research 42:A346, 2001.
- Thorland EC, Myers S, **Smith DI**. HPV16 integrations in cervical tumors exhibit specificity for common fragile sites, cluster at specific chromosome regions, and may disrupt genes important in cervical carcinogenesis. Amer. Assoc. for Cancer Research 42:A616, 2001.
- Chien J, Staub J, Avula R, **Smith DI**. Aberrant expression of perlecan in ovarian cancer. Amer. Assoc. for Cancer Research 42:A1723, 2001.
- Avula R, Stuab U, Sen A, Lee J, **Hartmann L,** Lillie J, **Smith DI, Shridhar V**. Identification of differnetially expressed genes in early and late stage primary ovarian tumors by suppression subtaction hybridization. Amer. Assoc. for Cancer Research 42:A1729, 2001.
- **Shridhar V,** Pandita A, Lee J, **Iturria S**, Staub J, Avula R, Sen A, Calhoun E, **Couch F**, James CD, **Hartmann L**, Lillie J, **Smith DI**. Comprehensive analysis of genetic alterations in ovarian cancer. Amer. Assoc. for Cancer Research 42:A2307, 2001
- Phillips LA, **Becker N, Hartmann L, Smith DI**. A relationship between common fragile site expression and nucleotide excision repair? Amer. Assoc. for Cancer Research 42:A4866, 2001.

Presentations by David I Smith:

- Transcriptional Profiling of Ovarian Tumors. Presented at Eli Lilly, Indianapolis, IN, November 13, 2000.
- Transcriptional Profiling of Ovarian Tumors- Presented at the Department of Defense Annual Meeting on Ovarian Cancer, Dulles Hilton, Maryland, November 30, 2000.
- Generation of a Molecular Profile for Ovarian Cancer- Presented at the Huntsman Cancer Center, Salt Lake City, Utah, December 15, 2000.
- Common Fragile Sites and Cancer- Presented at Case Western Reserve, Cleveland, Ohio, January 19, 2001.
- Celebration of Research Keynote Address: Mayo Foundation, Rochester, MN. Impact of the human genome project on biology. January 31, 2001.
- The Ovarian Cancer Program of the Mayo Clinic Cancer Center - Presented at the University of Minnesota, April 13, 2001.
- Impact of the Human Genome Project on Biology - Presented to the NCCTG Annual Meeting, April 25, 2001, Mayo Foundation.

Conclusions:

We feel that we have made excellent progress towards our stated goals in the last year of support for this Program Project. We have, in collaboration with our colleagues at Millenium Predictive Medicine, generated expression profiles of a large number of primary ovarian tumors. We have analyzed gene expression in the ovarian tumors using both 25,000 gene cDNA microarrays and by the construction of subtraction suppression hybridization (SSH) cDNA libraries. Millenium has generated ovarian-specific microarrays containing 6,000 genes which were identified from the cDNA microarrays or from the SSH cDNA libraries, and have analyzed over 150 primary ovarian tumors with these arrays. Dr. Viji Shridhar has conducted much of the work in collaboration with researchers at Millenium to do the cDNA microarrays and the SSH cDNA libraries and she has now gone on to examine in greater detail a number of consistently down-regulated genes that were identified. A number of the genes that Dr. Shridhar has identified have already been sent to the Biological Function Core for functional analyses. Dr. Fergus Couch has analyzed many of the primary ovarian tumors for mutations in the BRCA1 and BRCA2 genes

and has now identified familial and sporadic ovarian tumors for further analysis. In collaboration with the Cytogenetics Core facility, Dr. Couch has begun to analyze for gene amplification events that occur in the familial and sporadic ovarian tumors. This has been coupled with data generated from transcriptional profiling and several candidate amplicons are now being looked at in further detail. In the third project, the P.I. of this Program Project has continued his work examining the role that the common fragile sites, and the genes contained within them play in ovarian cancer development. Dr. Smith's lab has characterized the FRA6E (6q26) common fragile site in great detail and they've determined that this site is a large region of instability that contains at least 8 genes. However, the apparent target of deletions (detected using loss of heterozygosity) appears to be within the very large Parkin gene, which is frequently not expressed in ovarian tumors. This is the gene which is mutated in autosomal recessive juvenile Parkinsonism. Dr. Smith's laboratory has also found that many of the consistently under-expressed genes in ovarian tumors are derived from within common fragile site regions of the genome, including NOEY2 and tsg101 (two putative tumor suppressor genes).

What are the implications of this work? Our work with Millennium has enabled our collaborators to find a number of consistently over-expressed genes in ovarian tumors and these are currently being tested as markers for the early detection of ovarian tumors. The researchers within this Program Project have focused their efforts on the identification of early genetic events in the development of ovarian cancer. Since so little is known about early genetic events in this disease we believe that this is a useful investment. In the past year, we've learned that most of the expression alterations that are observed in late stage ovarian tumors are also found in early stage tumors. This indicates that even early stage tumors are quite advanced and that we need to focus on events that occur before early stage tumors develop. Dr. Shridhar has found a number of very exciting candidate genes that are consistently down-regulated in ovarian tumors and the Biological Function Core is now characterizing several of these genes to better understand how they might contribute to ovarian cancer development. Dr. Couch now has sporadic and familial ovarian tumors and these are being analyzed using molecular cytogenetic techniques in conjunction with transcriptional profiling to identify regions of amplification that are different between sporadic and familial tumors. This will give us greater insights into the potentially distinct pathways of sporadic versus familial ovarian cancer development. Finally, Dr. Smith's group is characterizing the common fragile sites and the genes contained within them to determine the role that they play in ovarian cancer development. Parkin is but one example of a very large common fragile site gene which is consistently under-expressed in ovarian tumors, but we now have several other very interesting genes which have been determined to reside within common fragile site regions, including NOEY2 and tsg101. In the next year we will examine whether alterations in the common fragile site genes are an early or a later event in the development of ovarian cancer.

We are not proposing any major changes at the present time from our original proposal. However, since we've now identified a number of very interesting genes that are consistently down-regulated in ovarian tumors and are derived from within common fragile site regions, we are proposing to examine these genes in greater detail, in addition to analyzing the large Parkin gene from FRA6E.

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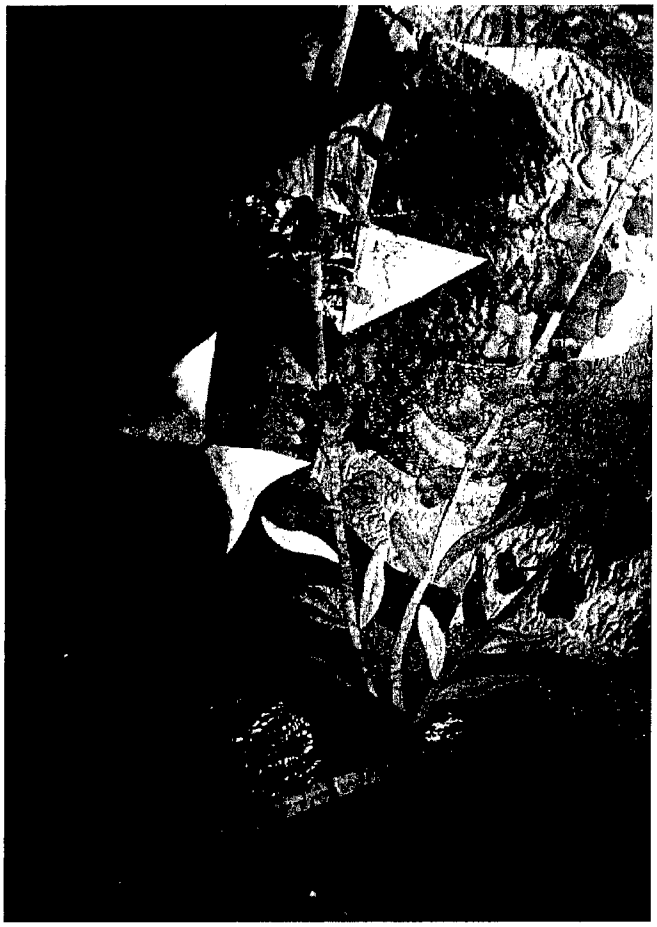
Mayo Clinic Cancer Center

Women's Cancer Program:
Research Update 2001



Rochester Marriott Hotel, Ballroom, Mezzanine Level
6:30 to 8:00 p.m.
Friday, September 28, 2001

Program

Welcome

Lynn C. Hartmann, M.D.
Women's Cancer Program

Presentations

A Half Century of Trying — What the patient with ovarian cancer can achieve now, as compared with 1951
John H. Edmonson, M.D.
Medical Oncology

Molecular Portraits of Breast Cancer
Patrick C. Roche, Ph.D.
Experimental Pathology

Genetic Studies of Familial Breast Cancer Progression
Robert B. Jenkins, M.D., Ph.D.
Cytogenetics

7:30 p.m. Poster displays about selected research activities
Opportunity to visit with individual researchers

Sponsored by
Women's Cancer Program, *Mayo Clinic Cancer Center*
A Comprehensive Cancer Center designated by the National Cancer Institute

Cover art: "Nepenthe ©" Quilted wallhanging presented to Women's Cancer Program by artist Eunice M. Hill.

Posters

Feel free to ask questions of the research persons at any of the posters on display.

Breast Pathology in Women with a Personal and Family History of Breast Cancer, Including BRCA 1/2 Mutation Carriers: A Model for Cancer Progression

Camilo P. Adem, MD
Divisions of Anatomic Pathology and Cytogenetics

We studied the pathologic characteristics of cancer patients with and without a family history of breast and/or ovarian cancer who underwent therapeutic and prophylactic mastectomy. We found that BRCA 1/2 carriers had lower incidences of precursor lesions and higher values of proliferation compared to sporadic cases.

Functional Insulin Receptors on Human Epithelial Ovarian Carcinoma Cells

Laurie K. Bale
Endocrinology Research

Ovarian carcinoma cell lines express insulin receptors that cause cells to grow in response to low doses of insulin and IGF-II. This finding has implications for development of tumor-specific therapeutics.

Psychological Changes Associated With Exercise Participation Among Women With Breast Cancer

Matthew M. Clark, PhD
Psychiatry and Psychology

Twenty-four women who completed treatment for breast cancer were randomly assigned to an exercise intervention. Those who participated in the on-site exercise program demonstrated improvements in mood and body image.

Ovarian Cancer, A Half Century of Trying
What the patient with ovarian cancer can achieve now, as compared with her expectations in 1951

John H. Edmonson, MD
Medical Oncology

A contrast of the current situation with that of a half century ago.

Psychosocial Research in the Women's Cancer Program

Marlene Hanson Frost, RN, PhD, AOCN, Julie Ponto, RN, MS, AOCN,, Romayne Thompson, Teresa Allers, DeAnne Smith, RN, CNP, Katie Zahasky, RN, MA, CNP, and Lynn Hartmann, MD
Women's Cancer Program, and Department of Medical Oncology

The Women's Cancer Program has made the study of psychosocial issues a priority and has targeted women with breast and gynecologic, as well as women at risk of developing cancer. We will provide an overview of select studies that we have recently completed or are either currently proposing or conducting.

Outcomes in Patients with Leiomyosarcoma of the Uterus

Robert L. Giuntoli, II, MD, Daniel S. Metzinger, MD, Connie S. DiMarco, MD, Jeff A. Sloan, PhD, Gary L. Keeney, MD, Bobbie S. Gostout, MD
Gynecologic Surgery

We reviewed the characteristics of 210 patients evaluated at the Mayo Clinic for treatement of leiomyosarcoma of the uterus between 1975 and 1999. Lower stage, lower grade, and ovarian preservation were associated with improved disease specific survival. Adjuvant therapy did not appear to improve disease specific survival.

Benign Breast Disease: Toward Molecular Prediction of Breast Cancer Risk

L. C. Hartmann MD, T. Sellers PhD, F. J. Couch PhD, P. C. de Groen MD, S. J. Iturria PhD, R. B. Jenkins MD, PhD, W. L. Lingle PhD, L. Newman MD*, C. A. Reynolds MD, P. C. Roche PhD, V. Shridhar PhD, V. Suman PhD, T. Tlsty MD, D. W. Visscher MD, S. Hillman, MS
Mayo Clinic and *Wayne State University

Better risk prediction is essential for early detection and prevention strategies. Benign breast disease (BBD) is very common, especially with increasing use of mammography, but we lack good studies clarifying just how common the condition is in the population. It is known that women with BBD have an increased risk of a later breast cancer and that breast cancer can occur in either breast. This study will provide further information to better identify that 10% of women who will develop breast cancer.

Breast Cançer Clinical Trials

James N. Ingle, MD
Medical Oncology

Clinical trials evaluating new therapies for patients with breast cancer have been a major focus of the Mayo Comprehensive Cancer Center since the establishment of the National cancer Program three decades ago. This tradition continues to this day and involves the full spectrum of clinical settings from prevention to the treatment of metastatic disease. In addition to promising new hormonal agents and chemotherapy regimens, protocols are available examining monoclonal antibodies and truly novel approaches such as treatment of primary tumors with ultrasound (sound waves). Translational research is actively developing new treatments involving immunotherapy and gene therapy of breast cancer.

Dronabinol Versus Megestrol Acetate Versus Both for the Treatment of Cancer-Associated Anorexia: A North Central Cancer Treatment Group Study

Aminah Jatoi, MD
Medical Oncology

Over 50% of all patients with advanced cancer lose their appetite and cite loss of appetite as one the top three most distressing symptoms they experience. Does marijuana or one of its derivatives help? In this study, Mayo researches tested the marijuana derivative, dronabinol, and found it was less effective in boosting appetite than another, more standard treatment.

Spiritual Well Being and Overall Quality of Life in Women with Ovarian Cancer and Their Spouses/Partners

Mary E. Johnson, MA
Chaplaincy Services

How does ovarian cancer impact a woman's spiritual well being and that of her spouse/partner? Does this impact change over time? How does spiritual well being influence overall quality of life? In an effort to better understand how one copes spiritually with ovarian cancer we will track the experience of 60 women and their spouses/partners over the course of the disease.

The Granulin-Epithelin Precursor (GEP): a Putative Biomarker for Ovarian Cancer

Monica B. Jones, MD, Jacqueline Lafky, Elise C. Kohn, Karl C. Podratz, MD, and Nita J. Maihle, PhD
Tumor Biology, Department of Biochemistry and Molecular Biology; Department of Gynecologic Surgery, Mayo Clinic and Molecular Signaling Section, Department of Pathology, National Cancer Institute

For patients presenting with advanced ovarian cancer the 5 year survival is 25 - 40% whereas patients with disease limited to the ovary have survivals upwards of 95%. The discovery of new markers may increase the number of cases detected early and improve overall mortality rates. The granulin-epithelin precursor (GEP), a recently defined secreted growth factor, for ovarian cancer cells may be a candidate biomarker for this disease. Characterization of this growth factor and future development of GEP as a biomarker for ovarian cancer is presented.

Fenretinide-Induced Apoptosis in Epithelial Ovarian Carcinoma Cell Lines Is Initiated by Procaspase-8

Kimberly R. Kalli, PhD, Kathryn E. Devine, Phyllis A. Svingen, BS, Charles Erlichman, MD, Lynn C. Hartmann, MD, Cheryl A. Conover, PhD, and Scott H. Kaufmann, MD, PhD
Endocrine Research Unit

Fenretinide, a synthetic retinoid, causes ovarian cancer cell lines to undergo apoptosis, or programmed cell death. Fenretinide activates different intermediates in the apoptotic pathway than other chemotherapeutic agents used to treat ovarian cancer, thus, it is hoped that cross-resistance with these agents will not occur.

Chromosomal Instability and Loss of Tissue Differentiation Correlate With Different Features of Centrosome Amplification in Breast Cancer

Wilma L. Lingle, PhD
Experimental Pathology

In these studies we demonstrate that centrosome size and number correlate with aneuploidy, MT nucleation capacity correlates with loss of differentiation, and centrosome amplification is present in pre-invasive lesions. These results support the hypothesis that centrosome amplification is an early event in tumorigenesis that drives aneuploidy and loss of differentiation through independent centrosome functions.

Mayo Clinic Cancer Center Gynecology Oncology Group(GOG) Clinical Trials Program

Harry Long, MD, Prema Peethambaram, MD, Julie Ponto, RN, MS, AOCN
Medical Oncology and Women's Cancer Program

Mayo Clinic Cancer Center participates in multiple Gynecology Oncology Group (GOG) clinical trials. This poster describes current areas of involvement with the GOG and gynecologic studies that are currently available.

Breast Cancer Adjuvant Therapy: How to Communicate Benefits

Charles L. Loprinzi, MD
Medical Oncology

Understanding the benefits from adjuvant systemic therapy (tamoxifen and/or chemotherapy) for patients with resected breast cancer has been quite confusing for physicians and patients alike. Given this, a methodology of better elucidating individual patient prognoses and individual patient benefits from these therapies has been undertaken. This poster will describe how the results of this work allow physicians and patients to have better information with which to make informed decisions.

Cancer Education Center: A Portal to the Mayo Clinic Cancer Center

Teresa Mettler
Cancer Education Program

The Cancer Education Center, part of the Mayo Clinic Cancer Center, will provide reliable, relevant and current information about all aspects of cancer. Annually, 40,000 patrons are anticipated to visit the center located in the west lobby of the Gonda Building. Books, brochures, audiovisual tools, internet access for consumer health research, and interactive educational classes will help patients become active participants in dealing with the challenges of cancer.

Methodologic Lessons Learned from Oncology Patient Hot Flash Studies

Paul J. Novotny
Mayo Clinic Cancer Center Statistics

A description of methods used to assess the efficacy of agents to reduce hot flash activity in cancer survivors

Using the Computer for Breast Cancer Information and Support; Usefulness, Value and Barriers to Use

Julie Ponto, RN, MS, AOCN, Laurie Jo Vlasak, RN, BSN,
Becky Smith, RN, MA
Women's Cancer Program and Patient Education Center

This poster highlights preliminary results from a study of women who used a computer program, geared specifically to women with breast cancer, to obtain information and support related to their diagnosis of breast cancer.

The Study of Tamoxifen and Raloxifene for the Prevention of Breast Cancer (STAR)

Sandhya Pruthi, MD and Jean M. Jensen
Breast Clinic

Mayo Clinic Rochester and Jacksonville are participating, in conjunction with the NCI/NSABP, to recruit high risk women to the STAR trial for the prevention of breast cancer. This study will determine if raloxifene is as effective as or better than tamoxifen in reducing the risk of developing breast cancer. Secondary aims include evaluating the effect of these drugs on heart disease and osteoporosis.

Evaluation of Soy Phytoestrogens for Treatment of Hot Flashes in Breast Cancer Survivors: An NCCTG Trial

Susan K. Quella, RN, BSN, OCN, Charles Loprinzi MD, Debra Barton, RN,
J Knost, J Sloan PhD, J Gerstner, B LaVasseur, D Swan, B. Stevens, K. Miller,
P. Novotny
Mayo Clinic Cancer Center

Hot flashes are a major problem in some breast cancer survivors, for which safe, effective treatment is needed. Based on anecdotal information, we hypothesized that soy-derived phytoestrogens might abrogate hot flashes. We currently report on data from 132 randomized women. We did not detect evidence to substantiate that this soy prepara-tion in this dose and schedule, could abrogate hot flashes.

Pilot Study of Accelerated Hyperfractionated Radiation Therapy Following Lumpectomy and Axillary Lymph Node Dissection in Patients with Stage I and II Breast Cancer Receiving Adriamycin Based Chemotherapy

P Schomberg, MD, V Suman, PhD, J Brindle, MD, J Ingle, MD,
R Hawkins, MD, J Donohue, MD, W Jarvis, MD, L Wold, MD,
C Wisseman, MD, R Drummond, MD, M Halyard, MD, A Hatfield, MD,
W McGinnis, MD, J Bollinger, MD, and C Clausen, MD
Radiation Oncology

The poster presents the results of a North Central Cancer Treatment Group pilot study of a new radiation schedule for the treatment of early stage breast cancer patients undergoing breast conservation therapy and receiving adriamycin based chemotherapy. The new schedule shortens the time required to complete radiation therapy and potentially lessens the burden of the usual long treatment course.

Alcohol, Folate, and Breast Cancer

Thomas A. Sellers, PhD
Epidemiology

This poster summarizes work from the Iowa Women's Health Study. We observed that alcohol increases risk of postmenopausal breast cancer when folate intake is low. Suggests a mechanism for risk reduction.

Genetic Analysis of Early Versus Late Stage Ovarian Tumors

Viji Shridhar[1], PhD, John Lee[2], Ajay Pandita, PhD[1], Steve Iturria, PhD[1],
Rajeswari Avula[1], Julie Staub[1], Mike Morrissey, PhD[2], Eric Calhoun[1],
Kimberly Kalli, PhD[1], David James, PhD[1], Fergus J. Couch, PhD[1],
Lynn C. Hartmann, MD[1], Jim Lillie, PhD[2] and David I. Smith, PhD[1]
[1] The Mayo Foundation, Rochester, MN
[2] Millennium Predictive Medicine, Cambridge, MA

In the United States, ovarian cancer is the fourth most common cause of cancer-related deaths among women. Although women with low stage tumors have a relatively good prognosis, most women diagnosed with late stage disease eventually succumb to their cancer. In an attempt to understand early events in ovarian carcinogenesis, and to explore steps in its progression, we have applied multiple molecular genetic techniques such as expression profiling with cDNA microarrays and comparative genomic hybridization. Results from the analysis indicate that "early stage" ovarian cancers exhibit profound alterations in gene expression, many of which are similar to those identified.

TBX2 Amplification Plays a Key Role in BRCA1 and BRCA2 Related Breast Cancers

Colleen S. Sinclair, Camilo P. Adem, MD, Cheryl L. Soderberg, Guojun Wu, PhD, Daniel J. Schaid, PhD, Jeffrey M. Slezak, Carol Reynolds, MD, Lynn C. Hartmann, MD, Robert B. Jenkins, MD, PhD, and Fergus J. Couch, PhD
Mayo Clinic Cancer Center

Breast cancers arising in BRCA1/BRCA2 mutation carriers are genetically different from sporadic breast tumors. We found the TBX2 gene to be amplified in approximately 70% of BRCA1/BRCA2 mutation carriers as compared to 25% of sporadic controls. TBX2, when overexpressed, allows cells to become immortal which is an early step of tumorigenesis and therefore makes this an ideal candidate oncogene in breast cancer.

Gender Differences in 5FU-induced Toxicities

Jeff A. Sloan, PhD
Biostatistics

An analysis of gender differences in toxicity reporting by colon cancer patients.

HER2/neu and c-myc Oncogene Alterations in Breast Lesions from Women with BRCA 1/2 Gene Mutations

Cheryl L. Soderberg
Molecular Cytogenetics

Breast cancer arising in women with BRCA 1/2 gene mutations has particular clinical and pathologic features. We studied HER2/neu and c-myc oncogene amplifications by FISH. Our results showed a higher incidence of gain/amplification in the BRCA 1/2 related cases compared to sporadic cases.

Investigations into the Mammographic Breast Density Response to Hormone Replacement Therapy (HRT)

Celine M. Vachon, PhD
Department of Health Sciences Research, Division of Clinical Epidemiology and Mayo Clinic Cancer Center

A subset of women who initiate HRT experience an increase in breast density (approximately 20-35%). We performed a case-control study to investigate how this subgroup of women, who respond to HRT initiation via increased breast density, differs from women who do not experience a change in density with regard to breast cancer risk factors and type of HRT.

The Women's Cancer Program is committed to:

- Advancing the scientific understanding of breast and gynecologic cancers
- Educating patients, professionals and the public about breast and gynecologic cancers
- Optimizing the care of patients with these diseases

Mayo Clinic investigators study the entire spectrum of issues in women's cancers, including the following types of research activities:

Basic Laboratory Research explores how genetic and hormonal factors cause tumors to form and grow.

Applied Research projects seek to identify markers of tumor behavior so that therapies may be tailored to an individual's needs. Laboratory work to develop improved and more targeted therapies that destroy tumor cells while preserving normal cells is a high priority.

Clinical Trials are available for all stages of breast, ovarian, endometrial and cervical cancers. The goal of these trials is to improve the care of women with these diseases.

Epidemiologic Research seeks to identify individuals and populations at risk for women's cancers.

Psychosocial Research studies the psychological, social and spiritual effects of cancer on a woman and her family.

This is an event to share information about current research in women's cancers at Mayo Clinic Cancer Center with patients, staff, benefactors and interested public who make these programs possible. Mayo Clinic Cancer Center researchers will be available to discuss their specific studies and will be pleased to answer your questions. The insert provides a preview of some of the posters that will be presented.

Please join us and talk with our staff about progress in cancer research at Mayo Clinic Cancer Center. Your interest and support is appreciated.

Mayo Clinic Cancer Center

Director – Franklyn Prendergast, M.D., Ph.D.
Deputy Director – Michael O'Connell, M.D.
Associate Director, Basic Sciences – Edward Leof, Ph.D.
Associate Director, Translational Research – Charles Erlichman, M.D.
Associate Director, Population Sciences – Thomas Sellers, Ph.D.
Associate Director, Education – Lynn Hartmann, M.D.
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Women's Cancer Program-Related Research

Tumor Study Groups
Breast – James Ingle, M.D.
Familial Cancer – Noralane Lindor, M.D.
Gynecologic – Lynn Hartmann, M.D.

Cancer Genetics
David Smith, Ph.D.

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Native American Programs
Judith Kaur, M.D.

Population Sciences
Thomas Sellers, Ph.D.

Psychosocial Research
Marlene Hanson Frost, Ph.D.

For more information contact the Women's Cancer Program at 507-284-4300.

MC1604-24/0801

Breakout Sessions **September 29**

Debra Barton

Options for Turning Down the Heat: Hot Flash Management

Debra Barton, RN, PhD(c), Department of Gynecology Mayo Clinic

This session will review the current information about treatment options for hot flashes. Time will be allotted for questions and discussion.

Jennifer Hazelton

What Do I Tell My Children?

Jennifer Hazelton, RN, MS, Mayo Breast Clinic

Deciding what to tell your children about your cancer treatment can be complicated. In this session, you will learn how cancer and its treatment may affect children during their various developmental stages. Suggestions for talking with your children, including time for discussing your questions, will be provided.

Lori Thicke

Cancer? Now What?

Lori Thicke, RN, MS, Mayo Breast Clinic

Receiving a cancer diagnosis can be devastating. During this session, you will discuss common reactions to cancer and learn ways to deal with the potential chaos. Group participation will be encouraged to learn from each other's experiences.

Educational Exhibits on Women's Cancers

Hage Atrium, Siebens Building
September 28 to October 5

Educational posters will be available for viewing on the day of the program and throughout the following week. Exhibits will cover a variety of topics related to women's cancers, including the latest research developments, and coping with cancer and its treatments.

Event Sponsors

Support for this educational event has been provided by the following organizations:

- Novartis Oncology
- Amgen
- Pharmacia Oncology

Women's Cancers 2001: Merging Science and Care

Purpose

- To provide women, their friends and family members and the public with information about women's cancers
- To provide a forum for women with cancer and their support people to talk with others concerned about cancer issues
- To recognize our patients who have had cancer and learn from their experiences

Overview

Living through a diagnosis of cancer is challenging. The physical, mental and emotional effects can be overwhelming for a woman with cancer. In addition, cancer may impact relationships with spouses, children, friends and co-workers. Finding accurate information and taking care of yourself may require new ways of communicating with those around you and performing your daily activities.

In recognition of Gynecological Cancer Awareness Month in September and Breast Cancer Awareness Month in October, and to pay tribute to the many women facing cancer, the Women's Cancer Program of Mayo Clinic Cancer Center presents *Women's Cancers 2001: Merging Science and Care.*

During this program, you will learn about exciting ways scientists and clinicians are investigating new methods of diagnosing and treating women's cancers. You will hear how these discoveries influence the care that is provided to women and their family members. You also will learn strategies for coping with cancer and its physical, mental and social side effects.

Join us and learn how you can deal more effectively with cancer in your life or the life of a loved one.

Location

Phillips Hall
Siebens Medical Education Building, First Floor
100 2nd Ave. S.W.
Mayo Clinic
Rochester, Minnesota

Friday evening event

Reception With Researchers

September 28, 6:30 to 8 p.m.

Ballroom on the Mezzanine Level of the Marriott Hotel

A special reception will give you the opportunity to talk with researchers who are investigating new ways of detecting, treating or preventing cancers of concern to women. Informational posters describing their work will be available for you to view and refreshments will be provided.

Saturday event

Program Schedule

September 29, 8 a.m. to 3 p.m.

Phillips Hall, Siebens Building

8:00 a.m.	Registration and Continental Breakfast
8:30 a.m.	Welcome
8:45 a.m.	*"Advances in Breast Radiology"* Marilyn Morton, D.O. Department of Radiology
9:30 a.m.	*"Current Issues and Future Directions in Gynecologic Cancers"* William Cliby, M.D. Department of Gynecologic Surgery
10:15 a.m.	Break
10:45 a.m.	Breakout Sessions
11:45 a.m.	Lunch
1:00 p.m.	Breakout Sessions
2:00 p.m.	*Surviving with Hope* Panel discussion
3:00 p.m.	Closing Remarks

Keynote Presenters

Marilyn Morton, D.O.

Marilyn Morton, D.O.
Department of Radiology, Mayo Clinic
Dr. Morton is a consultant in the Department of Diagnostic Radiology at Mayo Clinic and an assistant professor of medicine at Mayo Medical School. She is Mayo Clinic's spokesperson for mammography and breast cancer imaging.

Along with her responsibilities as a physician and teacher, Dr. Morton is involved in breast cancer research. She has authored articles for medical journals about her research and she lectures about mammography and imaging at medical meetings across the country.

William Cliby, M.D.

William Cliby, M.D.
Department of Gynecologic Surgery, Mayo Clinic
Dr. Cliby is a consultant in gynecologic surgery at Mayo Clinic and an assistant professor of obstetrics and gynecology at Mayo Medical School. His specialty is surgical treatment of gynecologic cancers.

In addition to his work as a surgeon and teacher, Dr. Cliby's research interests lie in striving to better understand the molecular biology of gynecological cancers and molecular genetics of ovarian cancer. He has published numerous journal articles about his research. He also speaks about gynecologic cancers at national medical meetings.

Panel Discussion

Mary Johnson

Mary Johnson
Chaplain Services, Mayo Clinic
Surviving with Hope
Family members and women with cancer will discuss their experiences and provide insights into dealing with the many challenges of cancer. Suggestions for maintaining hope with a sense of humor and dignity will be offered.

Women's Cancers 2001: Merging Science and Care
September 28 & 29, 2001

Research Reception – 6:30 to 8 p.m., Friday

Education Event – 8 a.m. to 3 p.m., Saturday

Registration Fee: $20 per person
(Includes Friday reception, Saturday program, continental breakfast and box lunch)

Mail registration form and payment to:

Women's Cancers 2001: Merging Science and Care
Matrix Meetings, Inc.
P.O. Box 1026
Rochester, Minn. 55903-1026

Please print or type all information below. You may duplicate this form.

Name:_____

Address:_____

City:_____ State:_____ Zip:_____

Telephone:_____

Please mark your two choices for the breakout sessions with an "X" below. Please also indicate one alternative choice with an "A".

____ **Options for Turning Down the Heat: Hot Flash Management**

____ **What Do I Tell My Children?**

____ **Cancer? Now What?**

____ **The Effect of Cancer on the Family**

____ **I Have Cancer? How Could God Let This Happen to Me?**

____ **Colon Cancer in Women**

Registration Deadline: *September 17, 2001*

Will you be attending the Friday evening reception? __Yes __No

Please make checks payable to Matrix Meetings, Inc. No refunds will be given after September 14 (postmark date).

For more information on this event, call 507-266-4886.

Early registration is encouraged because this event is expected to fill quickly.

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Women's Cancers 2001: Merging Science and Care

Saturday, September 29, 2001
8 a.m. - 3 p.m.

Phillips Hall
Siebens Medical Education Building, First Floor
Mayo Clinic
Rochester, Minnesota

MC1604-17/R0501

[CANCER RESEARCH 61, 4258–4265, May 15, 2001]

Loss of Expression of a New Member of the DNAJ Protein Family Confers Resistance to Chemotherapeutic Agents Used in the Treatment of Ovarian Cancer[1]

Viji Shridhar,[2] Keith C. Bible, Julie Staub, Rajeswari Avula, Yean Kit Lee, Kimberly Kalli, Haojie Huang, Lynn C. Hartmann, Scott H. Kaufmann, and David I. Smith

Division of Experimental Pathology, Department of Laboratory Medicine and Pathology [V. S., J. S., R. A., H. H., D. I. S.], Division of Medical Oncology and Oncology Research [K. C. B., L. C. H.], Departments of Oncology [K. C. B., Y. K. L., S. H. K.] and Molecular Pharmacology [K. C. B., S. H. K.], and Endocrine Research Unit [K. K.], Mayo Clinic/Foundation, Rochester, Minnesota 55905

ABSTRACT

Differential display-PCR between ovarian tumor cell lines and short-term cultures of normal ovarian epithelial cell brushings was used to isolate a differentially expressed transcript and its corresponding gene. The gene, which mapped to 13q14.1, has partial homology in the DNAJ domain to a number of proteins with a similar domain and was designated as *methylation-controlled J protein* (*MCJ*). MCJ has the highest similarity to a functionally undefined protein from *Caenorhabditis elegans*. *MCJ* is expressed as a 1.2-kb transcript in several adult tissues, with testis showing the highest level of expression. Expression of *MCJ* was absent in three of seven ovarian cancer cell lines. Similarly, expression analysis using semiquantitative reverse transcription-PCR indicated that 12 of 18 primary ovarian tumors examined had either a complete absence or lower levels of expression of this gene. 5-Aza-2′-deoxycytidine treatment of the OV202 cell line induced *MCJ* expression in a dose-dependent manner, implicating methylation in this induction. Loss of heterozygosity and methylation-specific PCR analysis revealed that the loss of *MCJ* expression in primary tumors and cell lines was attributable to deletion of one allele and methylation of the other. To assess the potential functional significance of *MCJ* down-regulation, the sensitivity of parental (*MCJ*-nonexpressing) and MCJ-transfected OV167 cells to antineoplastic agents was evaluated. *MCJ* expression was associated with enhanced sensitivity to paclitaxel, topotecan, and cisplatin, suggesting that *MCJ* loss may play a role in *de novo* chemoresistance in ovarian carcinoma. These observations raise the possibility that *MCJ* loss may: (*a*) have potential prognostic significance in ovarian cancer; and (*b*) contribute to the malignant phenotype by conferring resistance to the most commonly used chemotherapeutic agents for ovarian cancer.

INTRODUCTION

The DNAJ proteins are a highly conserved family of proteins with the *Escherichia coli* heat shock protein, DNAJ (the human HSP40[3] orthologue), as its founding member (1). The defining feature of the HSP40 family is a highly conserved 70-amino acid residue, termed the DNAJ domain, that includes a signature tripeptide, HPD, that is critical for the function of the DNAJ domain (2). DNAJ proteins belonging to the HSP40 family contain four distinct domains including the DNAJ domain, whereas other proteins from this superfamily only possess the DNAJ domain (2). J-domains are present in diverse proteins and participate in complex biological processes. For example, HSP40 family J-domain proteins serve as cochaperones by recruiting HSP70 and accelerating ATP hydrolysis (3, 4). The DNAJ proteins participate in processes such as protein folding and translocation (5), cell cycle control by DNA tumor viruses (6–12), and regulation of protein kinases (13).

In this report, we describe the molecular cloning of a new member of the DNAJ domain protein family designated as MCJ. Collectively, our studies demonstrate that MCJ loss is common in human ovarian cancer, results from the deletion of one allele (LOH) and the silencing of the other by hypermethylation, and confers resistance to the three drugs most commonly used in the treatment of ovarian cancer. Here we show that stable transfectants expressing *MCJ* in OV167 are more sensitive to cisplatin, paclitaxel, and topotecan than parental and vector-transfected controls, implicating *MCJ* down-regulation in processes leading to decreased drug sensitivity.

MATERIALS AND METHODS

Cell Culture. Five of eight ovarian carcinoma cell lines (OV167, OV177, OV202, OV207, and OV266) were low-passage primary lines established at the Mayo Clinic (14), whereas OVCAR-5, SKOV-3, and the PC3 prostate cancer cell line were purchased from American Type Culture Collection (Manassas, VA). All cells were grown according to the provider's recommendations.

Assessment of Methylation Control. The OV202 cell line was treated with varying concentrations of 5-aza-2′-dC, ranging from 1 to 5 μM the day after plating. After a 48-h exposure to 5-aza-2′-dC, the cells were harvested in Trizol (Life Technologies, Inc., Rockville, MD) for RNA extraction.

mRNA Differential Display. DD-PCR was performed on the short-term cultures of normal OCEs and tumor cell lines as described by Liang and Pardee (15). Total RNA was extracted from the cell lines using Trizol and treated with RNase-free DNase I to eliminate genomic DNA contamination. Differential display of the expressed transcripts was performed using the RNA Image kit (GenHunter Corp., Nashville, TN) according to the manufacturer's instructions. Of the several bands identified that were differentially expressed, band 13 was absent in the tumor lane. This band was excised from the gel, reamplified with T11G and AP6 primers, and sequenced using dye terminator technology by the Molecular Biology Shared Resource of the Mayo Foundation.

Strategy for Cloning the Gene. BLAST search of the isolated sequence identified several homologous ESTs in the database EST. The homologous ESTs were assembled into a contig with the use of Sequencher 3 (Gene Codes Corp., Ann Arbor, MI) software. The integrity of the full-length cDNA obtained by this electronic walking was confirmed by PCR analysis using PCR primers flanking each junction between EST clones. The entire cDNA contig was sequenced twice with overlapping primers.

MS-PCR. The methylation state of *MCJ* was determined using the recently described technique of MS-PCR (17). DNA was modified with sodium bisulfite according to Herman *et al.* (17) with the following modifications. DNA (1–1.5 μg) was digested with *Eco*RI in a 50-μl reaction overnight. The digested DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 0.1 volume of 5 M ammonium acetate and

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[2] To whom requests for reprints should be addressed, at Mayo Clinic/Foundation, Division of Experimental Pathology, 200 First Street SW, Rochester, MN 55905. Phone: (507) 266-2775; Fax: (507) 266-5193; E-mail: shridhar.viji@mayo.edu.

[3] The abbreviations used are: HSP40, heat shock protein 40; DD-PCR, differential display-PCR; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; MS-PCR, methylation-specific PCR; RACE, rapid amplification of cDNA ends; 5-aza-2′-dC, 5-aza-2′-deoxycytidine; EST, expressed sequence tag; ORF, open reading frame; BAC, bacterial artificial chromosome; FISH, fluorescence *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCE, ovarian epithelial cell; FACS, fluorescence-activated cell sorting; MCJ, methylation-controlled J-protein.

100% ethanol in the presence of 1 μl of 20 mg/ml glycogen (Boehringer Mannheim, Indianapolis, IN). The DNA pellet was washed twice with 70% ethanol, and the DNA was taken up in 90 μl of 10 mM Tris (pH 7.5) containing 1 mM EDTA (TE buffer). Ten μl of freshly prepared 3 M NaOH were added to each sample, and the DNA was denatured at 42°C for 30 min. After the addition of 10 μl of distilled water, 1020 μl of 3.0 M sodium bisulfite (pH 5.0), and 60 μl of 10 mM hydroquinone, the samples were incubated in the dark at 55°C overnight (16–20 h). Modified DNA was purified using the Wizard purification system (Promega Corp., Madison, WI) according to the manufacturer's instructions, followed by denaturation with 0.3 M NaOH for 15 min at 37°C. The DNA was eluted in 50–100 μl of TE and stored at −20°C in the dark.

We sequenced portions of BAC 251N23 and obtained an additional 361 bp 5′ of the reported cDNA sequence (GenBank accession no. AF126473). Restriction site analysis of this additional sequence revealed the presence of a *Sma*I site 75 bases upstream of the reported cDNA sequence. A pair of primers, MCJ-WTF (5′-CGTGAGCCACCGCACCGGC-3′) at 108 bp upstream of the *Sma*I site and MCJ-WTR (5′-CTTTCCTGACCCCCTTCCG-3′) at 86 bp downstream of the *Sma*I site, were used to detect unmodified DNA. Nucleotide sequences of primers specific for methylation-mediated, modified DNA were MCJ-MF (5′-CGTGAGTTATCGTATTCGGT-3′) and MCJ-MR (5′-CTTTC-CTAACCCCCTTCCG-3′), which yielded a product of 195 bp. Primers used for the analysis of unmethylated sequences in the modified DNA were MCJ-UF (5′-GTTTTTAAAGTGTTGGGAT-3′) at 101 bp upstream of the *Sma*I site and MCJ-UR (5′-TAAACTTACCTAAACTTTCC-3′) at 100 bp downstream of the *Sma*I site, which yielded a product of 234 bp. The primers for amplifying unmethylated sequences were specifically chosen not to contain any CpG-rich sequences at the 3′ end of the primer. PCR was performed by the "hot-start" method (Taq gold; Perkin-Elmer) with an initial denaturation of 10 min, followed by 30 cycles of amplification at 56°C, annealing with primers amplifying methylated sequences and 50°C, and annealing for amplifying nonmethylated/modified DNA with UF/UR primers. Controls without DNA and positive controls with unmodified DNA were performed for each set of reactions.

5′ RACE. To obtain the missing 5′ end sequences, 5′ RACE was performed with poly(A)$^+$ RNA isolated from PC3 cells. Adaptor ligation and PCR were performed according to the instructions provided in the Marathon Ready cDNA amplification kit (Clontech, Palo Alto, CA). Primers used for 5′ RACE were 5′-GCAAGTACTCAGCGTAGCGC-3′ and MCJ-nested 5′-CCGTAGGGACAAACTAGTTACGC-3′.

Northern Blot Analysis. Fifteen μg of total RNA were fractionated on 1.2% formaldehyde agarose gels and blotted in 1× SPC buffer (20 mM Na_2HPO_4, 2 mM CDTA pH 6.8) onto Hybond-N membranes (Amersham, Piscataway, NJ). The probes were labeled using the random primer labeling system (Life Technologies, Inc.) and purified using spin columns (100 TE) from Clontech. Filters were hybridized at 68°C with radioactive probes in a microhybridization incubator (Model 2000; Robbins Scientific, Sunnyvale, CA) for 1–3 h in Express Hybridization solution (Clontech) and washed according to the manufacturer's guidelines.

Semiquantitative RT-PCR. Fifty-100 ng of reverse transcribed cDNA were used in a multiplex reaction with the forward MCJ-4 (5′-GCGC-TACGCTGAGTACTTGC-3′) and reverse primer MCJ-5 (5′-AGATAA-GACTGTGGTCAATC-3′) to yield a 595-bp product and *GAPDH* forward (5′-ACCACAGTCCATGCCATCAC-3′) and reverse (5′-TCCACCACCCT-GTTGCTTGTA-3′) primers to yield a 450-bp product. The PCR reaction mixes contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 400 μM concentration of each primer for *MCJ* and 50 μM for the *GAPDH* primers, and 0.5 unit of Taq polymerase (Promega) in a 12.5-μl reaction volume. The conditions for amplification were 94°C for 3 min and then 29 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s in a Perkin-Elmer-Cetus 9600 Gene-Amp PCR system. The products of the reaction were resolved on a 1.6% agarose gel. Band intensities were quantified using the Gel Doc 1000 photo documentation system (Bio-Rad, Hercules, CA) and its associated software.

BAC Library Screening and FISH. MCJ primers (5′-AAGCTCCCT-GAGGGTCCGCGTTG-3′ with 5′-GGGTAACGTGTCCCGTGCAAG-3′ and 5′-GCGCTACGCTGAGTACTTGC-3′ with 5′-GCGTAGCGACCTGCA-AAT-3′) were used for the isolation of two BACs (420G23 and 251N23) by screening a BAC library from Research Genetics, Inc. (Huntsville, AL) according to the manufacturer's instructions. BAC DNA was extracted from an overnight 500-ml culture with TIP500 from Qiagen (Valencia, CA) according to the protocol provided by the manufacturer. The 251N23 BAC was labeled with biotin-16-dUTP using the Nick Translation kit (Boehringer Mannheim). FISH analysis was then performed with this labeled BAC clone. Primers used to determine the exon/intron junctions in the genomic BAC clone by direct sequencing of the BAC DNA are shown in Table 3.

LOH Analysis of Primary Ovarian Tumors. We used eight pairs of microsatellite markers on chromosome 13q obtained from Research Genetics in addition to the *MCJ*-associated microsatellite (MCJ3′NF, 5′-GATTGAC-CACAGTCTTATCT and MCJ18, 5′-TAAGAGGTCTACTCATTGCTCAC). The markers used in this study are listed in Table 1 along with their chromosomal locations. The PCR reaction mix contained 50 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, a 200 μM concentration of each primer, 0.05 μl of [^{32}P]CTP (10 μCi/μl), and 0.5 unit of Taq polymerase (Promega) in a 10-μl reaction volume. The conditions for amplification were 94°C for 2 min and then 30 cycles of 94°C for 30 s, 52°C-57°C for 30 s, and 72°C for 30 s in a Perkin-Elmer-Cetus 9600 Gene-Amp PCR system in a 96-well plate. The PCR products were denatured and run on 6% polyacrylamide sequencing gels containing 8 M urea. The gels were dried and autoradiographed for 16–24 h and scored for LOH. Multiple exposures were used before scoring for LOH. Allelic imbalance indicative of LOH was scored when there was >50% loss of intensity of one allele in the tumor sample with respect to the matched allele from normal tissue. The evaluation of the intensity of the signal between the different alleles was determined by visual examination by two independent viewers (V. S. and J. S.).

Establishment of MCJ Stable Transfectants. On the basis of the cDNA sequence of MCJ, two primers were synthesized to amplify a 536-bp fragment of MCJ from base 367-903 containing the entire ORF. A *Hin*dIII site was introduced into the forward primer 5′-CCTGAAGCTTACTAGTTTGTC-CCT-3′ and a *Bam*HI site into the reverse primer 5′GCGGGATCCTTCCT-TCAGTGTG-3′ (restriction sites are underlined in the sequences). The PCR product was digested with both *Hin*dIII and *Bam*HI, gel purified, subcloned into the cloning sites of the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* DH5α (Life Technologies, Inc.) cells. Mini-preparations of the ampicillin-resistant colonies were sequenced and verified. Exponentially growing cells of OV167 in 100-mm dishes were washed with serum-free medium and treated with a mixture of 5 μg of plasmid, 30 μl of LipofectAMINE, and 20 μl of Plus reagent. After a 3-h incubation, complete medium with serum was added. Beginning 24 h after the start of transfection, G418 was added to select the transfectants. Two stable clonal transfectants, MCJ 6 and MCJ 13, were subsequently generated. For controls, cells were similarly transfected with vector [pcDNA3.1(+) only] and selected.

Tissue Culture and Colony-forming Assays. Topotecan was kindly provided by the Pharmaceutical Resources Branch of the National Cancer Institute. Paclitaxel and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were obtained as described previously (16, 18, 19). Stock (1000-fold concentrated) solutions of paclitaxel and topotecan were prepared in DMSO and stored at −20°C prior to use. Cisplatin was prepared immediately before use as a 1000-fold concentrated solution in DMSO.

OV167 cell lines were cultured in MEM with Earle's salts and nonessential amino acids containing 20% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A). Cells were passaged once weekly and maintained at 37°C in an atmosphere containing 95% air/5% CO_2 (v/v). To determine population doubling times, 1×10^5 cells were seeded in triplicate 100-mm tissue culture plates, incubated for intervals between 24 and 240 h, trypsinized, and counted on a hemacytometer. Colony-forming assays were performed as described previously (16).

Table 1 *Results of LOH analysis in primary ovarian tumors*

Marker	13q	% LOH (no. of cases with LOH/no. of informative cases)
898	14.1	0% (0/10)
325	14.1	35% (6/17)
263	14.1	48% (12/25)
MCJ	14.1	48% (10/21)
1272	14.2	8% (2/25)
887	14.2	25% (2/8)
328	14.3	35% (6/17)
168	14.3	44% (7/16)

In brief, subconfluent cells were released with trypsin, plated at a density of 4000 cells/plate in multiple 35-mm dishes containing 2 ml of medium A, and incubated for 14–16 h at 37°C to allow cells to attach. Graded concentrations of each drug or equivalent volumes of DMSO (0.1%) were then added to triplicate plates. After a 24-h treatment, plates were washed twice with serum-free MEM and incubated in drug-free medium A for an additional 14 days. The resulting colonies were stained with Coomassie Blue and counted manually. Diluent-treated control plates typically contained 75–200 colonies and served as a basis for estimates of colony-forming efficiency for the four lines.

Flow Cytometry. Flow cytometry for cell cycle analysis was performed as reported previously (18). Briefly, cells were grown to 30–40% confluence in 100-mm tissue culture dishes, released by trypsinization, and sedimented at 200 × *g* for 5 min. All additional steps were performed at 4°C unless otherwise indicated. Samples were fixed in 50% ethanol, treated with RNase A, stained with propidium iodide, and analyzed by flow cytometry on a Becton Dickinson FACScan (San Jose, CA) using an excitation wavelength of 488 nm and an emission wavelength of 585 nm as described (19). Histograms were analyzed using ModFit software (Verity Software House, Topsham, ME). Cellular accumulation of topotecan was assessed by FACS analysis as described previously (20). Briefly, cells grown to 50–60% confluence in 100-mm dishes were incubated for 1 h in the presence of 20 μM topotecan, trypsinized in the continued presence of topotecan, and examined by FACS using an excitation wavelength of 488 nm and an emission wavelength of 585 nm.

Assessment of Cell Viability. To directly assess cell viability, cells were grown to 30% confluence in 100-mm dishes, treated with 100 nM paclitaxel for 24 h, harvested at the indicated time points, and assessed for either their ability to exclude trypan blue or apoptotic morphology by staining with Hoescht 33258, as described previously (16, 18). Floating and adherent cells from each dish were combined prior to evaluation with trypan blue or Hoescht staining.

Statistics. Reliabilities of differences in sample means (statistical significances) were calculated using the *t* distribution (two-sided) and pooled estimates of sample variances.

RESULTS

Isolation and Characterization of a Novel cDNA Containing the DNAJ Domain. We performed DD-PCR with primers HT11G and AP6 from the RNA Image kit (GenHunter Corp.) against low-passage cell lines established from primary ovarian tumors and short-term cultures of normal OCEs. Several fragments were isolated. One fragment (13) was expressed exclusively in the normal cell line and absent in three of seven tumor cell lanes. This band was isolated from the gel by standard procedures, reamplified with the same set of primers, and sequenced. Comparative sequence analysis of this fragment using the BLAST alignment revealed that the 150-bp fragment showed considerable homology to several ESTs. Computer-based walking with the available ESTs (National Center for Biotechnology Information/ BLAST) using Sequencher 3 software generated a contig of 720 bp of sequence.

```
MCJ:          LIAVGLGVAALAFAGRYAFR----IWKPLEQVITETAKKISTPSFSSYYKGGFEQKMSRR 95
              LI  GLG+AA+ F  RY  R    I K +E +       ++  +FS+YY+GGF+QKMSR
C. elegans:   LIVAGLGLAAVGFGARYVLRNQALIKKGMEAI------PVAGGAFSNYYRGGFDQKMSRA 58

MCJ:          EAGLILGVSPSAGKAKIRTAHRRVMILNHPDKGGSPYVAAKINEAKDLLETTKH 149
              EA  ILGV+PSA  AKI+ AH++VMI+NHPD+GGSPY+AAKINEAKDL+E++
C. elegans:   EAAKILGVAPSAKPAKIKEAHKKVMIVNHPDRGGSPYLAAKINEAKDLMESS 110
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Fig. 2. GAP-BLASTP alignment of MCJ (residues 40–149) with a DNAJ-like protein from *C. elegans* (GenBank accession no. U80438; cDNA CEESD64F). *Double underline,* J-domain. *Underline,* predicted transmembrane domain. +, a conservative substitution.

Analysis of this sequence for the presence of an ORF with the National Center for Biotechnology Information ORF search revealed a protein with a predicted ORF of 150 amino acids (Fig. 1). A BLAST search of protein sequences (GAP-BLASTP; Ref. 21) revealed that this putative protein had the highest homology to a *C. elegans* DNAJ containing M_r 16,500 protein of unknown function (GenBank accession no. U80438 and cDNA CEESD64F), and this homology extended beyond the DNAJ domain. The alignment of the putative protein encoded by the isolated sequences and CEESD64F (Fig. 2) showed 56% (63 of 112) identity and 73% (83 of 112) overall similarity. These two proteins are similar in two respects. In contrast to the majority of DNAJ-containing proteins, both MCJ and CEESD64F contain their DNAJ domains in the COOH-terminal half of the protein. In addition, both have a potential membrane-spanning domain (between residues 36–58 in MCJ and residues 5–23 in CEESD64F) at the NH_2 terminus of each respective protein (Fig. 2).

Expression of MCJ in Ovarian Tumor Cell Lines and Normal Tissues. Primers MCJ1 (5′-TAACTAGTTTGTCCCTA-3′) and MCJ2 (5′-CAGTGTGGTCCTTAAGC-3′) were synthesized based on the 720-bp sequence flanking the ORF. RT-PCR was performed on short-term cultures of normal OCEs and epithelial cell brushings from patients without cancer (Fig. 3*A*) and seven ovarian tumor cell lines including OV167, OV177, OV202, OV207, OV266, OVCAR5, and SKOV3. With the exception of OV167, OV202, and OV266, all other cell lines amplified the expected 540-bp fragment (Fig. 3*B*). Northern blot analysis confirmed the results obtained with RT-PCR (Fig. 3*C*). The probe used for Northern analysis was the full-length ORF probe generated by RT-PCR in the normal cell line. Expression analysis with the multiple-tissue Northern blot revealed that MCJ is expressed as ~1.2-kb message in all tissues examined (data not shown). Testis showed the highest level of expression, whereas expression in other normal tissues, including adrenal gland, total brain, fetal brain, kidney, lung, pancreas, prostate, and uterus, was much lower.

```
                                                GGTCAGGAAAGCTCAGGCAAGCCCACCCTCAGGCATTACAGCTAGACTCCGAGCTTACTGGGCAGTCATCTGATTC  75
GACCAACATCAGTTCGCAGGGCTTAAGCCCAGTCCCTTACGGCGGCTGGGGAGGGACCAGGCCCAAGTATATAAAGCTCCCTGAGGGTCC 164
GCGTTGGCTTTGCGCCTGTGAGTGTGATTCAAGAACGTCCCAGTGCCCTTGGCTCCTTTCGGAGTGTGACCCCGTGCTTGCACGGGACAC 253
GTTACCCAGCTCGGGTGAGAAGGGTATCTTCCGGGAACCTCGCCTTTAATAGCACAACGAGCGCACGGCCCCCTGGATCTGCGAGAAGGA 342
TCTGCGAGAAGAAACCGCGCTAACTAGTTTGTCCCTACGGCCGCCTCGTAGTCACTGCCGCGGCGCCTTGAGTCTCCGGGCCGCCTTGCC 431
ATGGCTGCCCGTGGTGTCATCGCTCCAGTTGGCGAGAGTTTGCGCTACGCTGAGTACTTGCAGCCCTCGGCCAAACGGCCAGACGCCGAC 520
 M  A  R  R  G  V  I  A  P  V  G  E  S  L  R  Y  A  E  Y  L  Q  P  S  A  K  R  P  D  A  D  30
GTCGACCAGCAGGGACTGGTAAGAAGTTTGATAGCTGTAGGACTGGGTGTTGCAGCTCTTGCATTTGCAGGTCGCTACGCATTTCGGATC 609
 V  D  Q  Q  G  L  V  R  S  L  I  A  V  G  L  G  V  R  A  L  A  F  A  G  R  Y  A  F  R  I  60
TGGAAACCTCTAGAACAAGTTATCACAGAAACTGCAAAGAAGATTTCAACTCCTAGCTTTTCATCCTACTATAAAGGAGGATTTGAACAG 698
 W  K  P  L  E  Q  V  I  T  E  T  A  K  K  I  S  T  P  S  F  S  S  Y  Y  K  G  G  F  E  Q  90
AAAATGAGTAGGCGAGAAGCTGGTCTTATTTTAGGTGTAAGCCCATCTGCTGGCAAGGCTAAGATTAGAACAGCTCATAGGAGAGTCATG 787
 K  M  S  R  R  E  A  G  L  I  L  G  V  S  P  S  A  G  K  A  K  I  R  T  A  H  R  R  V  M 120
ATTTTGAATCACCCAGATAAAGGTGGATCTCCTTACGTAGCAGCCAAAATAAATGAAGCAAAAGACTTGCTAGAAACAACCACCAAACAT 876
 I  L  N  [redacted]  K  G  G  S  P  Y  V  A  A  K  I  N  E  A  K  D  L  L  E  T  T  T  K  H 150
TGATGCTTAAGGACCACACTGAAGGAAAAAAAAAAGAGGGGACTTCGAAAAAAAAAAAAAGCCCTGCAAAATATTCTAAAACATGGTCTTCT 965
 *
TAATTTTCTATATGGATTGACCACAGTCTTATCTTCCACCATTAAGCTGTATAACAATAAAATGTTAATAGTCTTGCTTTTTATTATCTT 1054
TTAAAGATCTCCTTAAATTCT 1076
```

Fig. 1. cDNA nucleotide sequence and the putative protein sequence of MCJ. *Highlighted,* transmembrane domain at the NH_2 terminus and DNAJ domain at the COOH terminus of the protein. *Boxed,* signature tripeptide (*HPD*). *Underlined,* polyadenylation signal. *Small arrowheads,* positions of the introns.

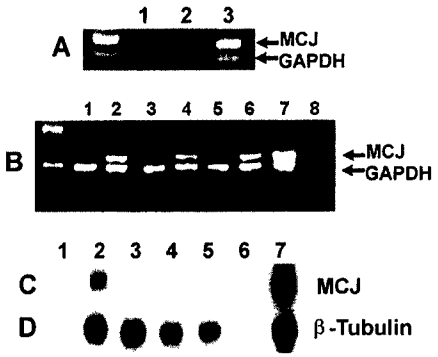

Fig. 3. *A*, agarose gel showing the products of the MCJ ORF region. *Lanes 1* and *2*, normal epithelial cell brushings from patients without cancer. *Lane 3*, short-term cultures of normal OCEs. *B*, agarose gel showing the products of the MCJ ORF region by semiquantitative RT-PCR in the ovarian cell lines. *Lane 1*, OV167; *Lane 2*, OV177; *Lane 3*, OV202; *Lane 4*, OV207; *Lane 5*, OV266; *Lane 6*, OVCAR5; *Lane 7*, SKOV3; *Lane 8*, water control. The *lane* to the *left* of *Lane 1* is a marker. *C*, autoradiograph showing the Northern hybridization results in the same cell lines (with MCJ ORF as probe) as in *A*. *Lane 1*, OV167; *Lane 2*, OV177; *Lane 3*, OV202; *Lane 4*, OV207; *Lane 5*, OV266; *Lane 6*, OVCAR5; *Lane 7*, SKOV3. *D*, tubulin hybridization of the corresponding samples.

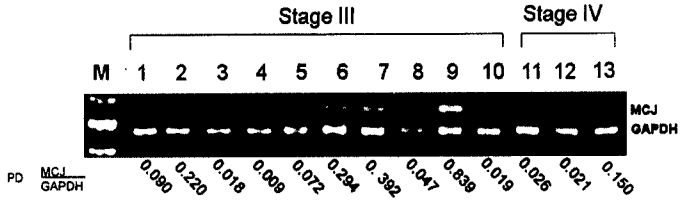

Fig. 4. Agarose gel showing the products of semiquantitative RT-PCR resolved on a 1.6% agarose gel. Sample numbers are indicated at the *top* of the figure, and the staging information for these tumors is indicated *above* the tumor numbers. *M*, 100-bp ladder. *Top band*, product of amplification with MCJ-4 and MCJ-5 primers. *Bottom band*, product of amplification with *GAPDH* primers F and R. Ratio of the band intensities of MCJ: GAPDH in pixel density are shown *below* each lane.

Cloning the Full-Length cDNA by 5′ RACE. The cDNA generated by EST-based walking was 720 bp in length. This cDNA contained a polyadenylation signal, AATAAA. However, the size of the transcript estimated by Northern analysis was 1.2 kb. To generate the missing 5′ end of this cDNA, 5′ RACE was performed with the Marathon Ready cDNA kit using RNA isolated from PC3 cells under conditions recommended by the manufacturer. We obtained an additional 354 bp of sequences with 5′ RACE. Reanalysis of the 5′ RACE sequences revealed that the ORF generated from the original 740-bp sequence was not changed (GenBank accession no. AF126743). In addition, the putative initiation codon occurs within a strong Kozak context (22) and is preceded by a stop codon (Fig. 1).

Expression Analysis of MCJ in Primary Ovarian Tumors. To determine whether MCJ was expressed in primary ovarian serous adenocarcinomas, we analyzed the expression of MCJ in 18 stage III and 3 stage IV serous ovarian carcinomas by a semiquantitative RT-PCR analysis using MCJ primers MCJ-4 (5′-GCGCTACGCTGAGTACTTGC-3′) and MCJ-5 (5′-AGATAAGACTGTGGTCAATC-3′; expected product size of 595 bp). GAPDH primers served as controls. We found that there was complete loss of MCJ expression in 5 of 15 stage III tumors and in 2 of 3 stage IV tumors. Also, 4 of 15 stage III and 1 of 3 stage IV tumors showed lower levels of MCJ expression (representative examples are shown in Fig. 4). Taken together, two-thirds (12 of 18) of the primary ovarian tumors showed either loss or diminished levels of expression by semiquantitative RT-PCR analysis.

LOH Analysis MCJ in Primary Ovarian Tumors. Sequencing a portion of the 420G23 BAC revealed a dinucleotide repeat consisting of 14 CA repeats ~80 bases downstream of the 3′ end of MCJ. We made primers (MCJ3′NF and MCJ18) flanking this repeat and found that this sequence was polymorphic in humans. We then performed LOH analysis with this new microsatellite in the primary ovarian tumor samples that showed lower levels or absence of expression of MCJ. In the tumors tested, this marker was 75% informative. None of the benign tumors but 48% of the high-stage tumors showed loss of this marker (Fig. 5).

To further delineate the region of loss, we analyzed seven other markers (*D13S263, D13S325, D13S898, D13S1272, D13S887, D13S328,* and *D13S168*) for LOH in these tumors (see Table 1 for band location and LOH data). According to the LDB database (Genetic Location database at Southampton, United Kingdom), markers *D13S1272* and *D13S887* map to 13q14.2, whereas *D13S328* and *D13S168* are in 13q14.3. The closest marker to the MCJ marker is *D13S263* (in 13q14.1), which is 100-kb distal to MCJ (23). The next highest frequency of loss was seen with the markers *D13S328* and *D13S168* in 13q14.3, which are in close proximity to the *esterase D* locus. These two markers are 10 cM away from *D13S263*.

Transcriptional Induction in the OV202 Cell Line by 5-aza-2′-dC Treatment. Because there was an absence of expression of *MCJ* mRNA in the OV202 cell line by both RT-PCR and Northern analysis, we were interested in whether methylation of this gene resulted in absence of its expression in this cell line. Therefore, we treated the OV202 cell line with the methyltransferase inhibitor 2′-deoxy-5-azacytidine to determine its effect on the transcription of the *MCJ* gene. After 2-day exposure to concentrations of 5-aza-2′-dC ranging from 1 to 5 μM, RNA was extracted from control and subjected to RT-PCR to assess *MCJ* mRNA expression. There was a dose-dependent increase in the expression of this message after treatment with 5-aza-2′-dC (Fig. 6), which is an inhibitor of DNA methyltransferases (24, 25). Because the reexpression of this message seems to be linked to the methylation status of this gene or to some other regulatory gene controlling the expression of this gene, we named this gene *MCJ*.

MS-PCR of MCJ in Cell Lines and Primary Tumors. On the basis of the results obtained with 5-aza-2′-dC, we tested cell lines lacking MCJ expression for CpG island methylation using MS-PCR (17). To distinguish unmodified from modified DNA, primers that encompassed regions containing multiple cytosines were chosen. In addition, restriction site analysis revealed the presence of rare restriction sites, such as *Sac*II and *Eag*I, in these regions. Thus, CpG pairs

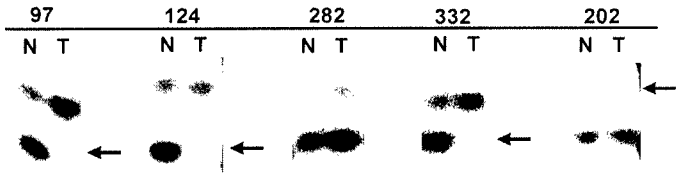

Fig. 5. Autoradiograph of LOH results of selected tumor samples with the MCJ-associated marker. For each panel, the tumor number is shown *above* the rule. *N*, normal DNA; *T*, tumor DNA. *Arrow*, loss of allele in the tumor.

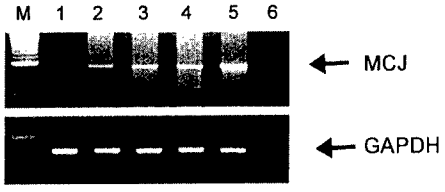

Fig. 6. Agarose gel electrophoresis of RT-PCR amplification product of *GAPDH* and *MCJ* ORF region in OV202 cell line after 5-aza-2′-dC treatment. *Lane 1*, control untreated OV202; *Lanes 2–5*, 0.1, 0.5, 1.0, and 5.0 μM 5-aza-2′-dC, respectively; *Lane 6*, water control.

near the 3′ end of the primers could provide maximum discrimination between methylated *versus* unmethylated sequences. Primers (wild-type, methyl-specific, and primers that would amplify unmethylated sequences) were synthesized flanking these restriction sites at nucleotide position 331 and at nucleotide position 484 of the MCJ cDNA sequence to amplify a 154-bp product. PCR amplification of bisulfite-modified DNA with these methyl-specific primers yielded a product both in matched normal (WBC) and tumor DNA. Sequencing of these products revealed no differences in the methylation status of either the *Sac*II, *Eag*I, or other CpG sites within this sequence between the normal and tumor samples. This indicated clearly to us that the methylation site specific for lower levels of expression was probably present 5′ to this sequence.

To check for other potential CpG sites, we sequenced the BAC 251N23 and obtained an additional 361 bp of sequences. Restriction site analysis of this additional sequence revealed the presence of a *Sma*I site 75 bases upstream of the reported cDNA sequence. Primers were designed to amplify the methylated and unmethylated sequences at this position, as described in "Materials and Methods." Using this set of primers, we amplified methylation-specific products both in normal and tumor DNA (data not shown). However, sequencing these products with the reverse primer revealed that the *Sma*I site showed the presence of both methylated unconverted Cs as well as Ts (Gs and As, respectively, in the sequence Fig. 7, *A* and *D*) in all of the normal blood DNA samples. In tumor samples expressing MCJ (tumors 183 and 270), only the unmethylated fully converted Ts (A in the opposite strand) are seen. Panels *B* and *C* in Fig. 7 show the sequence of the MS-PCR product amplified with methyl-specific primers in the blood and tumor DNA, respectively, of patient 183. In tumor samples with complete loss of MCJ expression (tumors 202, 220, 332, 485, 97, and 107), only the nonconverted methylated Cs (G as seen in Fig. 7*E*) were visible at the *Sma*I site. The sequence of the MS-PCR product amplified with methyl-specific primers in the blood and tumor DNA, respectively, of patient 485 is shown in Fig. 7, *D* and *E*. In addition, in tumor samples with complete loss of MCJ expression, we saw the loss of the other allele by LOH (Fig. 7*E, inset,* for tumor 485). Table 2 lists the results of the RT-PCR expression analysis, along with the MSP-PCR results and LOH status, in 18 high-stage tumors with and without the loss of expression of MCJ. In tumors 202, 220, 332, 485, and 107 (which have all lost MCJ expression), there is a loss of one allele (LOH analysis) and loss of expression of the other allele, attributable to methylation in the same tumor. In tumors with lower levels of expression (tumors 121, 124, 323, and 282) or normal MCJ expression (tumors 183, 417, and 531), we did not see LOH of the *MCJ* allele (Fig. 7*C, inset*). This marker, however, was uninformative in some of the samples. In tumors 183 and 270, the presence of a clear RT-PCR product also corresponded with the presence of only unmethylated alleles at this site (Fig. 7*C*). In samples with lower levels of expression and no LOH, the presence of both methylated and unmethylated alleles was seen at this site (Table 2).

Genomic Organization and FISH Mapping of MCJ. We isolated two different BACs by screening the Research Genetics BAC pools. Sequencing of these two BACs with cDNA-specific primers revealed that the coding region of *MCJ* is interrupted by introns. We assembled the exonic sequences to the sequence of the BAC 335G18 that was available on the HTGS database. *MCJ* spans ~83 kb of genomic DNA and is interrupted by five introns. The primers spanning the intron/exon sequences are listed in Table 3. The 3′ end BAC 421G23 was used to map *MCJ* to chromosome 13q14.1 by FISH analysis (data not shown). This mapping reconfirmed the EST-based mapping of one of the ESTs (AA812596) used to build the cDNA contig.

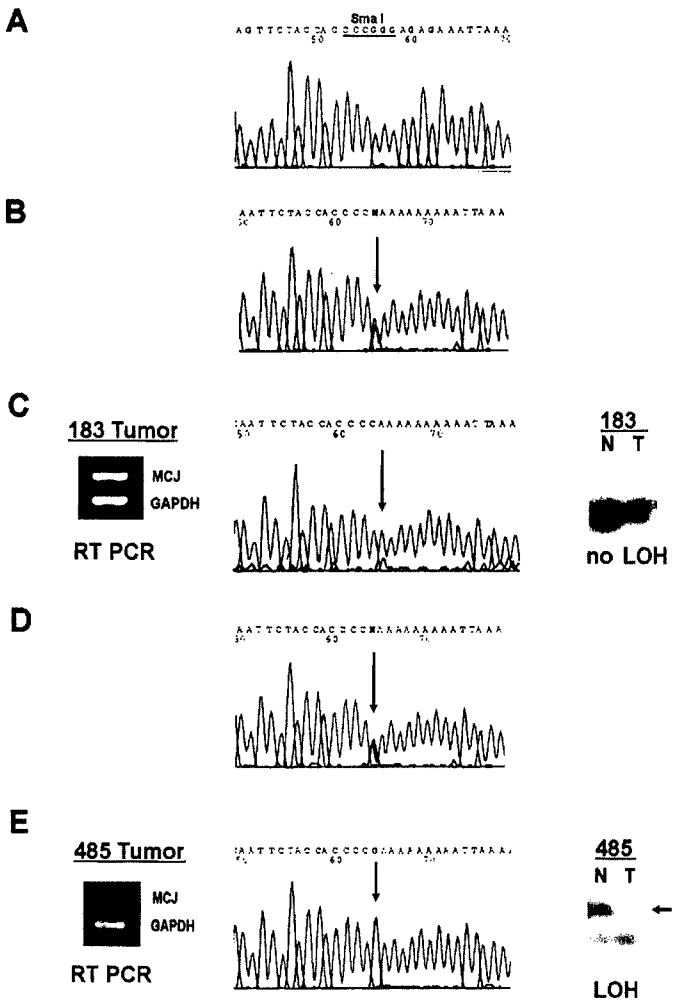

Fig. 7. *A*, 5′ end sequence of MCJ. *Underlined, Sma*I site. *B–E,* sequence of the MS-PCR product amplified with methylation-specific primers (*B* and *D*, in the blood of patient 183 and 485, respectively). *Arrow,* presence of both methylated and unmethylated alleles at the *Sma*I site. *C*, tumor of patient 183. *Arrow,* presence of only unmethylated alleles at the *Sma*I site. *Left inset,* result of semiquantitative RT-PCR of *MCJ* and *GAPDH*. Notice the expression of *MCJ* in the tumor. *Right inset,* result of LOH analysis of this marker in this tumor. *E,* tumor of patient of 485. *Arrow,* presence of only the methylated allele at the *Sma*I site. *Left inset,* result of semiquantitative RT-PCR of *MCJ* and *GAPDH*. Notice the absence of expression of *MCJ* in the tumor. *Right inset,* result of the LOH analysis of this marker in this tumor. Notice that there is LOH of this marker in this tumor. Because the products of the MS-PCR were sequenced with the reverse primer only, the observed Gs and As correspond to Cs and Ts in the opposite strand.

Mutational Analysis of MCJ in Primary Tumors. Primers (Table 3) were synthesized from intronic sequences flanking individual exons. Individual exons were amplified from matching blood and tumor DNA from several patients and sequenced directly to check for mutations within the coding sequences. Whereas several sequence polymorphisms were seen, no tumor-specific mutations were detected in any of the exons.

Functional Analysis of MCJ in OV167. A parental MCJ-nonexpressing primary ovarian carcinoma cell line (OV167), vector transfected control, and two stable MCJ clones (6 and 13) were tested for the expression of MCJ by semiquantitative RT-PCR. Only the two MCJ transfectants expressed the MCJ transcript (Fig. 8*A*). Examination of the four OV167 lines demonstrated no consistent differences between MCJ-high (clones 6 and 13) and MCJ-nonexpressing (OV167 and empty vector transfectant) lines with respect to doubling time (*i.e.,* proliferation rate). In particular, doubling times for the parental OV167 and the vector control were 5.0 and 3.0 days, respectively, whereas doubling times for the two MCJ transfectants (clones

Table 2 *Results of RT-PCR, LOH, and MSP analysis in high-stage tumors*

Sample	Stage	RT-PCR	LOH	MSP *Sma*I site
98	III	+[a]	−	NT[b]
183	III	+	−	UNM
209	III	+	UI	NT
270	III	+	UI	UNM
417	III	+	−	M/UNM
531	III	+	−	NT
121	III	L	−	M/UNM
124	III	L	+[c]	NT
285	III	L	UI	M/UNM
305	III	L	UI	M/UNM
323	III	L	UI	M
282	IV	L	−	M/UNM
202	III	−	+	M
220	III	−	+	M
332	III	−	+	M
485	III	−	+	M
97	IV	−	+	M
107	IV	−	+	M

[a] NT, not tested; L, lower levels of expression; UI, uninformative; M, methylated *Sma*I site; UNM, unmethylated *Sma*I site; M/UNM, presence of both methylated and unmethylated allele at the *Sma*I site.
[b] +, the presence of a product in RT-PCR.
[c] +, LOH.

6 and 13) were 3.0 ± 1.0 and 3.5 ± 0.5 days, respectively (not significant). Colony-forming efficiencies were also similar in the MCJ-nonexpressing (OV167, 5.33 ± 1.1%; vector control, 2.95 ± 0.83%) and MCJ-high (clone 6, 0.95 ± 0.62%; clone 13, 2.55 ± 0.84%) lines (not significant), although transfection tended to somewhat reduce colony-forming ability of the parental line.

Despite the above similarities, the four lines displayed clear differences in their sensitivities to paclitaxel, topotecan, and cisplatin, as assessed by colony-forming assays. The two MCJ-nonexpressing lines displayed IC_{50}s that were 3.5-fold higher for paclitaxel ($P < 0.005$; Fig. 8*B*), 2.2-fold higher for topotecan ($P < 0.0005$; Fig. 8*C*), and 2-fold higher for cisplatin ($P < 0.005$; data not shown) than the IC_{50}s of the MCJ-expressing lines (clones 6 and 13).

To confirm that the observed differences in colony formation were reflective of differences in cell killing, we also examined the sensitivities of the lines to paclitaxel by directly assessing cell death (using trypan blue staining) and apoptosis (using Hoescht 33258 staining). Trypan blue staining confirmed that the two MCJ-expressing clones were more sensitive to paclitaxel-induced cytotoxicity (data not shown). Hoescht staining showed that the MCJ-expressing lines were similarly more sensitive to paclitaxel-induced apoptosis (Fig. 8*D*).

Although the MCJ-high and MCJ-nonexpressing lines did not vary significantly with respect to doubling time, we had some concern that the observed resistance of the MCJ-deficient lines to the cell cycle-dependent agents paclitaxel and topotecan might be attributable to differences in cell cycle distributions. To evaluate this possibility, we examined cell cycle distribution in all four lines. As shown in Fig. 8*E*, the cell cycle distributions of the four lines were similar and could not, therefore, explain the observed differences in drug sensitivity.

In an effort to determine whether differential drug accumulation

Table 3 *Intron/exon primer sequences*

Lowercase letters are intronic sequences, and uppercase letters represent exon sequences. Splice donor and acceptor sites, in lowercase letters, are underlined. The polyadenylation (AATAAA) sequence in 3′ untranslated region is underlined.

Intron	Exon #	Exon Size	Intron	Size (bp)
	1	531	GCAGAGACTGgtgatccctgcca	41963
ttttcttccatagGTAAGAAGTT	2	54	GCATTTGCAGgtaagataaagaat	3189
cttttactatttagGTCGCTACGC	3	72	TCAACTCCATgtaagttaaacgtgg	9609
ctttcctatacagAGCTTTTCATC	4	77	TAGGTGTAAGgtaggtgtgcaataa	7078
atttctttttacagCCCATCTGCTG	5	73	CCAGATAAAGgtaggtagaattcct	21336
tttgttctctccagGTGGATCTCCTT	6	267	TAACAATAAAATGTTAATA	
			GTCTTGCTTTTTATTATCTTT	
			TAAAGATCTCCTTAAATTCT+polyA	

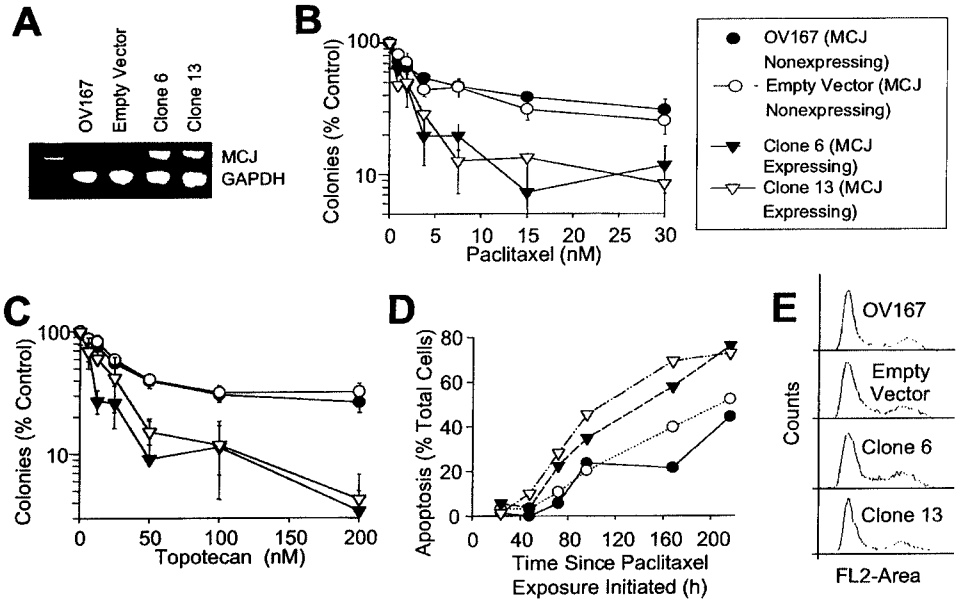

Fig. 8. Effects of paclitaxel and topotecan in MCJ-nonexpressing and MCJ high-expressing ovarian cell lines. *A*, agarose gel electrophoresis of RT-PCR amplification product of GAPDH and MCJ. The *lane* to the *left* of *Lane 1* is the marker. *Lane 1*, parental OV167; *Lane 2*, vector transfected OV167; *Lanes 3* and *4*, stable MCJ clones 6 and 13. *B* and *C*, effects of paclitaxel (*B*) or topotecan (*C*) on colony formation in OV167 cell lines. Cells were exposed to the indicated drug for 24 h, followed by incubation in drug-free medium for 12–14 days to allow colonies to form. Each *data point* represents the mean colony count from triplicate plates. *B* and *C*, $P < 0.005$ and $P < 0.0005$, respectively, as indicated in the text. The results are each representative of four independent experiments; *bars*, ±1 SD. *D*, effects of paclitaxel on induction of apoptosis in OV167 cell lines. Cells were exposed to paclitaxel for 24 h and incubated in drug-free medium. After combining floating and adherent cells from each plate, apoptosis was assessed by Hoescht 33258 staining. Data shown are representative of duplicate experiments. *E*, cell cycle distributions of subconfluent OV167 cell lines. Cells were grown to 30% confluence, harvested, fixed, stained with propidium iodide, and examined using FACS as described in the text.

might be responsible for the observed differences in drug sensitivities of MCJ-high and MCJ-low lines, we also examined topotecan accumulation in the four lines by FACS analysis (20). These studies showed no significant difference between the lines (data not shown), eliminating the possibility that differential drug accumulation was responsible for the observed differences in drug sensitivities.

DISCUSSION

In the United States, ovarian cancer is the fourth most common cause of cancer-related deaths among women. Approximately 23,000 women are diagnosed with and ~14,000 women die from ovarian cancer annually in the United States (26). Although women with low-stage ovarian cancer have a good prognosis, most women are diagnosed with late-stage disease and eventually succumb to their cancer (27). Much progress, therefore, remains to be made in the early diagnosis and treatment of ovarian cancer. A major concern in treating ovarian cancer patients is the frequent development of resistance to chemotherapy. Whereas most patients initially respond to the commonly used chemotherapeutic drugs, resistance to these drugs usually develops, and the patients eventually succumb to the disease. Many mechanisms have been postulated to explain this resistance (28–33), but these remain to be tested in clinical materials. Accordingly, there is considerable interest in identifying genes that could differentiate between chemosensitive and chemoresistant ovarian tumors.

Similar to cancers of other tissues, multiple genetic alterations are common in ovarian carcinomas. Alterations in tumor suppressor genes such as *p53* (34), *pRB* (35), and *NOEY2* (36) have been implicated in ovarian carcinogenesis. Chromosomal regions of loss have frequently identified new tumor suppressor genes involved in either the initiation, progression, or metastasis of cancer-related genes. In the present study, we report the discovery of a novel gene (*MCJ*) that we identified using DD-PCR between ovarian tumor cell lines and short-term cultures of normal OSEs. Expression of this gene was either absent or reduced in a majority of primary ovarian tumors and ovarian carcinoma cell lines. In specimens lacking *MCJ* expression, one allele was lost and the other silenced by methylation. Interestingly, a comparison of the *MCJ*-expressing and *MCJ*-nonexpressing low-passage primary ovarian carcinoma cell lines implicates *MCJ* loss in conferring resistance to the three drugs most commonly used in the treatment of ovarian cancer. These findings have potentially important implications for ovarian cancer development and treatment.

After *MCJ* was identified by DD-PCR, analysis of the ORF revealed that *MCJ* is a new member of the DNAJ family of proteins with sequence identity between MCJ and other DNAJ domain-containing proteins ranging from 30 to 50%. The major difference between MCJ and most other DNAJ-like proteins is the location of the DNAJ domain. Expression analysis of MCJ on a multiple-tissue Northern blot showed that this gene was highly expressed in testis. In this respect, it is similar to the *Drosophila melanogaster* DNAJ protein, DNAJ60 (37). Iliopoulos *et al.* (37) have shown that DNAJ60 encodes a putative protein of 217 amino acids with a molecular mass of 27.7 kDa and a pI of 10.5 that may play an important function during spermatogenesis and/or in the male genital tract. Whereas we have no evidence at present about a testis-specific function of MCJ, it is interesting to note that both MCJ and DNAJ60 are extremely basic proteins with similar pIs of 10.35. Another member of the DNAJ family of proteins with testis-specific expression is MSJ-1 (38). However, sequence analysis of MCJ revealed that it had no significant homology to MSJ-1.

We have shown that the absence of expression of *MCJ* is related directly to the methylation status of this gene. In the OV202 cell line, induction of *MCJ* is observed after 5-aza-2′-dC treatment. This is the first report linking methylation to the absence of expression of a DNAJ-like protein. The cell lines with loss of expression of *MCJ* were all cell lines derived from primary tumors harvested at the time of surgery, and therefore, the methylation pattern seen in these cell lines is a *de novo* effect and not the result of following exposure to chemotherapeutic agents *in vivo*. LOH analysis on 13q14.1 identifies this as a new region of LOH in ovarian cancer in the region of *MCJ*. We have shown LOH of the marker identified only 80 bases downstream of the 3′ end of the *MCJ* gene and that there is loss of an *MCJ* allele in some of the tumors not expressing *MCJ*. Whereas we have not found any tumor-specific mutations in the *MCJ* coding region, we have seen loss of expression of this gene both by LOH and hypermethylation in the same tumor. Although there are no reports of a DNAJ domain protein acting as a tumor suppressor, our data clearly indicate the mechanism for loss of function of this gene is probably attributable to loss of DNA sequences by deletion (LOH) and to hypermethylation. In this regard, this gene fits the criteria as a class II tumor suppressor.

To examine the effects of differences in *MCJ* expression, we evaluated the cytotoxic effects of cisplatin, paclitaxel, and topotecan in cells lacking or expressing *MCJ*. Cisplatin/carboplatin and Taxol are the most effective drugs in the treatment of ovarian cancer, and the combination of carboplatin/paclitaxel has been widely accepted as standard treatment for advanced ovarian cancer (28). Several lines of evidence support the idea that there is a direct correlation between the induction of apoptosis and drug sensitivity. For example, inactivation of the *p53* gene could confer resistance to cisplatin and DNA-damaging agents as measured by both the induction of apoptosis and resulting antiproliferative effects (33, 34). It is clear from several studies that there may be multiple mechanisms involved in determining drug resistance (30, 31). Our preliminary studies suggest that loss of *MCJ* in ovarian carcinoma may be of potential functional significance. In particular, *MCJ* loss appears to be associated with *de novo* resistance to the antineoplastic agents paclitaxel, topotecan, and cisplatin in the OV167 cell line. On the other hand, it is important to note that the magnitude of resistance (2–3.5-fold) conferred by *MCJ* loss is less than resistance conferred by some other means. For example, overexpression of P-glycoprotein can confer a much greater level of resistance to paclitaxel (16, 39). On the other hand, the 2–3.5-fold resistance to these agents might be significant in the clinical setting, particularly when combined with other resistance-inducing changes. We speculate, therefore, that MCJ loss may have potential prognostic significance in ovarian cancer. The impact of MCJ loss within the context of the full spectrum of genetic alterations in ovarian cancer, however, remains to be more fully elucidated. It would be very informative to look at sequential tumor specimens derived from patients undergoing multiple surgeries to try to correlate the *in vitro* and *in vivo* chemoresistance characteristics.

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Genetic Analysis of Early- *versus* Late-Stage Ovarian Tumors[1]

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ABSTRACT

In the United States, ovarian cancer is the fourth most common cause of cancer-related deaths among women. The most important prognostic factor for this cancer is tumor stage, or extent of disease at diagnosis. Although women with low-stage tumors have a relatively good prognosis, most women diagnosed with late-stage disease eventually succumb to their cancer. In an attempt to understand early events in ovarian carcinogenesis, and to explore steps in its progression, we have applied multiple molecular genetic techniques to the analysis of 21 early-stage (stage I/II) and 17 advanced-stage (stage III/IV) ovarian tumors. These techniques included expression profiling with cDNA microarrays containing approximately 18,000 expressed sequences, and comparative genomic hybridization to address the chromosomal locations of copy number gains as well as losses. Results from the analysis indicate that early-stage ovarian cancers exhibit profound alterations in gene expression, many of which are similar to those identified in late-stage tumors. However, differences observed at the genomic level suggest differences between the early- and late-stage tumors and provide support for a progression model for ovarian cancer development.

INTRODUCTION

Of the cancers unique to women, ovarian cancer has the highest mortality rate. Over 26,000 women are diagnosed with this disease in the United States annually, and 60% of those diagnosed will die of the disease (1). There has been little change in ovarian cancer incidence and mortality over the past 5 decades and unfortunately there are a number of significant barriers to progress in its treatment. These include poor understanding of the underlying biology of this disease, inadequate screening tools as well as few early warning signs. The 5-year survival for patients with stage I disease can exceed 90%, but it is less than 25% for advanced-stage disease (2). These statistics underscore the need for better tools for the screening and staging of ovarian cancer.

Like other solid tumors, ovarian cancer is thought to result from an accumulation of genetic alterations. These events lead to changes in expression of many genes. Alterations in tumor suppressor genes such as *p53* (3, 4), *pRB* (3, 5), *NOEY2* (6), *BRCA1* (7), and oncogenes such as *K-ras* (8), *c-myc* (9) and *c-erbB-2* (10) have been shown to play an important role in ovarian carcinogenesis. There is very little information, however, on the sequence of genetic changes that are associated with progression of disease from early to advanced stage.

Examining tumors for alterations in gene expression is a potentially useful approach to identifying molecular differences between early- and late-stage ovarian carcinomas. Although there are several approaches to investigate differential gene expression in tumors, here we have used cDNA microarrays to develop expression profiles of early- and late-stage ovarian cancer (11–13). To extend our understanding of any stepwise genetic alterations that may underlie the assumed clinical progression from early- to late-stage ovarian cancer, CGH[3] (14) was used to identify regions of genome loss and gain in the same series of tumors.

MATERIALS AND METHODS

Tissue Processing and Tumor Selection. Surgically removed ovarian tumors were snap-frozen in the surgical pathology unit of the Mayo Clinic. The tumor content of the specimens was assessed by H&E-stained sections. Only high-grade specimens containing more than 75% tumor were used for these experiments. Twenty normal OSEs from patients without cancer were used as normal controls. The epithelial nature of these brushings was verified by cytokeratin staining. The majority of these patients were between 45 and 65 years old, undergoing incidental oophorectomy at the time of pelvic surgery for other indications. All of the ovaries were examined pathologically and found to be benign or they were excluded. RNA from five such pooled brushings were profiled on cDNA microarrays along with five stage I, two stage II, and seven stage III ovarian tumors. The histology, grade, and stage of each tumor used in cDNA microarray, semiquantitative RT-PCR, and CGH studies are listed in Tables 1 and 5, respectively. Tumors were staged according to International Federation of Gynecology and Obstetrics criteria.

Ovarian Cell Lines. Five of seven ovarian-carcinoma cell lines (OV 167, OV 177, OV 202, OV 207, and OV 266) were low-passage primary lines established at the Mayo Clinic (15). OVCAR-5 is a NIH cell line (16) and SKOV-3 was purchased from American Type Culture Collection (Manassas, VA). All of the cells were grown according to the provider's recommendations.

cDNA Microarrays. The cDNA microarray consisted of 25,000 elements from the Unigene set from Research Genetics Inc. (Huntsville, AL), which included 10,000 known genes; 13,000 ESTs; and 2,000 elements made up of control genes, dyes, bacterial genes, and water controls. After consolidating genes or ESTs belonging to the same Unigene clusters, we estimated that there were a total of 18,304 unique genes or ESTs. All of the genes and ESTs on the array were sequence verified before being arrayed on a high-precision robotics platform. Each 3- × 5-inch membrane contained up to 6,000 elements and each probe was hybridized to five sets of membranes in duplicate.

Total RNA Isolation and Labeling. Total RNA was extracted from specific tumor samples using Trizol (Life Technologies, Inc., Rockville, MD) as recommended by the manufacturer after estimating the tumor content by standard histological methods. The integrity of the RNA was assessed by ethidium bromide staining after agarose gel electrophoresis. Total RNA was labeled with [α-^{33}P]CTP after reverse transcription with oligo(dT)$_{30}$. Fifteen μg of total RNA was annealed to 3 μg of oligo-dT$_{30}$ primer in 13 μl of total volume and incubated at 70°C for 2 min in a PCR machine and held at 4°C.

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[3] The abbreviations used are: CGH, comparative genomic hybridization; OSE, ovarian epithelial cell brushing; EST, expressed sequence tag; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 *Tumor cohort*

Histology	Stage	Grade	cDNA microarray	RT-PCR
Cl cell OV 106	I	3	+[b]	+
Cl cell OV 267	I	3		+
Cl cell OV 496	I	3	+	+
Endo OV 51	I	3		+
Endo OV 78	I	3		+
Endo OV 88	I	3		+
Endo OV 105	I	3		+
Endo OV 338	I	3		+
Endo OV 647	I	3	+	+
Serous OV 6	I	3		+
Serous OV 17	I	3	+	+
Serous OV 20	I	3		+
Serous OV 90	I	3		+
Serous OV 234	I	3		+
Serous OV 363	I	3		+
Serous OV 526	I	3	+	+
Cl cell OV 102	II	3		+
Endo OV 296	II	3		+
Serous OV 149	II	3		+
Serous OV 354	II	3		+
Serous OV 401	II	3	+	+
Serous OV 402	II	3	+	+
Serous OV 414	II	3		+
Cl cell OV 176	III	3		+
Endo OV 93	III	3		+
Endo OV 110	III	3		+
Endo OV 259	III	3		+
Serous OV 4	III	3	+	+
Serous OV 11	III	3	+	+
Serous OV 13	III	3	+	+
Serous OV 16	III	3	+	+
Serous OV 29	III	3	+	+
Serous OV 150	III	3	+	+
Serous OV 167	III	3		+
Serous OV 206	III	3		+
Serous OV 208	III	3		+
Serous OV 461	III	3		+
Serous OV 472	III	3	+	+
Serous OV 97	IV	3		+

[a] Cl, clear; OV, ovarian; Endo, endometrioid.
[b] +, the tumors used in each analysis.

Reverse transcription of this annealed template was carried out in a final volume of 50 μl, 1× first strand buffer (DTT, 40 units of Rnase inhibitor, 400 units of Superscript II reverse transcriptase, 2 μl of 10 mM dNTP mix with dCTP at 0.1 mM concentration, 100 μCi of [α-^{33}P] dCTP at 42°C for 60 min. The reaction was stopped by consecutive addition of the following reagents: 4 μl of 50 mM EDTA, 4 μl of 0.5 M NaOH, and 2 μl of diethylpyrocarbonate water. Heating at 65°C for 10 min degrades the RNA template in the reaction. Unincorporated nucleotides were removed from the labeled probe using a Clontech (Palo Alto, CA) Chroma Spin TE-30 spin column. The labeled probe with an estimated specific activity of 1×10^6 cpm/μl was preannealed to 100 mg of COT1 DNA to block repetitive sequences in a final volume of 100 μl. The blocked probe was heated for 5 min at 95°C followed by 65°C for 20 min and then added to the duplicate filters along with 5 μg/ml of denatured salmon sperm DNA. The filters were hybridized overnight at 65°C in a Hybaid hybridization oven. Each probe was hybridized to duplicate filters. The next day, the filters were washed for 15 min at 65°C with 4% SDS solution in 20 mM sodium phosphate (pH 7.2) with 1 mM EDTA followed by 1% SDS solution in the same buffer with three changes at 65°C. After the wash, the filters were dried between whatman papers and baked at 80°C for 1 h. The filters were imaged on a storm 840 phosphoimager after 24–48 h exposure.

Image Analysis and Data Recovery. The ArrayVision program (Imaging Research Inc., St. Catharines, ON, Canada) was used to quantify the spot intensities of the scanned images for each membrane. For each membrane, the spot intensities were normalized by the median intensity for the entire array, resulting in a new median intensity of 1.0. Spots that were <0.1 after normalization were thresholded to 0.1 to account for the level of background noise. After normalization and thresholding, spot intensities were averaged across the duplicate filters for each tissue sample. Fold-regulation for each tumor, at each gene, was determined by calculating the ratio of tumor expression:average expression for the five (pooled) control normal OSEs.

Semiquantitative RT-PCR. Reverse transcribed cDNAs (50–100 ng) were used in a multiplex reaction with gene-specific primers and GAPDH, forward primer (5′-ACCACAGTCCATGCCATCAC-3′) and reverse primer (5′-TCCACCACCCTGTTGCTTGTA-3′) yielding a 450-bp product. The PCR reaction mixtures consist of 50 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 400 μM concentration of each primer for the specific gene to be analyzed (50 μM for the GAPDH primers), and 0.5 units of Taq polymerase (Promega, Madison, WI), in a 12.5-μl reaction volume. The conditions for amplification were as follows: 94°C for 3 min, then 29 cycles of 94°C for 30 s, 50–62°C for

Table 2 *Differential gene expression in ovarian tumors: number of sequences with expression changes >2-fold, 5-fold, 10-fold, and 20-fold*

No. of tumors	Down-regulated				Up-regulated			
	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold
14	148	24	9	2	11	1	0	0
13	262	48	16	6	30	6	1	0
12	409	56	19	6	43	9	2	1
11	565	68	21	7	67	11	5	1
10	719	87	24	8	108	12	5	1
9	945	108	27	11	168	15	6	1
8	1261	135	34	12	265	25	8	1
7	1750	179	46	12	435	36	12	3
6	2346	248	56	18	794	51	18	5
5	3115	409	115	42	1417	72	27	7
4	4159	565	144	52	2510	116	32	12
3	5718	781	187	65	4303	198	51	25
2	8245	1159	279	81	7051	388	105	42
1	12106	2940	1044	414	11178	1552	480	183

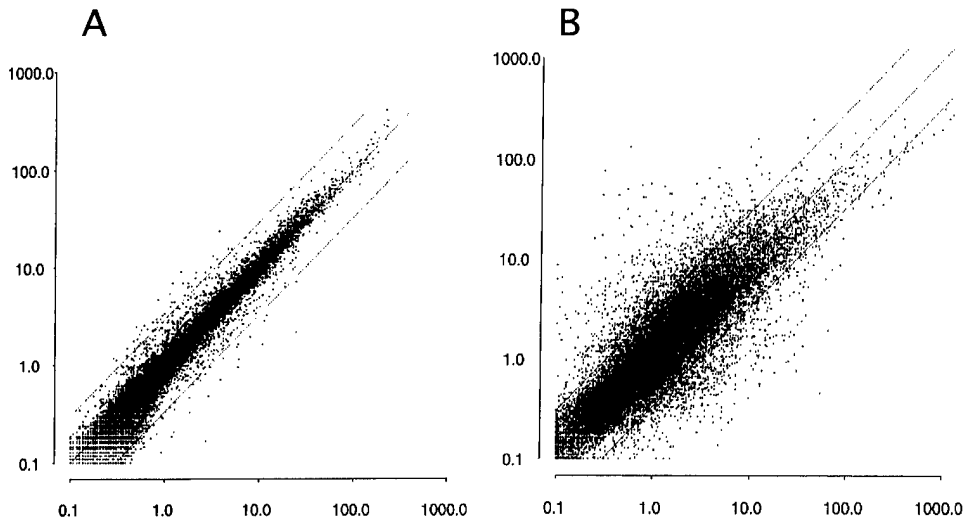


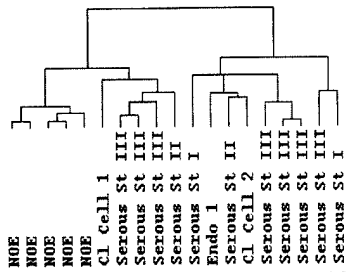
Fig. 1. Representative scatter plots of cDNA microarray analysis. *A*, duplicate filters of OV 106 hybridization (Pearson correlation, 0.97). *B*, scatter plot of an early-stage tumor (OV106) *versus* one of the pooled normal epithelial brushings (Pearson correlation coefficient, 0.76). The *lines*, 2-fold differences in expression levels between samples.

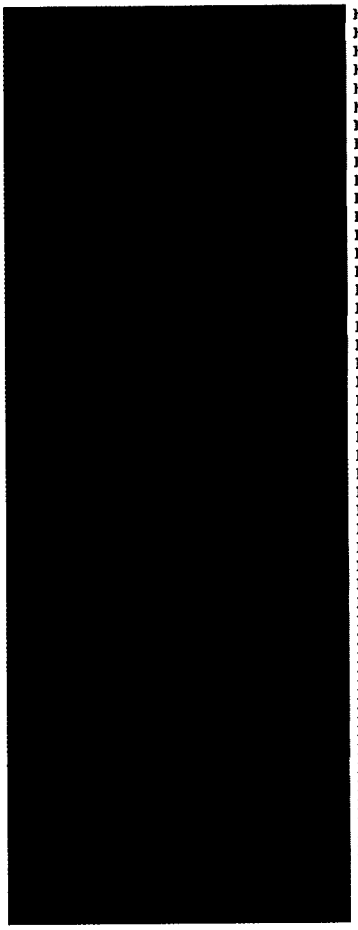
Table 3 *Differential gene expression as a function of tumor stage*

	Stage I/II down				Stage III/IV down				Stage I/II up				Stage III/IV up			
No. of tumors	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold	2-fold	5-fold	10-fold	20-fold
7	200	35	11	2	406	56	18	6	19	2	0	0	48	10	3	0
6	413	62	19	6	739	91	22	9	64	8	2	1	99	12	4	1
5	759	86	26	8	1393	154	36	11	185	19	7	1	239	16	5	1
4	1322	152	38	13	2360	321	99	42	498	39	15	3	714	27	8	2
3	2500	275	65	16	3365	508	127	47	1311	92	36	9	1615	65	17	8

30 s depending on the gene-specific primers being tested, and 72°C for 30 s in a Perkin-Elmer-Cetus (Norwalk, CT) 9600 Gene-Amp PCR system. The products of the reactions were resolved on a 1.6% agarose gel. Band intensities were quantified using the Gel Doc 1000 photo-documentation system (Bio-Rad, Hercules, CA) and its associated software. Gene specific primers were as follows: for *Gas 1*, forward, CGC GCC TCG TCT CCT TTC CC, and reverse, GGC GCG TGG GCT AAA AGA GC; for *PAI1*, forward AAT CGC AAG GCA CCT CTG AG, and reverse, GAT CTG GTT TAC CAT CTT TT; for *StAR*, forward, GAC CCC ACC ACT GCC ACA TT, and reverse, GAT CTT AGA CTT GCA GGC TT; for *AREG*, forward, CCG CTG CGA AGG ACC AAT GA, reverse, CTA TGA CTT GGC AGT CAG TC; for *FGF7*, forward, TAA TGC ACA AAT GGA TAC, and reverse, ATT GCC ATA GGA AGA AAG; for *HPR6*, forward, TGC CTA GCG CGG CCC AAC, and reverse, CAG ACT GGA CTG TTA CAA ATG; for *ITM2A*, forward, CGC AGC CCG AAG ATT CAC TAT G, and reverse, R-CTT ATT ACC AAG GAC ACT CTA TCT; and for *decorin*, forward, CCT GGT TGT GAA AAT ACA TGA, and reverse, TGA CAT TAA CAA GAT TTT GCC.

CGH. Metaphase spreads from normal human lymphocytes were prepared using standard protocols (17). The slides were aged for 2–3 days prior to denaturation at 70°C by 70% formamide in 2× SSC, followed by dehydration in a series of ethanol-water mixtures. The slides were treated with proteinase K, at a concentration of 0.1 μg/ml in 20 mM Tris (pH 7.5)-2 mM $CaCl_2$ prior to hybridization. The CGH procedure was similar to published standard protocols (14, 17). Twenty images were captured using a Nikon Labophot-2





Hs.2296 apolipoprotein C-I
Hs.1257 amphiregulin
Hs.75284 CD36 antigen
Hs.41585 ARP2 (actin-related protein 2, yeast)
Hs.38586 hydroxy-delta-5-steroid dehydrogenase
Hs.70008 low density lipoprotein receptor
Hs.37012 hydroxysteroid (11-beta) dehydrogenase 1
Hs.3281 neuronal pentraxin II
Hs.105806 granulysin
Hs.21858 CAG repeat domain
Hs.93769 CAG repeat domain
Hs.113779 cytochrome P450, subfamily XIX
Hs.113405 diacylglycerol kinase, alpha (80kD)
Hs.106194 cytochrome P450, subfamily XIX
Hs.94107 glutathione S-transferase A2
Hs.102484 glutathione S-transferase A3
Hs.1363 cytochrome P450, subfamily XVII
Hs.3132 steroidogenic acute regulatory protein
Hs.117848 hemoglobin, epsilon 1
Hs.12409 somatostatin
Hs.1119 nuclear receptor subfamily 4, group A,
Hs.101664 interleukin enhancer binding factor 3,
Hs.28441 ring finger protein
Hs.1279 complement component 1, r subcomponent
Hs.3076 MHC class II transactivator
Hs.9348 general transcription factor II, i
Hs.94804 adenylate cyclase 1 (brain)
Hs.89040 prepronociceptin
Hs.347 lactotransferrin
Hs.2256 matrix metalloproteinase 7 (matrilysin,
Hs.80395 mal, T-cell differentiation protein
Hs.59889 3-hydroxy-3-methylglutaryl-Coenzyme A
Hs.75741 amiloride binding protein 1
Hs.7614 calmodulin 2 (phosphorylase kinase, delta)
Hs.99348 distal-less homeo box 5
Hs.1494 msh (Drosophila) homeo box homolog 1
Hs.25195 endometrial bleeding associated factor
Hs.28988 glutaredoxin (thioltransferase)
Hs.14958 phytanoyl-CoA hydroxylase (Refsum disease)
Hs.82009 cytochrome P450, subfamily I
Hs.111661 aspartylglucosaminidase
Hs.119708 glutathione peroxidase 3 (plasma)
Hs.103176 small inducible cytokine subfamily B
Hs.75758 immunoglobulin gamma 3 (Gm marker)
Hs.111572 immunoglobulin lambda gene cluster
Hs.105678 H.sapiens gene encoding kappa light chain
Hs.118641 immunoglobulin gamma 3 (Gm marker)
Hs.140 immunoglobulin gamma 3 (Gm marker)
Hs.109646 NADH dehydrogenase (ubiquinone)
Hs.36137 hepatocyte nuclear factor 3, gamma

Fig. 2. Hierarchical clustering of 19 ovarian epithelial samples, using Eisen's Stanford clustering package. Eighteen thousand expressed sequences were profiled against five normal ovarian epithelial (NOE), two early-stage clear cell, one early-stage endometrioid, four early-stage serous, and seven late-stage (stage-III) serous samples. The 50 annotated clones with maximal normalized expression variance (SD divided by mean of expression) are shown. Data were log-transformed after addition of a baseline noise compensation term and were clustered using centered Pearson correlation. Whereas the normal samples neatly cluster together, separation between early- and late-stage samples is less compelling.

Fig. 3. Hierarchical clustering of 14 ovarian tumor samples, using Eisen's Stanford clustering package. Data were log-transformed and clustered by centered Pearson correlation. Cluster tree analysis of five times or more up-regulated genes in early- and late-stage tumors. *Red*, fold up-regulation of five times or more. *Green boxes*, fold regulation between 1 and 2. *Different shades of green*, values above 2 but less than 3.

microscope equipped with an automatic filter wheel and an 83,000 filter set (Chroma, Brattleboro, VT) with single band pass exciter filters for UV/FITC (490 nm), 4′,6-diamidino-2-phenylindole (360 nm), and rhodamine (570 nm), and were analyzed using the QUIPS CGH software version 3.12 (Vysis Inc., IL). Using this software, the ratio of rhodamine:FITC signal is expressed as a red:green ratio, with deviations from a 1:1 ratio indicative of gain or loss of chromosome material. The lower and upper limits for gain and loss were established by performing control CGH experiments with a well-characterized tumor cell line, IMR32 (18), and DNA derived from male or female normal tissue. On the basis of these findings, a 95% confidence interval for gain and loss was set at 1.20 and 0.80, respectively, with gene amplification defined as gain >1.5.

Cluster Analyses. Hierarchical cluster analyses were performed using the Cluster software package (19). Genes were clustered using the Pearson correlation coefficient as the distance metric, and clusters were agglomerated using the average linkage criterion. Graphical displays of our cluster analyses were obtained using the TreeView program (19).

RESULTS

Gene Expression Patterns of Early-Stage (I/II) and Late-Stage (III/IV) Tumors. We examined gene expression in seven early- and seven late-stage ovarian tumors (Table 1) using cDNA microarray filters containing approximately 25,000 members of the Unigene set and control probes. Each tumor was examined in duplicate, with results indicating excellent reproducibility (Pearson correlation, >0.97 in all cases). The overall degree of similarity in gene expression between duplicate microarray experiments and among different tissue specimens was assessed using scatter plots. A representative scatter plot for two duplicate filters is shown in Fig. 1*A* (Pearson correlation, 0.97), and Fig. 1*B* displays the scatter plot of an early-stage tumor (OV106) *versus* one of the pooled normal epithelial brushings (Pearson correlation coefficient, 0.76).

Analysis of the Differential Expression in Early- and Late-Stage Ovarian Tumors. Table 2 displays the number of genes for which we found varying levels of differential expression relative to normal ovarian epithelial cell brushings. Generally speaking, more genes were determined to be down-regulated than up-regulated. For example, 46 genes are at least 10-fold down-regulated in at least seven of the tumors that were expression-profiled. The number of genes that are 10-fold up-regulated in at least seven tumors is only 12. A similar trend was evident when the tumors were grouped by stage (Table 3). Heirarchical clustering for a set of genes selected for maximal variance (without prejudice toward differential expression) are shown in Fig. 2. The dendrogram in Fig. 2 shows the tight clustering of normal samples, whereas separation between early- and late-stage samples is far less compelling. Figs. 3 and 4 display heirarchical clustering for a set of genes that are ≥5-fold up- and down-regulated in a significant number of early- and late-stage tumors, respectively, relative to the pooled epithelial cell brushings. It is evident from these analyses that several of the genes are aberrantly regulated to the same extent in both early- and late-stage tumors.

To assess the extent to which genes in specific functional categories are differentially expressed in both early- and late-stage tumors, expressed sequences showing five times the expression changes in a majority of early-and late-stage tumors were grouped in one of four categories: cell-cell interactions; intermediate filament markers; cell cycle and growth regulators; and genes involved in invasion and metastasis (Fig. 5). This analysis revealed that genes involved in cell-cell interactions such as *cadherin 11* (20), *cadherin 2* (21), and

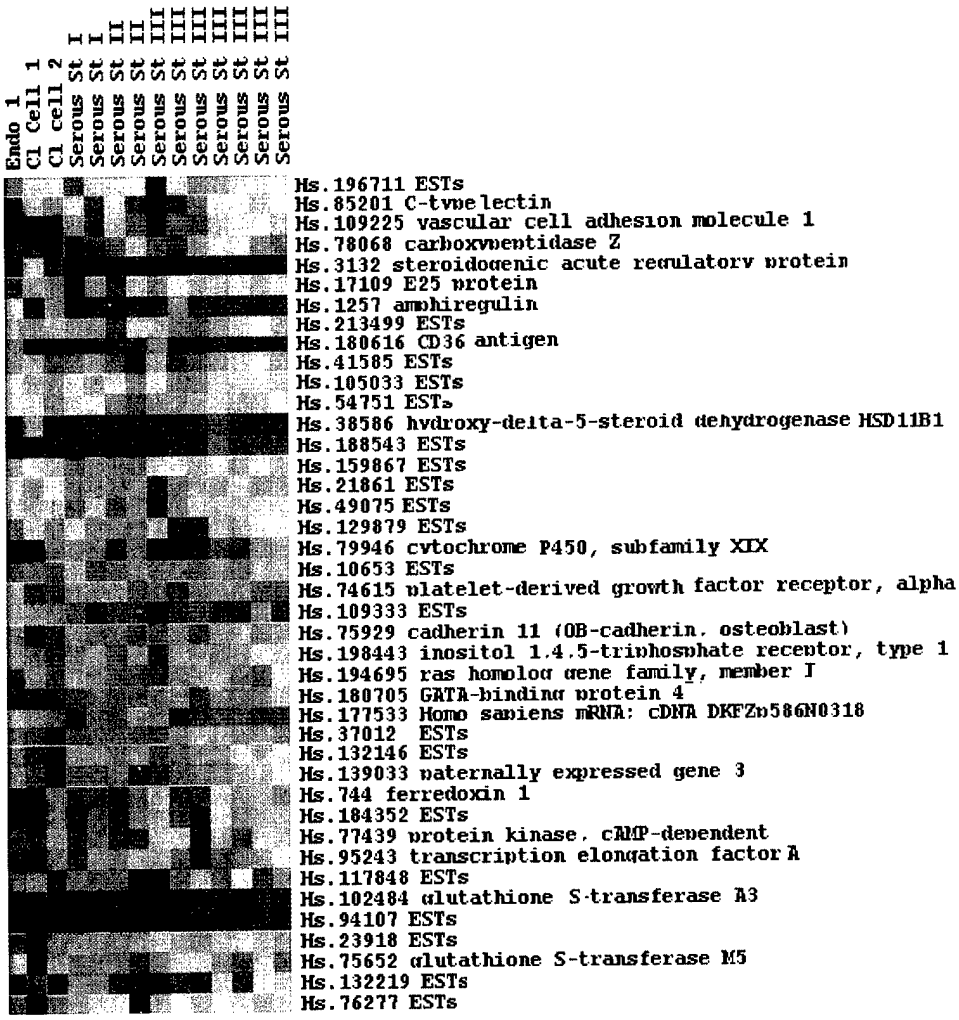


Fig. 4. Hierarchical clustering of 14 ovarian tumor samples, using Eisen's Stanford clustering package. Data were log-transformed and clustered by centered Pearson correlation. Cluster tree analysis of five times or more down-regulated genes in early- and late-stage tumors. *Green boxes*, fold down-regulation of five times or more. *Red boxes*, fold regulation between 1 and 2. *Different shades of red*, values above 2 but less than 3.

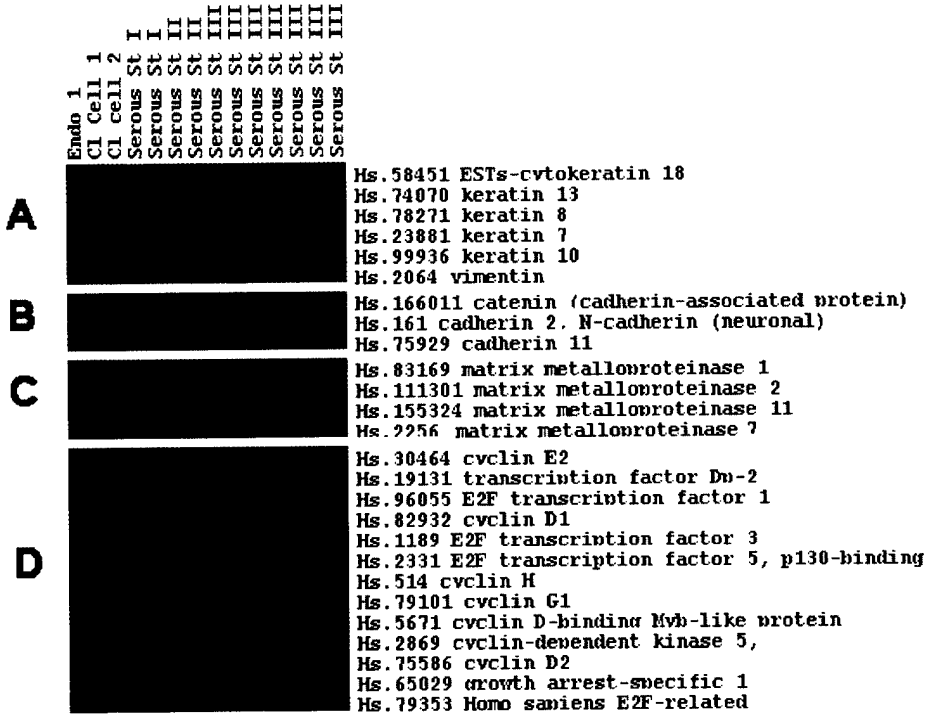


Fig. 5. Cluster tree analysis of gene categories. five times or more differentially regulated genes in early- and late-stage tumors. *Green boxes*, fold down-regulation of five times or more. *Red boxes*, fold up-regulation. *A*, intermediate filament genes; *B*, cell-cell interactions; *C*, cell invasion and metastasis; and *D*, cell cycle and growth regulators.

Table 4 *Genes with expression level changes >10-fold in both early- and late-stage tumors*

Cluster identification	Cluster title
10-fold down-regulated genes in early- and late-stage tumors	
Hs.[a]38586	*Hydroxy-δ-5-steroid dehydrogenase, 3 β- and steroid δ-isomerase 1*
Hs.213499	*ESTs*
Hs.49075	*Human DNA sequence from clone 22D12 on chromosome Xq21.1-21.33*
Hs.188543	*ESTs*
Hs.129879	*ESTs*
Hs.79946	*Cytochrome P-450, subfamily XIX (aromatization of androgens)*
Hs.180616	*CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1*
Hs.159867	*ESTs*
Hs.1275	*Amphiregulin (schwannoma-derived growth factor)*
Hs.41585	*ESTs*
Hs.94107	*ESTs*
Hs.3132	*Steroidogenic acute regulatory protein*
Hs.105033	*ESTs*
Hs.109333	*ESTs*
Hs.117848	*ESTs*
Hs.102488	*Glutathione S-transferase A3*
Hs.54751	*ESTs*
Hs.17109	*Integral membrane protein 2A*
Hs.21858	*CAG repeat containing (glia-derived nexin 1 α)*
Hs.54751	*ESTs*
Hs.9914	*Follistatin*
Hs.139033	*Paternally expressed gene 3*
Hs.105806	*Granulysin*
Hs.169228	*δ (Drosophila)-like 1*
Hs.37012	*Homo sapiens DNA sequence from PAC 434O14 on chromosome 1q32.3-41*
Hs.148493	*Cathepsin B*
10-fold up-regulated genes in early and late stage tumors	
Hs.5372	*Claudin 4*
Hs.2719	*Epididymis-specific, whey-acidic protein type, HE 4*
Hs.16696	*E74-like factor 3 (ets domain transcription factor)*
Hs.19222	*Ecotropic viral integration site 1*
Hs.896603	*Mucin 1, transmembrane*
Hs.24743	*ESTs*
Hs.111461	*Ceruloplasmin (ferroxidase)*
Hs.206297	*ESTs*
Hs.104472	*ESTs*
Hs.23291	*ESTs*
Hs.73149	*Paired box gene 8*
Hs.2256	*Matrix metalloproteinase 7 (matrilysin, uterine)*
Hs.204238	*Lipocalin 2 (oncogene 24p3)*
Hs.76550	*ESTs*
Hs.36451	*Glutamate receptor, ionotropic, N-methyl D-aspartate 2C*
Hs.155097	*Carbonic anhydrase II*

[a] Hs., unigene cluster IDs.

nidogen (*enactin*; Ref. 22) were all down-regulated in a majority of tumors, whereas genes involved in invasion and metastasis, including *matrilysin* (*MMP 7*; Refs. 23, 24), *gelatinase* (*MMP 9*; Ref. 25), *matrix metalloproteinase 10* and *12* (Ref. 23) were up-regulated in a majority of tumors. Genes belonging to the intermediate filament category, such as *vimentin* (26) and *keratin 10* (27) were down-regulated in both early- and late-stage tumors, whereas *keratins 8* (28), *13* (29), and *18* (28) were up-regulated in both stages. For the cell cycle/growth regulators, *cyclin D2* (30, 31), *cyclin dependent kinase 5*, *growth arrest-specific 1* (32), and the *E2F*-related transcription factor *DP1* (33) were all down-regulated. Other genes in this category, such as *cyclin E2* (34), proliferating cell nuclear antigen (35), and transcription factor *E2F5* (36), were up-regulated in both early and late-stage tumors.

The genes with an average-fold change of at least 10-fold change across all tumors are listed in Table 4. Several of these genes, such as *mucin 1* (37), *ceruloplasmin ferroxidase* (38), *claudin 4*, and *HE4* (39, 40) have been previously identified as up-regulated in ovarian tumors.

Validation of Microarray Results by Semiquantitative RT-PCR. To validate the expression levels of genes from the profiling analysis, we performed semiquantitative RT-PCR, with GAPDH as a control in seven ovarian tumor cell lines, on 20 early (I/II)- and 16 late (III/IV)-stage tumors (Table 1) with a set of genes that showed 5-fold differential regulation in at least 50% of the tumors. Fig. 6*A* shows the expression levels of a subset of the genes in ovarian tumor cell lines compared with short-term cultures of ovarian epithelial cells. Fig. 6*B* shows the expression levels of four of these genes in primary ovarian tumors compared with normal epithelial cell brushings. Among the genes with the most striking difference in expression in tumors relative to normal epithelial cell brushings, *FGF7* showed complete loss of expression in all of both early- and late-stage serous tumors and in six of the seven ovarian tumor cell lines. *Decorin*, a small proteoglycan involved in the activation of *EGFR* (41) pathway showed lower levels of expression in 22 of 31 tumors and in five of seven cell lines. The expression of plasminogen activator inhibitor 1 (*PAI1*; Ref. 42), a well-characterized serine protease inhibitor, was also lost in most (25 of 36) of the tumors tested. Among the genes showing frequent up-regulation by microarray analysis, *PUMP-1* (24, 43) and *HE4*, an epididymis-specific whey acidic protein (40), showed overexpression in almost all of the tumors tested (data not shown).

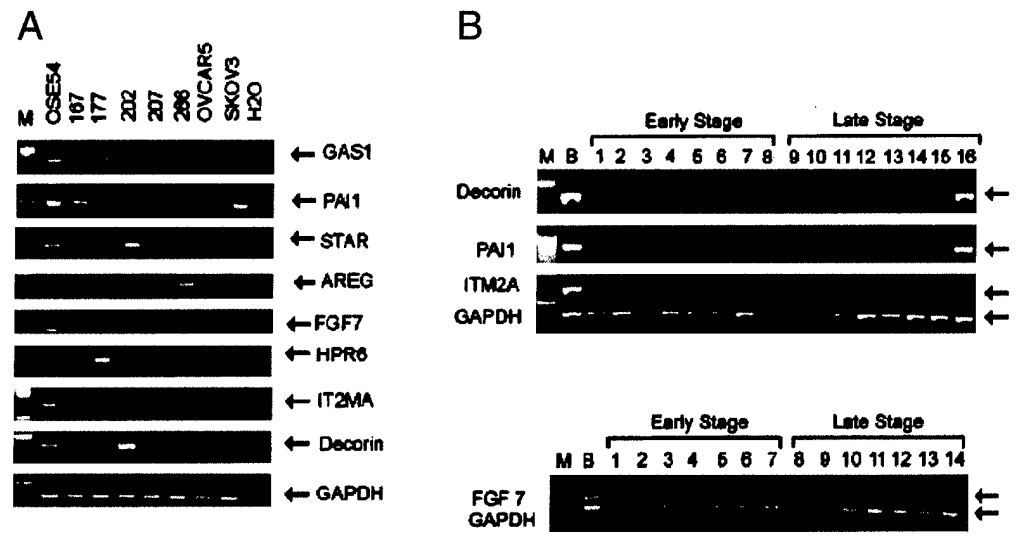


Fig. 6. *A*, agarose gel showing the products of semiquantitative RT-PCR in the ovarian cell lines. *M*, 100-bp ladder; *Lane 1*, short-term cultures of normal ovarian epithelial cells (*OSE54*); *Lane 2*, OV 167; *Lane 3*, OV 177; *Lane 4*, OV 202; *Lane 5*, OV 207; *Lane 6*, OV 266; *Lane 7*, OVCAR 5; *Lane 8*, SKOV; *Lane 9*, water control (*H2O*). Probes: *panel 1*, growth arrest-specific gene 1 (*GAS1*); *panel 2*, plasminogen activator inhibitor 1 (*PAI1*); *panel 3*, steroidogenic acute regulatory protein (*STAR*); *panel 4*, amphiregulin (*AREG*); *panel 5*, fibroblast growth factor 7 (*FGF 7*); *panel 6*, human progesterone binding protein (*HPR6*); *panel 7*, integral membrane protein 2A (*ITM2A*), *panel 8*, decorin; *panel 9*, GAPDH. *B*, agarose gel showing the products of the result of semiquantitative RT-PCR resolved on a 1.6% agarose gel. *On top of the figure*, sample numbers; *on top of the tumor numbers*, the staging information for these tumors. *M*, 100-bp ladder; *Lane 1*, normal epithelial cell brushings (*B*). *Panel 1*, decorin; *panel 2*, plasminogen activator inhibitor 1 (*PAI1*); *panel 3*, integral membrane protein 2A (*ITM2A*); *panel 4*, fibroblast growth factor 7 (*FGF 7*) and GAPDH.

Table 5 *CGH results*

Patient no. (histology; stage)	CGH changes
OV106 (Clear Cell; I)	−1p32-pter;+1q;+**3q22-26**[a];−4q;−6q24-27;+8q;+12p;−13q21-qter;−18q12-21;−Xq21-23
OV496 (Clear Cell; I)	+1p32-pter;−2q21-qter;−3;−4q12-28;+5p;−5q;−6;−12q;−13q21-33;+16;+18;+19;−Xq
OV51 (Endo; I)	NAD
OV105 (Endo; I)	+1q31-42;−2q22-24;−4q13-24;−5q12-23;−6q21-23;+11q22-24;+12q23-qter;−13q21-31;−15q21-24;+16p12-13;+19;+20q;−21
OV338 (Endo; I)	+1q21-qter;+6;+8;−9p;−21q
OV647 (Endo; I)	−4q;−5q;+6q24-27;−7q21-31;+16p12-13.2;+17;−18q;+20
OV6 (Serous; I)	+1p35-qter;−1p21-31;−2q23-32;−4q21-31.3;−5q14-32;+8q;−13q13-33;+19;+20q;+22q;−Xq21-qter
OV90 (Serous; I)	−1q24-31;+3q24-qter;−4q13-25;−5q21-23;−6;+8q23-qter;−9p13-23;+12p11.2-qter;−13q21-qter;−15q21;+17q24-25;−18q12-21; +20q12-qter;+22q
OV234 (Serous; I)	−1p33-35;+2q22;−4q22-31;−5q14-21;−6q14-16;−10p;+12p12-13;+17q;+20q
OV102 (Clear Cell; II)	−1p12-31;+1q31-42;−2q34-36;**+3q24-26**;−17p
OV401 (Serous; II)	+3q;**+3q24**;+**3q26**;−4q;+5p;−5q;−6;−7p;+**8q24**;+**11q23-24**;−18q21-22;+20;−Xq21-qters
OV402 (Serous; II)	+1q41-44;−2q24-32;+3q24-27;−5q21-23;−6q21-23;+11q13;−13q21-31;−18q12-22;+19;+20q;−Xq
OV176 (Clear Cell; III)	+**3q25-26**;+8q;+17q;+**20q12-13**
OV453 (Clear Cell; III)	+3q24-26.3;+17q24-25
OV522 (Clear Cell; III)	+**2p24-25**
OV623 (Clear Cell; III)	+8q
OV78 (Endo; III)	+1q21-41;−2p14-16;+3q13.1-13.3;−4q;+5p;+8q21.1-24;+9p21-23;+11q12-13;+17q23-25;−18q21-22;+20q;−X
OV93 (Endo; III)	+1q32-44;+2p24-25;+2q23-24;+3q13.3-qter;+3q24-26;−4q;−5;−6q22-qter;−8p;+8q;+9p22-23;+12p12-13;−13q31-34;−18q12-23;+20q13
OV110 (Endo; III)	+1p36;−4q13-32;−5q13-22;+8q24;+10;+11q24-25;+12p12-13;−14q23-24;+16q21-23;+19q;+20q
OV259 (Endo; III)	−1p13-22;+1q;+2q35-37;+**3q13**;+**3q25-27**;−4q23-qter;+5p;−5q14-qter;−6q;−7p;−11;+**12p12-13**;−13q;+19q;+**20q12-13**;+**22q12.2-12.3**;−Xq
OV4 (Serous III)	−2q;+**3q26.1-26.3**;−4q22-24;−5q13-23;−6q16.3-25;+**7q33-35**;+**8q23-24**;+**11q13-14**;+**12p11-13**;−18q12.3-22;+20q12-13.2
OV11 (Serous III)	−4q;−5q13.3-23.2;+**8q23-24**;+**11q23-24**;+12p12-13;−Xq21-qter
OV16 (Serous III)	+1q;−18q22;+22;−Xq23-25
OV29 (Serous III)	+**1q21-22**;−2q22-24;−5q;+8q;+**8q23-24**;+**11q12-13**;−18q12-qter;+**18p11.2-11.3**;+20q;−Xq
OV72 (Serous III)	+1p32-36.3;+1q31-32;−2q22-34;+5p13-15;−5q;+6p21.1-pter;+**7p21**;+8;+**8q23-24**;−9p;+**11q12-13**;+**12p12-13**;−18q12-qter;+19;+20;−Xq
OV150 (Serous III)	+**1q21-24**;+**2p24**;−3p;+**3q26-1-26.3**;−4p;+**5p14-15**;−6q13-21;+**8q24**;−9p24;+**11q13**;+**12p12-13**;−13q21-22;16p12-13.2;−18q;+20q12-13;−Xq
OV392 (Serous III)	+3q26;−4q221;−5q12-21;−6q24-27;+7q32-qter;+8q24.1-24.3;+12p13;−13q14-22;+20q;−X
OV448 (Serous III)	+**1p36**;−2q31-34;−5q14-23.3;+**17q24-25**;+19;+**20q12-13.2**;−X
OV319 (Endo; IV)	−1p31;+1q;+**2p24**;+2q24;+**3q21-26**;−4q32-qter;+**8q11.2-21.2**;+**8q23-24**;−9p23;−13q31;−15q21-qter;−18q;+**20q13**;−X

[a] Boldface denotes amplification.

CGH Analysis. To investigate whether any of the results from the gene expression analysis could be associated with genomic alterations, we performed CGH on a set of 29 ovarian tumors. The overall trend suggested similar changes in both early- and late-stage tumors (Table 5; Fig. 7). There were, however, conspicuous differences between the two tumor groups. For instance, the CGH results indicated that losses are more common than gains in the early-stage tumors. Consistent losses were observed at chromosomal regions of 2q (5 of 12), 4q (8 of 12), 5q (8 of 12), 6q (6 of 12), 13q (6 of 12), 18q (3 of 14), and Xq (5 of 12). Additional losses were also observed on chromosomes 7, 9, and 15 (2 cases); and 3, 12, 17, and 21 (1 case each). Gains involving chromosomes 20 (7 of 12); 1 (6 of 12); 3 and 8 (5 of 14); 19 (4 of 12); 11, 12, 16, 17, and 22 (3 of 14); 2, 5, 6, and 18 (1 of 12) were also observed. The chromosomal arms that showed frequent loss in the late-stage tumors were nearly identical to those observed in the early-stage tumors, and included 2q (5 of 17), 4q (9 of 17), 5q (9 of 17), 6q and 13q (5 of 17), 18q (6 of 13), and Xq (10 of 17). Less frequent losses were seen on chromosomes 9 (3 cases); 1p (2 cases); and 3, 7, 8, 11, 14, and 15 (1 case each). Gains on chromosomes 8 (76%), 20 (70%), 1 and 3 (58%), 12 (52%), 11 (41%), and 2 (35%) were evident in at least six of the late-stage tumors. Less frequent gains were observed on chromosomes 19 and 17 (4 of 17); 5 and 7 (3 of 17); 9, 16, and 22 (2 of 13); and 6 and 10 (1 case each).

Amplifications were mainly confined to the late-stage ovarian tumors. These involved regions at 8q23–24 (8 cases); 20q12–13 (6 cases); 3q24–26, 11q12–13, and 12p11–13 (5 cases each); 2p24–25 (3cases); 1p36, 9p21–23, 11q24–25, and 17q24–25 (2 cases each); and 1 case each on 1q32–44, 2q23–24, 3q13, 5p14–15, 7p21, 7q35, 8q11–21, and 22q12. In contrast, amplifications in the early-stage cancers were observed in just six instances: three times at chromosome 3q24–26 and one case each on 3q23, 8q23–24, and 11q24–25.

DISCUSSION

This report represents the first communication of an investigation involving the genome-wide examination of changes in gene expression and chromosomal regions for early- and late-stage ovarian cancer. In our cDNA microarray analysis, we identified several differentially expressed genes the potential role of which in carcinogenesis have been described previously. However, this analysis also identified several genes, both of known and unknown function, the role of which in tumor development has yet to be elucidated.

We validated the differential expression of several of the genes identified through microarray analysis by semiquantitative RT-PCR of RNA from both ovarian tumor cell lines and primary tumors. The down-regulation (at least 5-fold) of genes such as hydroxy-δ-5 steroid dehydrogenase, 3β (*HSD3B1*; Ref. 44), steroidogenic acute regulatory protein (*StAR*; Ref. 45, 46), *amphiregulin* (47), *glutathione S-transferase A3* (48), paternally expressed *gene 3* (49), and *integral membrane protein 2A* (50) have not previously been associated with ovarian cancer. Other genes such as *decorin* (41, 51), *platelet-derived growth factor receptor* α (52), *cadherin 11* (21), *cyclin D2* (53), *E2F*-related transcription factor *DP1* (33), *NOEY2* (6), and secreted frizzled related protein (*SFRP*; Ref. 54), have functions that can be or have been linked with carcinogenesis. Our expression analysis further revealed the consistent up-regulation of several genes, including *HE4* (40), *matrilysin* (*MMP 7*), *ceruloplasmin ferroxidase* (38), *claudin 4* (39), *cyclin D1* (55), *mucin 1* (56) *protease serine 8* (57), *keratins 7* (58) and *8* (28), as well as several ESTs. We have also validated the expression levels of *HE4*, *MMP7*, *ITM2A*, *HSD3B1*, and *PEG3* by real time RT-PCR in a smaller panel of primary tumors, and the results were similar to the semiquantitative RT-PCR analysis (data not shown).

To address the issue of whether the changes that we observed at the expression level were present at the genomic level, CGH analysis was performed on 12 early- and 17 late-stage tumors. Chromosomes 2, 4, 5, 6, 13, and 18 showed regions of loss in both stages at a similar frequency (59–61). Previous genomic sequence copy number screenings of ovarian tumors by conventional and molecular cytogenetics have reported similar findings (62). High copy number gains or amplifications were mainly observed in the late-stage tumors (62).

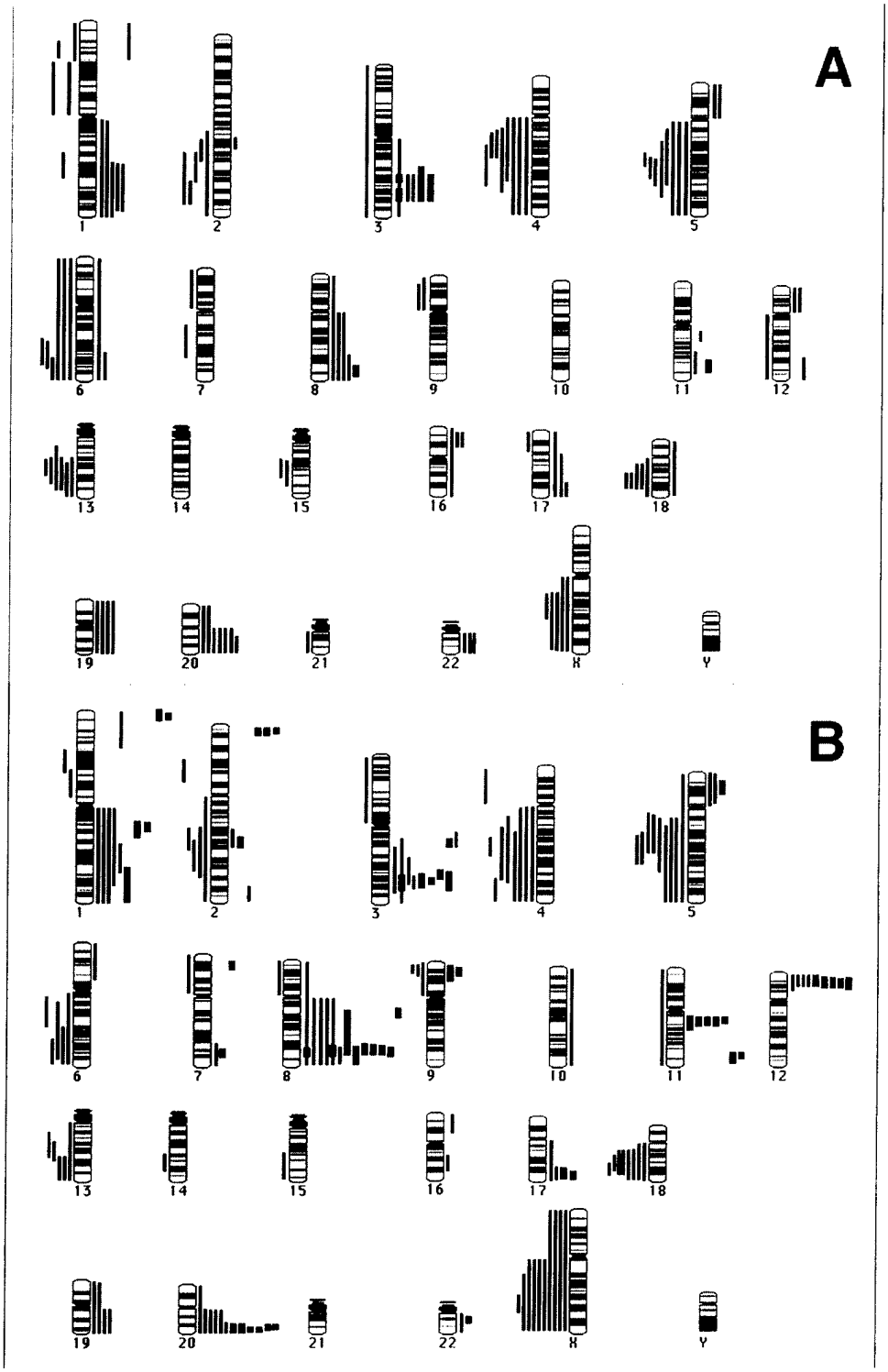


Fig. 7. Ideograms of the results of CGH analysis of 29 ovarian tumors. *Vertical lines to the right of schematic chromosomes*, gains; *vertical lines to the left*, losses. *Thicker line to the right*, the presence of high copy gain/amplification. *A*, CGH analysis of early-stage (I/II) tumors. Copy number changes of 12 tumors indicate a pattern of losses. In particular, loss of chromosomes 2, 4, 5, 6, 13, and 18 are frequent. Gains were less frequent, mainly involving chromosomes 1, 3, 6, 8 11, 19, and 20. *B*, CGH analysis of late-stage (III/IV) tumors. Copy number changes of 17 tumors indicate a pattern of losses and gene amplification; *thicker lines*, high level gains/amplifications (~>5 copy number). In contrast to the early-stage ovarian carcinomas, there are more chromosomal gains, and more tumors with localized gene amplification, predominantly involving the 3q24–26, 8q23–24, 11q12–13, 12p12–13, and 20q12–13 region. Other amplifications were detected on chromosomal arms 1p, 1q, 2p, 2q, 5p, 7q, 9p, 11q, 16p, 17q, and 18p. Losses were common on chromosomes 2, 4, 5,6, 13, and 18.

Chromosomal regions involved in amplification were 3q24–26 (63), 8q23–24 (64), 11q12–13 (55), 12p11–12 (65), 17q24–25 (66), and 20q12–13 (67).

Inspection of the data generated by CGH and expression profiling allows one to speculate on a mechanistic basis for the differential expression of at least some of the genes. For example, CGH results indicated high-level gains of 8q24 and 11q13 in multiple tumors (Fig. 8), and the microarray analysis of the same tumors indicated an increase in expression of genes/ESTs from these same regions: *i.e., cyclin D1 (PRAD1), uncoupling protein 2, folate receptor, and matrix metalloproteinase 7*, from 11q13; and *MYCC* (68), *PTK2 protein tyrosine kinase 2* (69), *RAD21* homologue (70), and several ESTs from 8q24 (data not shown), respectively. From the combined results, it seems reasonable to consider that the overexpression of these genes may be the result of gene amplification. Similarly, loss of genomic sequences from chromosomal region 4q could be responsible for the low expression of *LIM* (71), *Hevin* (72), *MAP kinase 10* and several ESTs detected by the expression profiling from the same region.

The data from the microarray analysis revealed that the majority of genes that were differentially expressed in ovarian cancer showed

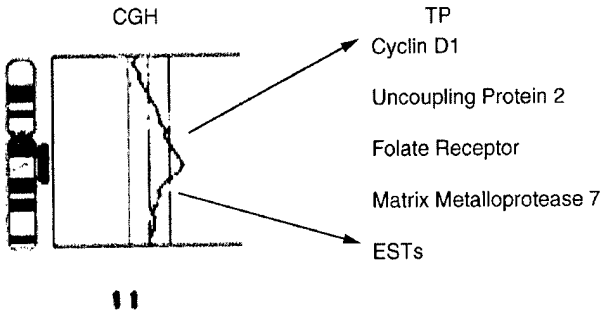


Fig. 8. CGH profile of an ovarian tumor illustrating a high copy gain/amplification in the 11q12–13 region of chromosome 11. The transcription profile of the same tumor by cDNA microarray analysis exhibited overexpression of cyclin D1, uncoupling protein 2, folate receptor, metalloproteinase 7, and some ESTs from this region.

aberrant transcript levels in both early- and late-stage tumors. At initial consideration, such results seem contrary to the genetic dogma associated with solid-tumor evolution that holds that gene alterations, and presumably, therefore, gene expression changes, become more prevalent as tumors become increasingly malignant. Interestingly, the CGH data indicate some differences between early- and late-stage tumors, especially the more common finding of regional gain and/or amplification in the late-stage tumors. Consequently, the CGH data provide some support for the genetic evolution of this solid tumor.

The inconsistency observed between the expression profiling and the CGH data could potentially be attributable to epigenetic changes such as methylation. Inactivation of genes attributable to CpG island methylation is very well documented (73). Evidence seems to indicate that these changes are probably very early events in carcinogenesis (74, 75). This may partially explain why the majority of genes are aberrantly regulated in early-stage tumors. It is also well documented that amplification events in carcinogenesis are late events (76–78) and is consistent with what is observed from the CGH data. Abnormal expression of genes is not attributable only to gene amplification or gains. Other genomic changes like rearrangements (translocations) are also responsible for alteration in gene expression. CGH can only aid in detecting net chromosomal losses or gains in tumor cells and does not account for rearrangements, thus overlooking changes that could be responsible for abnormal expression detected by microarray analysis.

An aspect of our finding that may be especially important to the clinical treatment of ovarian cancer is the determination of extensive, aberrant gene expression in early-stage tumors. One of the assumptions of most screening strategies is that a significant fraction of stage III/IV disease metastasizes from stage I lesions. These data are consistent with that possibility. In addition, poorly differentiated stage I cancers have been treated with cytotoxic chemotherapy. These data suggest that early-stage lesions have most of the genetic changes required for metastatic spread, consistent with a need for aggressive therapy. Further study of the genes that show consistent, high-level expression changes may result in the identification of markers useful for early cancer detection and may further point to potential therapeutic targets.

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Identification of under-expressed genes in early and late stage primary ovarian tumors by suppression subtraction hybridization.

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ABSTRACT

In order to identify novel tumor suppressor genes involved in ovarian carcinogenesis, we generated four down regulated suppression subtraction cDNA libraries from two early stage (Stage I/II) and two late stage (Stage III) primary ovarian tumors each subtracted against cDNAs derived from normal ovarian epithelial cell brushings. Approximately 600-700 distinct clones were sequenced from each library. Comparison of down regulated clones obtained from early and late stage tumors revealed genes that were unique to each library suggesting tumor specific differences. We found 45 down regulated genes that were common in all four libraries. We also identified several genes whose role in tumor development has yet to be elucidated, in addition to several under expressed genes whose potential role in carcinogenesis has previously been described. The differential expression of a subset of these genes was confirmed by semi-quantitative RT-PCR using GAPDH as control in a panel of 15 Stage I and 15 Stage III tumors of mixed histological subtypes. Chromosomal sorting of library sequences revealed that several of the genes mapped to known regions of deletion in ovarian cancer. Loss of heterozygosity analysis revealed multiple genomic regions with a high frequency of loss in both early and late stage tumors. In order to correlate the loss of expression of some of the genes to loss of an allele by LOH, we utilized a microsatellite marker for one of the novel genes on 8q and have shown that loss of expression of this novel gene correlates with loss of an allele by LOH. In conclusion, our analysis has identified down regulated genes, which map to known as well as novel regions of deletions and may represent potential candidate tumor suppressor genes involved in ovarian cancer.

INTRODUCTION

Each year approximately 16,000 American women succumb to ovarian cancer, the deadliest of all gynecologic malignancies (1). Because ovarian cancer is frequently asymptomatic in its early stages, 75% of patients have advanced stage disease at the time of diagnosis. However, if the disease is caught in an early stage, the five-year survival rate jumps to 92%, while the anticipated 5-year survival for patients with advanced stage disease is less than 20%. If stage I disease is a precursor of late stage ovarian cancer, as is the case with many other tumor types, identifying molecular alterations in early stage tumors should provide insights into developing strategies for early detection.

There are several PCR-based approaches to analysis of gene expression changes including mRNA differential display PCR (DD-PCR) (2, 3), RNA fingerprinting by arbitrary primed PCR (AP-PCR) (4, 5) and representational difference analysis (RDA) (6-8). In RDA, several rounds of subtractions are needed. In addition, RDA does not resolve the problem of the wide differences in abundance of individual RNA species. While DD-PCR and AP-PCR are potentially faster methods of identifying expression differences between two populations, both of these methods have high levels of false positives and are biased for high copy number mRNAs. Suppression subtraction hybridization (SSH) (9-11) has the distinct advantage over other PCR based techniques in that SSH is used to selectively amplify target cDNA fragments (differentially expressed) while simultaneously suppressing non-target DNA amplification and generating a library of differentially expressed sequences. The normalization step equalizes the abundant cDNAs within a target population and the subtraction step excludes the common sequences between the driver and tester populations.

In this study, we report on down regulated genes identified from two early and two late stage primary ovarian tumors subtracted against normal ovarian epithelial cell brushings. Collectively our studies demonstrate that 1) several genes identified in the SSH libraries as down regulated genes map to known regions of deletions in ovarian cancer and 2) LOH analysis revealed novel regions of deletions not previously identified in early stage tumors.

METHODS

Tissue Processing

All specimens were snap frozen in the surgical pathology unit at the Mayo Clinic. The tumor content of the specimens was assessed by Hematoxylin and Eosin stained sections. Only specimens with greater than 75% tumor content were used for all experiments. Twenty normal ovarian epithelial cell brushings from patients without cancer were pooled and the epithelial nature of these brushings was verified by cytokeratin staining. Only brushings that contained >90% epithelial cell content were used. The majorities of patients providing normal ovaries was between 45 and 65 years old, and were undergoing incidental oophorectomy at the time of pelvic surgery for other indications. All ovaries were examined pathologically and found to be benign. The histology, grade, and the stage of each tumor used in SSH library construction, semi-quantitative RT-PCR and LOH studies are listed in Table 1. Tumors were staged according to the criteria proposed by International Federation of Gynecology and Obstetrics.

Cell culture

Five of seven ovarian-carcinoma cell lines (OV 167, OV 177, OV 202, OV 207, and OV 266) were low passage primary lines established at the Mayo Clinic (12), while SKOV-3 was purchased from American Type Culture Collection (Manassas, VA) and OVCAR 5 is a NIH human ovarian cancer cell line (13). All cells were grown according to the supplier's recommendations.

Suppression Subtraction Libraries

Four down-regulated libraries were generated from individual tumors. OV 338 (stage I endometrioid), OV 402 (stage II serous), and two stage III serous tumors (OV 4 and OV 13) were all subtracted against normal ovarian epithelial cell brushings.

Tester and Driver Preparations: Total cellular RNA from primary ovarian tumors (driver) and from 20 pooled normal ovarian epithelial cell brushings (tester) was prepared using Trizol reagent (Gibco-BRL, Rockville, MD) followed by purification by RNAeasy kit (Qiagen Inc, Valencia, CA. Cat # 74104). The integrity of the RNA was assessed by agarose gel electrophoresis. One μg of total RNA was then used for first and second strand cDNA synthesis in a 10 μl reaction volume using Smart II oligonucleotides and cDNA synthesis (CDS) primer (Clontech, Palo Alto, CA. Cat # K1052-1) following the manufacturer's instructions. The concentration of reverse transcribed cDNA was adjusted to 25 ng/μl. The resulting cDNAs were amplified and the cycle number was optimized for each sample after amplification with PCR primer (5'-AAGCAGTGGTAACAACGCAGAGT-3'). For cycle optimization, aliquots of the PCR reactions were removed after 15, 18, 21 and 24 cycles of amplifications. The resulting products were resolved on a 1.5% agarose gel and optimum cycle number was chosen after southern hybridization with GAPDH and transferrin receptor genes as probes. For most samples the optimum cycle numbers were between 17 and 19 cycles of amplification. The reaction was scaled up to generate 3 μg of double stranded cDNAs. The resulting cDNA was precipitated, washed with 70% ethanol dissolved in 40 μl of deionized water and digested with Rsa I in a 50 μl reaction mixture containing 100 units of enzyme (Boeringher Mannheim, Indianopolis, IN) for 3 h. The blunt ended cDNAs were then purified using PCR purification columns (Cat# A7048, Promega, Madison, WI). The driver cDNAs from primary tumors were adjusted to 300 ng/ μl in a final 7 μl volume. Fifty ng of digested double stranded tester (normal ovarian epithelial cell brushings) cDNA was ligated in two separate reactions with 2 μl of adapter 1 (10 μM) and adapter 2 (10 μM) (provided in the kit), respectively, and 1.0 unit of T4 DNA ligase (Life Technologies) in 10 μl total volume with buffer supplied by the manufacturer. After ligation, 1 μl of 0.2 M EDTA was added and the samples were heated at 75°C for 5 min to inactivate the ligase and stored at –20°C.

Subtractive Hybridization: SSH was performed between tester and driver mRNA populations using the PCR-select cDNA subtraction kit (Clontech, cat# K1804-1) according to the manufacturer's recommendations. Two µl of driver ds cDNA (150-200 ng/µl) was added to each of two tubes containing one µl of adapter-1 and adapter-2 ligated tester cDNA (10 ng) with 1X hybridization buffer in a total volume of four µl. The solution was overlaid with 10 µl of mineral oil and the cDNAs were denatured (1.5 min, 98°C) and allowed to anneal for 8-9 hrs at 68°C. After the first hybridization, the two samples were combined and an additional heat denatured driver (300 ng) in 1X hybridization buffer was added. The sample was allowed to hybridize for another 16 hrs at 68°C. The final hybridization reaction was diluted with 200 µl with dilution buffer provided by the manufacturer, heated at 68°C for 7 min and stored at –20°C.

PCR Amplification: For each subtraction, two PCR amplifications were performed. The primary PCR reaction in 25 µl contained 1 µl of subtracted cDNA, 1 µl of PCR primer1 (10 µM, 5'-CTAATACGACTCACTATGGGC-3') and 0.5 µl each of 50X advantage cDNA polymerase mix (Clontech) and 10 mM dNTP mix respectively. The cycling parameters were 75°C for 7 mins, followed by 27 cycles at 94°C for 30 secs; 68°C for 30 secs; 72°C for 2 mins. The amplified products were diluted 10-fold with deionized water and 2 µl was used in the secondary PCR reactions with NP1 and NP2 primers (provided in the kit). The cycling conditions were the same as in the primary PCR amplification except the reactions were in 50 µl volume for 11 cycles only with a final extension cycle for 7 mins at 68°C. The subtraction efficiency was determined by both PCR and Southern based methods as instructed by the manufacturer.

Cloning and Analysis of the subtracted cDNAs: Products from the secondary PCRs were inserted into PCR2.1-TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Prior to ligation, the subtracted cDNA mix was incubated for 1 h at 72°C with dATP and AmpliTaq DNA

polymerase (Perkin Elmer Cetus, Foster City, CA) to ensure that most of the cDNA fragments contained "A" overhangs. Approximately 100 ng PCR amplified cDNA were ligated into 50 ng of vector without further purification. Two µl of ligated products (10 ng of vector and 50 ng of cDNAs ligated in 10 µl volume) were transformed into 40 µl of DH10B cells by electroporation (BioRad, Hercules, CA). Routinely 50 and 200 µl aliquots of the transformed cells (grown in 1 ml of medium) were plated onto 150 mm LB/agar plates containing 100 µg/ml of ampicillin, with 100 µM IPTG and 50 µg/ml X-gal to discriminate white from blue colonies. The transformation efficiency was 2-4 x 10^6 colonies/µg of DNA.

Hybridization and Screening for Differentially Expressed Transcripts: The differential hybridization was performed initially on 96 randomly picked clones in order to determine subtraction efficiency. The inserts in the plasmid were amplified using NP-1 and NP-2 primers provided in the kit. The PCR conditions were 94°C for 4 min followed by 30 cycles of 94°C, 30 sec; 60°C, 30 sec; 72°C, 2 min followed by a final extension at 72°C for 5 min. The products of the PCR reactions were resolved on a 2% agarose gel run in duplicate. Following Southern blotting of the amplified inserts onto Hybond N membranes (Amersham, Piscataway, NJ). The membranes were stained with methylene blue in 0.2X SSC and visualized to ensure complete transfer of all the products. The blots were then hybridized with RsaI digested cDNA probes (reverse northern). Fifty ng of RsaI digested tester and driver cDNAs were labeled using random primer labeling kit (Stratagene, LA Jolla, CA) with 50 µCi of ^{33}P-dCTP following manufacturer's instructions. Equal counts (1-2 x10^7 cpm/µl) of the cDNA probes from normal and tumor tissues were heat-denatured and used to probe duplicate blots. Hybridization was performed at stringent conditions in 0.5 M Na_2PO_4, pH 7.2, 7% SDS at 65°C. The next day, the filters were washed twice in 2X SSC, 0.5% SDS at 68°C, then once in 0.1% SSC, 0.1% SDS at 68°C and exposed to phosphoimager screens overnight. The signal intensity of each spot in the membranes was compared between tester and driver hybridized duplicates. cDNA fragments displaying differential expression levels of >1.8-fold or higher were selected to estimate the efficiency of the differential hybridization.

Approximately 600-700 unique clones from each of the four libraries were successfully sequenced with M13 forward primer using an ABI Prism dye terminator cycle sequencing in the sequencing core at Millennium Predictive Medicine, Cambridge, MA. Sequences were compared with the National Center for Biotechnology Information sequence database using the BLAST program.

Semi quantitative RT-PCR

Fifty to 100 ng of reverse transcribed cDNAs were used in a multiplex reaction with a pair of gene specific primers and GAPDH forward (5'–ACCACAGTCCATGCCATCAC-3') and reverse primers (5'-TCCACCACCCTGTTGCTTGTA-3'), which yield a 450 bp product. The PCR reaction mixes contained 50 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 400 μM for gene specific primers, 50 μM for each of the the GAPDH primers, and 0.5 units of Taq polymerase (Promega, Madison, WI), in a 12.5 μl reaction volume. The conditions for amplification were as follows: 94°C for three minutes, then 29 cycles of 94°C for 30 seconds, 50-62°C for 30 seconds depending on the gene specific primers being tested, and 72°C for 30 seconds in a Perkin Elmer-Cetus 9600 Gene-Amp PCR system. The products of the reactions were resolved on a 1.6% agarose gel. Band intensities were quantified using the Gel Doc 1000 photo-documentation system (Bio-Rad, Hercules, CA) and its associated software.

The following Gene Specific Primers were utilized:

CTSK: F-GGA GAT ACT GGA CAAC CCA CTG
R-CCA ACT CCC TTC CAA AGT GC

PAI1: F-AAT CGC AAG GCA CCT CTG AG
R-GAT CTG GTT TAC CAT CTT TT

Cyclin D2: F-AGC TGC TGT GCC ACG AGG T
R-ACT GGC ATC CTC ACA GGT C

FGF7: F-TAA TGC ACA AAT GGA TAC
R-ATT GCC ATA GGA AGA AAG

EGR1: F-GAC ACC AGC TCT CCA GCC TGC
R-GGA AGG GCT TCT GGT CTG GGG

SPARC: F-CCA CTG AGG GTT CCC AGC AC
R-GGA AAC ACG AAG GGG AGG GT

Decorin: F-CCT GGT TGT GAA AAT ACA TGA
R-TGA CAT TAA CAA GAT TTT GCC

THBS2: F-TGG TCA CCA GGA CAA AGA CAC
R-ATC CTG CCA GCA AGC TGA CA

ITM2A: F-CGC AGC CCG AAG ATT CAC TAT G
R-CTT ATT ACC AAG GAC ACT CTA TCT

PEG3: F-CGG AGA ACT GTG AGA AGC TCG TC
R-GGT GGG GCT AGG CTA GAA GG

Northern Blot Analysis: Fifteen μg of total RNA was fractionated on 1.2% formaldehyde agarose gels and blotted in 1X SPC buffer (10 mM sodium phosphate, pH 6.8, 1 mM CDTA (Sigma, St Louis, MO) onto Hybond N membranes (Amersham, Piscataway, NJ). The control Small Ribosomal Protein S9, (*RPS9*) and gene specific probes were labeled using the random primer labeling system (Life Technologies, Gainsburg, MD) and purified using spin columns (100TE) from Clontech. Filters were hybridized at 68°C with radioactive probes in a micro-hybridization incubator (Model 2000, Robbins Scientific, Sunnyvale, CA) for 1-3 hours in Express Hybridization solution (Clontech, Palo Alto, CA) and washed according to the Supplier's guidelines.

RPS9: F-GCA ACA TGC CAG TGG CCC GG
R-ATC CTC CTC CTC GTC GTC TC

The above primers yield a 586 bp cDNA fragment and the conditions for amplification is similar to the semi quantitative RT-PCR conditions described above.

LOH analysis: Fifteen early and eighteen late stage tumors of differing histologies (Table 1) were analyzed. The 15 early stage tumors included three clear-cell, and six each of endometrioid and serous tumors. The eighteen late stage tumors included three clear- cell, four endometrioid and 12 serous tumors. The markers (Research Genetics, Huntsville, AL) used in this study are listed in Table 1 along with their chromosomal locations. Two new microsatellite markers, one near Methylation Controlled J

protein on 13q14.1 (14), and another marker within BAC CIT-B-470f8 on 19q14.3 (AC006115) are *MCJ*-NF-5'-GATTGACCACAGTCTTATCT-3' and *MCJ*-18-5'-TAAGAGGTCTACTCATTGCTCAC-3', 19-F-5'-GCACCTGGCCCAACTGTAAC-3' and 19R-5'-CCAGCTGCTGGCTCACCTT-3' respectively. The individual oligonucleotides were synthesized in the Mayo Molecular Technology Core at the Mayo Clinic, Rochester, MN. The PCR reaction mix contained: 50 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM concentration of each primer, 0.05 μl of ^{32}P dCTP (10 μCi/μl) and 0.5 units of Taq polymerase (Promega, Madison, WI) in a 10 μl reaction volume. The conditions for amplification were: 94°C for two min, then 30 cycles of 94°C for 30 seconds, 52-57°C for 30 sec, and 72°C for 30 sec in a Perkin Elmer-Cetus 9600 Gene-Amp PCR system in a 96 well plate. PCR products were denatured and run on 6% polyacrylamide sequencing gels containing 8 M urea. The gels were dried and autoradiographed for 16-24 hr and scored for LOH. Multiple exposures were used before scoring for LOH. Allelic imbalance indicative of LOH was scored when there was more than 50% loss of intensity of one allele in the tumor sample with respect to the matched allele from normal tissue. The evaluation of the intensity of the signal between the different alleles was determined by visual examination by two independent viewers (VS and JS).

RESULTS

In an attempt to identify novel tumor suppressor genes in ovarian cancer, we generated down regulated cDNA libraries from two early (Stages I and II; OV 338 and OV 402) and two late stage (Stage III; OV 4 and OV 13) tumors subtracted against normal ovarian epithelial cell brushings. The libraries were monitored at each stage of library construction to ensure that the clones generated from each of the four libraries truly reflected differentially expressed sequences. The subtraction efficiency was determined by both Southern and PCR based protocols. Figure 1A and 1B show the subtraction efficiency of libraries OV 402 (Panel 1) and OV 4 (Panel 2) by southern and PCR based methods (Fig 1B), respectively. We estimated a 60 to 70-fold enrichment of the differentially expressed genes in all four libraries. This was confirmed with the Southern based analysis, where we saw a complete subtraction of GAPDH in the subtracted cDNAs (Fig 1A). The subtraction efficiency of these libraries was also reflected in the fact that the housekeeping gene, GAPDH was only identified twice in over 2,000 clones sequenced in only one of the four SSH libraries.

We evaluated the differential expression of genes in each of the libraries by hybridizing tester and driver cDNAs to randomly amplify 96 cloned inserts by colony PCR. PCR products were resolved in duplicate. Care was taken to ensure equal loading of the PCR products onto 2% agarose gels to allow direct comparison of hybridization signal intensities (Fig. 2 A). Following transfer of the PCR products onto nylon membranes, we performed reverse Northerns to identify differentially expressed transcripts. The cDNA probes used for hybridization were restricted with RsaI to minimize background hybridization. Faint signals representing rare transcripts could easily be distinguished with this approach (Fig. 2 B). After densitometric analysis of each of the corresponding bands hybridized with tester and driver cDNAs, the percentage of these clones that showed the expected differential hybridization was 70-80%.

We sequenced approximately 2000 randomly picked clones from each of the four libraries. After consolidating for clones that appeared more than once in the libraries we estimated that there were approximately 600 distinct clones sequenced from each of the four libraries.

Analysis of SSH library genes

In order to discern the differences in gene expression in early versus late stage tumors, we compared the genes in each of the libraries to one another to verify how many of the differentially expressed genes were common among these four libraries. Of the 600 or so distinct clones in each library, 45 genes were common in all four libraries (Table 2). These potentially represent genes that may consistently be down regulated in both early and late stage ovarian tumors. Similar comparison of genes that were isolated from any three of four libraries revealed 80 common genes. There were 130 common genes in the two early stage tumors. Sixty of these130 genes were also present in either one of the two late stage tumors. A similar kind of analysis comparing sequences in the two late stage libraries revealed that there were 210 genes that were common between them. Only 55 of 210 genes were also identified in either one of the two libraries generated from early stage tumors.

Since we had randomly picked the clones for sequencing, we validated the differential expression of 20 genes ranging from clones that were highly represented to those that were infrequently occurring in the libraries to ensure that the sequences generated truly represented differentially expressed genes between normal and tumor cells. Initially, seven ovarian tumor cell lines were used for validation by semi-quantitative RT-PCR with GAPDH as control. The expression profile of these genes in tumor cell lines was compared to short-term cultures of normal ovarian epithelial cells. Several of these genes showed complete loss of expression in a number of cell lines (Fig.3A, Table 3). For example, hydroxy-delta-5-steroid dehydrogenase 3 beta- steroid delta-isomerase 1 (*HSD3B1*) (15), represented by only two clones in each of the four libraries, showed complete loss of expression in all the seven tested cell lines (Table 4). However, *PAI1* (16) that appeared several times (100-140) in each of the two late stage libraries

showed complete loss of expression in only two of seven cell lines. We also tested the differential expression of these genes in 20 early (I/II) and 16 late (III/IV) stage primary tumors of mixed histological subtypes by semi-quantitative RT-PCR comparing them to normal epithelial cell brushings. The twenty early stage tumors included five clear cell, six endometrioid and nine serous tumors. The late stage tumors included one clear cell, four endometrioid and eleven serous tumors (Table 1). Figure 3B shows the results of this analysis for *PAI1* (16), *ITM2A* (17), *FGF7* (18), *PEG3* (19) and a novel gene on 8q.

In addition we tested the expression of *HSD3B1* and *PRSS11*, a serine protease with an IGF binding domain (20) in cell lines and primary tumors by Northern analysis (Figs 4A and B). *HSD3B1* showed complete loss of expression in all the cell lines and the primary tumors. *PRSS11* showed complete loss of expression in 4 of 7 cell lines, three of eight primary tumors, and lower levels of expression in four of eight primary tumors. Control probe *RPS9* was hybridized to the cell line and primary tumor blots to indicate equal loading of RNA.

Chromosomal sorting of SSH genes

Genes from each of the four libraries were sorted based on their chromosomal positions. Several of the common genes identified in three or all four libraries mapped to known regions of deletions in ovarian cancer (21-24). For example, *ARHI* (*NOEY2*), a well-characterized imprinted tumor suppressor gene, with a reported loss of heterozygosity in 40-50% of ovarian cancer cases maps to 1p31(25). Another example, caveolin 1 (26) on 7q31.1-31.2, was identified in all four libraries that maps to a known region of deletion in ovarian cancer (27). Table 4 lists additional genes mapping to specific chromosomal regions of deletions in ovarian cancer. Of interest are chromosomal bands, 5q31-32, 10q11 and 10q25.3-26.2 because several of the down-regulated genes were isolated from these bands that were common to three of four or in all four libraries. The 5q31-32 genes are catenin (28), *FGF1* (29), *HDAC3* (30), selenoprotein P, plasma1 (*SEPP1*) (31), testican (*SPOCK*) (32),

transcription elongation factor B (SIII) polypeptide like (*TCEB1L*) (33), Transforming growth factor, beta induced, 68kD (*TGFβ1*) (34), *CDC23*, (35) early growth response 1 (*EGR1*) (36), and osteonectin (*SPARC*) (37). Down regulated genes from chromosomal band 10q11.2 and 10q25.3-26.2 that was identified from all four libraries were annexin A8 (*ANXA8*) (38) and *PRSS11* (39), respectively. Other genes such as nuclear receptor co-activator 4 (*ELE1*, 10q11.2) (40), proteoglycan, secretory granule (*PRG1*, 10q22.1) (41), vinculin (*VCL*, 10q22.1-23) (42), lipase A (*LIPA*, 10q23.3) (43), and protein phosphatase regulatory (inhibitor) subunit 5 (*PPP1R5*, 10q23-24) (44) were identified only from the two late stage libraries, OV 4 and OV 13.

LOH analysis of chromosomal regions 1p, 6q, 7q, 8p, 9p, 10q, 13q, 17p and 19q in stageI/II and stageIII/IV tumors:

Since many of the genes identified from the SSH libraries mapped to known regions of deletions in ovarian cancer, we analyzed a set of early and late stage tumors for loss of heterozygosity in regions of the genome where some of the down-regulated genes mapped. The chromosomal locations of the markers and the potential down regulated genes (identified in the SSH libraries) mapping to these regions are listed in Table 5. Figure 5 shows the overall LOH profile obtained. Down regulated genes mapping to chromosomal regions of loss identified from the libraries are *HSD3B1*, *EGR1*, serum glucocorticoid kinase (*SGK*) (45), and forkhead (Drosophila) homolog 1 (rhabdomyosarcoma) (FRKH) (46) mapping to 1p12-13, 5q31.1-31.2, 6q23.3, and 13q14.1 respectively. The approximate positions of these genes in relation to the markers of their respective chromosomes are also shown (Fig 5). The chromosome 1p11-13 and 6q 23.3 markers showed a higher frequency of loss in late stage tumors compared to early stage tumors. Other markers on chromosomes 8, 9, and 10 also showed more losses in high stage tumors. However, two markers on 5q31 and 13q14.1 and a marker within the BAC CIT-B-470f8 100 Kb distal to the *PEG3* locus on 19q13.4 had a higher frequency of LOH in early compared to late stage tumors. Markers D1S440, D1S534, D6S377 and D19S572 showed no LOH in early stage

tumors. In order to test if loss or lower levels of expression of a gene corresponded to a region of loss, we utilized a microsatellite repeat present within intron 2 of a novel gene on 8q that was identified in this study to determine the frequency of LOH in these tumors. This marker showed 50% loss both in low and high stage tumors (Figure 5). Figure 6 shows the pattern of loss of heterozygosity of this marker in ovarian tumors with loss of expression of this gene. For example, as shown in Figure 6, there was a direct correlation between lower levels of expression of this gene to loss of an allele by LOH (Tumor #684 and 208). In tumors with complete loss of expression (Tumor #182), the remaining allele could be inactivated either by hyper-methylation or by transcriptional inactivation due to other mechanisms. Tumors with ~~and~~ LOH, ~~no~~ loss of expression is seen as evidenced by semi-quantitative RT-PCR (Tumors #13 and #234).

Thus our LOH analysis revealed known and novel regions of loss where down regulated genes identified from the SSH libraries map to, lending support to the strength of the SSH technique to identify genes with low levels of expression in tumors. These genes could potentially represent candidate tumor suppressor genes involved in ovarian carcinogenesis.

DISCUSSION

This is the first report of down regulated genes in SSH libraries generated from primary ovarian tumors. The concept of identifying differentially expressed genes has been used before in techniques such as differential display PCR (DD-PCR) and representational differential hybridization (RDA). The strength of the SSH library is in the technique's ability to identify low abundance transcripts. While some of the genes identified from these libraries were also identified as down regulated genes by transcriptional profiling of the same tumors (47), we identified several known and unknown genes of very low abundance only in the SSH libraries.

Analysis of the differentially expressed sequences from early and late tumors allowed us to compare the library sequences to one another. In the four SSH libraries, we identified several genes whose function in carcinogenesis are known and others with no known roles in cancer. Some of the common genes such as tissue plasminogen activator inhibitor 1, *SPARC*, caveolin1 and *NOEY2* have been shown by others (16) (25, 26) to be differentially regulated in tumors. The potential tumor associated function of genes such as aldehyde oxidase, *HSD3B1* and *2*, *ITM2A*, alcohol dehydrogenase 2 (48), *PRSS11*, and PEG3 have not previously been linked with ovarian cancer. In addition, the function of several novel ESTs and genes identified from these libraries remain to be determined. Some of these same genes have been identified as down regulated genes by other techniques such as cDNA microarray analysis (47, 49) and differential display PCR (25).

It is a well-accepted concept that functional inactivation of both the alleles is a prerequisite for a tumor suppressor gene to be defined as such. The loss of expression of a gene could be due to deletion of both alleles (homozygous deletions), or deletion of one of the alleles and inactivation of the other allele either by inactivating mutations, by hypermethylation (50, 51) and/or altered activity of a transcriptional repressor (52). A combination of any one of these two events inactivating both the alleles will have the same result.

However, chromosomal sorting of approximately 600 genes and ESTs from each of the libraries revealed some interesting trends. Many of the genes identified from the SSH libraries were already mapped to known regions of deletions in ovarian cancer (21-24, 53, 54). We wanted to determine whether some of the known and novel genes identified from this screen would also map to regions of loss in ovarian cancer.

As evidenced in the LOH analysis, several of these coincided with regions of deletions observed in ovarian cancer. We identified several genes mapping to 5q31-32 in the SSH libraries. Of interest is early growth response gene 1 (*EGR1*), which has recently been identified as a down-regulated gene in ovarian cancer by cDNA microarray analysis (49). Two markers in the region, D5S396 and D5S500 showed a high frequency of LOH in early stage tumors not previously seen. *SPARC* is an acidic, cysteine-rich component of the extracellular matrix is directly regulated by progesterone and dexamethasone and indirectly by cytokines (55). Mok et al transfected the full length *SPARC* into SKOV3 cells and showed both reduced growth rate in cells expressing SPARC and reduced ability of these cells to form tumors in nude mice lending support to *SPARC* as a tumor suppressor. *SPARC* was identified as a down regulated gene in all four libraries from 5q31. Serum glucocorticoid kinase on 6q23.3, another region of deletion (23), was recently shown by Brunet et al (56) to act in concert with Akt in phosphorylating forkhead transcription factor, *FKHRL1*. This phosphorylation event leads to the activation of the PI3kinase cascade. Bagnoli et al (52) have shown a reciprocal negative regulation of α folate receptor *(αFR)* and caveolin 1 (*Cav-1*, on 7q31.1) proteins providing evidence for a new mechanism of *Cav-1* silencing in ovarian cancer. As indicated above, comparison of some of the down-regulated genes identified from the libraries corresponded with chromosomal regions of loss identified from the LOH studies. A marker 100Kb distal to paternally expressed gene 3 on 19q13.4 that was identified in all four libraries showed a higher frequency of deletion in low compared to high stage tumors. This is the first report of such a high frequency of deletion (33%) in early stage tumors in this region. Combining genomic with expression based analysis we were able to identify novel regions of loss in ovarian cancer. We have identified

several known and novel genes, including ESTs, whose functions in cancer have yet to be discerned. These genes could potentially lead to the identification of candidate tumor suppressor genes involved in ovarian cancer.

However not all the down regulated genes in these libraries could potentially represent tumor suppressor genes. This is essentially true for ribosomal genes. Other investigators also have reported the loss of expression of several ribosomal genes (CGAP-Digital Differential Display) in cancer and yet the functional consequence of loss of expression of these genes have not been directly linked to tumor suppression. While some of the genes such as *ARHI, caveolin 1 and SPARC*, that map to regions of deletions in ovarian cancer, have known tumor-suppressing functions, for other genes, neither the mechanistic basis for the loss nor the functional consequence of such a loss is known. One of the novel genes identified in this screen showed 50% LOH in both early and late stage tumors. Using a microsatellite marker associated with this gene we were able to correlate the loss and/or lower levels of expression of this gene to loss of an allele by LOH.

The data from the SSH library analysis revealed that there were many genes which were differentially expressed in both early and late stage tumors. Genes identified only in one library could potentially indicate tumor specific differences. We do not yet know what changes are critical in an early stage tumor to progress to a more malignant tumor. The genes identified from the SSH libraries are all based on expression differences. This technique cannot detect gross genomic changes, including chromosomal rearrangements or the mutator phenotype (57), unless they result in concomitant changes in transcript levels of genes. However, one very important epigenetic phenomenon, namely, methylation (58, 59) has been associated with changes in the levels of gene expression. Evidence seems to indicate that methylation changes are early events in carcinogenesis leading to the possibility that the majority of the genes inactivated in early stage tumors could be hypermethylated. Some of the genes isolated in this screen have been reported by others to be inactivated by hyper-methylation in ovarian cancer (60, 61).

For example, two of the down regulated genes identified from chromosomal band 12p13 from the library sequences such as cyclin D2 (62) and complement component 1 subcomponent (63) do not map to known regions of deletion in ovarian cancer. These genes could be inactivated by methylation. The inactivation of Cyclin D2 by methylation in Burkitt's lymphoma (62) and breast cancer (64) has previously been reported. Transcriptional inactivation can also result due to aberrant regulation of factors

In conclusion, we have identified several known and novel genes that are down regulated both in early and late stage tumors. Several of these genes were later mapped to the regions of loss by LOH analysis. However, we do not rule out the possibility that loss of expression of some of these genes could also be due to decreased transcriptional inactivation or through promoter hypermethylation. Thus combining expression- and genomic-based analyses has provided us with novel regions of alterations in ovarian cancer not previously reported. We are currently pursuing the cloning and characterization of some of the novel genes identified from these libraries to address the functional roles of these genes in ovarian cancer.

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HISTOLOGY	STAGE	GRADE	LOH	Northern	RT-PCR	SSH
Cl Cell OV 106	I	3	-	-	+	-
Cl Cell OV 267	I	3	+	-	+	-
Cl Cell OV 496	I	3	+	-	+	-
Endo OV 51	I	3	+	-	+	-
Endo OV 78	I	3	+	-	+	-
Endo OV 88	I	3	-	-	+	-
Endo OV 105	I	3	+	-	+	-
Endo OV 338	I	3	+	+	+	+
Endo OV 647	I	3	+	+	+	-
Endo OV 684	I	3	+	-	-	-
Serous OV 6	I	3	-	-	+	-
Serous OV 17	I	3	-	-	+	-
Serous OV 20	I	3	-	+	+	-
Serous OV 90	I	3	+	-	+	-
Serous OV 234	I	3	+	-	+	-
Serous OV 363	I	3	-	-	+	-
Serous OV 526	I	3	-	-	+	-
Cl Cell OV 102	II	3	+	-	+	-
Endo OV 296	II	3	+	-	+	-
Serous OV 149	II	3	+	+	+	-
Serous OV 354	II	3	+	-	+	-
Serous OV 401	II	3	-	-	+	-
Serous OV 402	II	3	+	-	+	+
Serous OV 414	II	3	+	-	+	-
Cl Cell OV 176	III	3	+	-	+	-
Cl Cell OV 453	III	3	+	-	-	-
Cl Cell OV 623	III	3	+	-	-	-
Endo OV 93	III	3	+	+	+	-
Endo OV 110	III	3	+	-	+	-
Endo OV 259	III	3	+	-	+	-
Serous OV 4	III	3	+	+	+	+
Serous OV 11	III	3	+		+	-
Serous OV 13	III	3	+	+	+	+
Serous OV 16	III	3	+	-	+	-
Serous OV 29	III	3	+	-	+	-
Serous OV 150	III	3	+	-	+	-
Serous OV 167	III	3	+	-	+	-
Serous OV 182	III	3	+	-	+	-
Serous OV 206	III	3	+	-	+	-
Serous OV 208	III	3	+	-	+	-
Serous OV 461	III	3	+	-	+	-
Serous OV 472	III	3	-	-	+	-
Serous OV 97	IV	3	+	-	+	-

TABLE 1

Table 2: Common down regulated genes in all four libraries

Cluster ID	Cluster Title
Hs.195851	Actin, alpha 2, smooth muscle
Hs.180952	Actin, gamma 1
Hs. 75442	Albumin
Hs. 4	Alcohol dehydrogenase 2, beta polypeptide
Hs. 87268	Annexin, A8
Hs.182183	Caldesmon
Hs. 83942	Cathepsin K
Hs. 74034	Caveolin 1
Hs. 169756	Complement component,1 s subcomponent
Hs. 78065	Complement component 7
Hs. 76053	DEAD/H box polypeptide 5 (RNA helicase,68kD)
Hs. 76152	Decorin
Hs. 58419	DKFZP586L2024 protein
Hs. 181165	Eukaryotic translation elongation factor 1, alpha 1
Hs. 2186	Eukaryotic translation elongation factor 1, gamma 1
Hs. 62954	Ferritin, heavy polypeptide 1
Hs. 89552	Glutathione S-transferase A2
Hs. 5662	G protein, beta polypeptide 2-like 1
Hs. 83381	Guanine nucleotide binding protein 11
Hs. 3297	H.sapiens Uba80, mRNA for ubiquitin
Hs. 180532	Heat shock 90kD protein 1, alpha
Hs. 158675	Heat shock factor binding protein 1
Hs. 155376	Hemoglobin, beta
Hs. 75445	Hevin
Hs.103391	IGFBP5
Hs. 38586	HSD3B1
Hs. 825	HSD3B2
Hs. 182187	IGF2
Hs. 107169	Insulin like growth factor binding protein 5
Hs. 17109	Integral membrane protein 2A
Hs. 184914	KIAA0471-myosin heavy chain
Hs. 181357	Laminin receptor 1(67kD, ribosomal protein SA)
Hs. 173714	MORF-related gene X
Hs. 153837	Myeloid cell nuclear differentiation antigen
Hs. 1255	Nuclear recptor subfamiliy 2, group F, member 2
Hs. 74615	PDGF, alpha polypeptide
Hs. 75111	PRSS11
Hs. 194695	Ras homolog gene family, member 1 (NOEY2)
Hs. 184108	Ribosomal protein L21
Hs. 180946	Ribosomal protein L5
Hs. 217493	Ribosomal protein S6
Hs. 151604	Ribosomal protein S8
Hs. 82448	Selectin L
Hs. 3314	Selenoprotein P, plasma, 1-Histidine rich
Hs. 56306	Small proline-rich pretein A
Hs. 46158	ESTs
Hs. 182643	Transcription factor B (SIII), polypeptide 1-like
Hs. 2064	Vimentin

Table 3: Chromosomal localization of down regulated genes from SSH libraries

Chromosome	Down Regulated Genes
1p12-13	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
1p12-13	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
1p31	ras homolog gene family, member I
1q21	cathepsin K (pycnodysostosis)
1q21-q22	small proline-rich protein 2A
2q33-36	insulin-like growth factor binding protein 5
2q33	aldehyde oxidase 1
3p21.3	ras homolog gene family, member A
4q12	insulin-like growth factor binding protein 7
4q21.2	alcohol dehydrogenase 2 (class I), beta polypeptide
5q31	selenoprotein P, plasma, 1
5q31	early growth response 1
5q31	secreted protein, acidic, cysteine-rich (osteonectin)
6p12.2	glutathione S-transferase A2
6q23.3	serum/glucocorticoid regulated kinase
6q27	thrombospondin 2
7p15-13	inhibin, beta A (activin A, activin AB alpha polypeptide)
7q21.3-22	plasminogen activator inhibitor, type I
7q31.1-31.2	caveolin 1, caveolae protein, 22kD
8p22-p21.3	platelet-derived growth factor receptor-like
8q21	Novel gene
9p21	ribosomal protein S6
9q11-q22	annexin A1
10q24.3	cytochrome P450, subfamily XVII (steroid 17-alpha-hydroxylase)
10q25.3-q26.2	protease, serine, 11 (IGF binding)
11p15.5	hemoglobin, beta
11p15.5	insulin-like growth factor 2 (somatomedin A)
12p13	cyclin D2
12q23	decorin
13q14.1	forkhead (Drosophila) homolog 1 (rhabdomyosarcoma)
13q14.3	integral membrane protein 2B
14q32.1	protease inhibitor 1 (anti-elastase), alpha-1-antitrypsin
14q22-q24	butyrate response factor 1 (EGF-response factor 1)
15q15-q21.1	fibroblast growth factor 7 (keratinocyte growth factor)
15q15	thrombospondin 1
16q13-q21	matrix metalloproteinase 2 (gelatinase A, 72kD)
16q24.2-q24.3	cadherin 13, H-cadherin (heart)
17	ESTs
19q13.4	paternally expressed gene 3
22q12.3	tissue inhibitor of metalloproteinase 3
Xq13.3-Xq21.2	integral membrane protein 2A
Xq21.1	ESTs

GENES	OSE	OV 167	OV 177	OV 202	OV 207	OV 266	OVCAR 5	SKOV 3	Chromosomal location
HSD3B1	+	-	-	-	-	-	-	-	1p13
CTSK	++	weak	-	-	-	-	-	-	1q21
IGFBP5	+	-	-	+	-	-	ND	ND	2q33-36
Hevin	+	-	-	-	-	-	-	-	4
SEPP1	+	+	+	-	-	+	+	+	5q31
TCEB1L	+	-	+	+	+	+	+	+	5q31
EGR1	+	weak	-	-	weak	+	weak	++	5q31
SPARC	+	-	-	weak	-	-	-	-	5q31
FGF1	+	-	-	-	+	-	-	+	5q31
Testican	+	-	+	+	+	-	+	-	5q31
THBS2	++	weak	-	-	-	-	-	-	6q27
PAI1	++	+	-	-	weak	weak	weak	+	7q21
Novel gene	+	+	-	+	-	-	-	-	8q21
PRSS11	++	-	-	+	-	-	+	+	10q25
Cyclin D2	+	-	-	+	-	-	-	-	12p13
Decorin	++	-	-	++	-	-	-	-	12q21.3
FGF7	+	-	-	+	-	-	-	-	15q15-21.1
PEG3	+		++	++	-	-	-	-	19q13.4
ITM2A	++	-	-	-	+	-	-	-	Xq13.3-21.1
Novel gene	+	-	-	-	-	-	-	-	Xq22

Table 4: Results of semi-quantitative RT-PCR analysis of down regulated genes in ovarian cancer cell lines. +: Presence of a product, -: Absence of a product, weak: Presence of a weak product, ND: Not determined.

TABLE 5: Markers used for LOH analysis and % LOH in early and late stage tumors

Markers	% LOH in Early Stage Tumors	% LOH in Late Stage Tumors	Cytogenetic band location	Down regulated genes from SSH libraries
D1S189	27 (4/15)	26 (5/17)	1p13.1	
D1S440	0 (0/13)	19 (3/16)	1p12	
D1S453	14 (2/14)	29 (5/17)	1p12	
D1S2863	27 (3/11)	29 (5/17)	1p12	HSD3B1
D1S534	0 (0/10)	22 (4/18)	1p11.2	
D1S514	14 (2/14)	33 (6/18)	1p11.2	
D5S396	50 (6/12)	50 (8/16)	5q31.1	EGR1, SPARC
D5S500	50 (5/10)	38 (5/13)	5q31.2	
D5S476	28 (2/7)	50 (6/12)	5q31.2	
D5S2119	30 (3/10)	25 (2/8)	5q31.2	
D6S311	0 (0/8)	54 (6/11)	6q23.1	SGK
D6S977	18 (2/11)	46 (6/13)	6q23.3	
D6S1008	10 (1/10)	37.5 (6/16)	6q25	
D7S1805	25 (2/8)	64 (7/11)	7q36	
D8S258	18 (2/11)	37.5 (6/16)	8p21	PDGFRL
8q NG	50 (6/12)	50% (4/8)	8q	8q Novel Gene
D9S259	36 (5/14)	44 (8/18)	9p21	
D10S215	9 (1/11)	20 (3/15)	10q23.1	LIPA, Actin α 2
D10S574	21 (3/15)	17 (3/18)	10q23.3	smooth muscle (ACTA2)
D17S1868	50 (4/8)	23 (3/13)	17q21.1	
D13S263	67 (6/9)	50 (9/18)	13q14.1	
MCJ*	62 (5/8)	46 (8/15)	13q14.1	FKHR
D19S180	17 (2/12)	28 (5/18)	19q13.3	
D19S572	0 (0/15)	14 (2/17)	19q13.3	
PEG3*	33 (4/12)	19 (3/16)	19q13.4	PEG3
D19S254	42 (5/12)	53 (9/17)	19q13.4	
D19S926	23 (3/13)	50 (8/16)	19q13.4	

The numbers in parenthesis are (# of tumors with LOH/Total # of informative tumors). * Primers are listed in the methods section.

FIGURE LEGENDS

Figure 1A: Southern Analysis: Equal amounts of unsubtracted and subtracted cDNAs were fractionated on 2% agarose gel, blotted and hybridized with (^{33}P) dCTP labeled GAPDH. 1; OV 402 library, 2; OV 4 library. Lanes 1 & 3: Unsubtracted cDNAs, Lanes 2 & 4: Subtracted cDNAs. **1B**: PCR based Analysis: 10 ng of Unsubtracted (Unsub) and subtracted (Sub) cDNAs were amplified with GAPDH primers as described in methods. 1; library OV 402 library, 2; library OV 4. Lanes 1-4; products after 18, 23, 28 and 32 cycles of GAPDH amplification, M: 100bp ladder.

Figure 2A: Products of colony PCR resolved on a 2% agarose gel. The gels were stained with ethidium bromide and photographed to ensure equal loading. Panels 1 & 3 and panels 2 & 4 are duplicates. **2B:** Duplicate filters hybridized with double stranded P^{33}-labeled tester (panels 1 & 2) and driver (panels 3 & 4) cDNAs of equal specificity under the same conditions as described in methods.

Figure 3A: Agarose gel showing the products of semi-quantitative RT-PCR in the ovarian cell lines. Lane 1: short term cultures of normal ovarian epithelial cells (OSE 54) Lane 2:OV 167, Lane 3:OV 177, Lane 4: OV 202, Lane 5: OV 207, Lane 6: OV 266, Lane 7: OVCAR 5, Lane 8:SKOV 3 and Lane 9: water control. Probes: Panel 1; CTSK-Cathepsin K, Panel 2; SPARC, Panel 3; EGR1-Early growth response gene 1, Panel 4; THBS2- Thrombospondin 2, Panel 5; PAI1-Plasminogen activator inhibitor 1, Panel 6; Decorin, Panel 7;Cyclin D2, Panel 8; FGF 7- Fibroblast growth factor 7, Panel 9: ITM2A-Integral membrane protein 2A, and Panel 10; GAPDH- Glyceraldehyde 3 phosphate dehydrogenase . **3B:** Agarose gel showing the products of the result of semi-quantitative RT-PCR resolved on a 1.6% agarose gel. Sample numbers are indicated on top of the figure and the staging information for these tumors are indicated on top of the tumor numbers. M:100 bp ladder, Lane 1: B-Normal epithelial cell brushings.

Panel 1: PAI1-Plasminogen activator inhibitor 1, Panel 2: ITM2A-Integral membrane protein 2A Panel 3: FGF 7- Fibroblast growth factor 7, Panel 4: A novel gene, Panel 5; PEG3 and GAPDH-Glyceraldehyde 3 phosphate dehydrogenase.

Figure 4: Autoradiograph showing the Northern hybridization results with probes: HSD3B1: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1, and PRSS11. RPS9: Ribosomal protein S9. **4A** (Cell lines); O: OSE, Lane 1: OV167, Lane 2: OV 177, Lane 3: OV 202, Lane 4: OV 207, Lane 5: OV 266, Lane 6: OVCAR 5, Lane 7: SKOV3. **4B** (Primary tumors): Lanes 1-4: Early stage, Lanes 5-8: Late stage tumors. The staging information for the primary tumors is listed in Table1.

Figure 5: Histogram of the results of LOH with the markers tested. □: Early stage, ■: Late stage. % LOH: Frequency of LOH with specific markers. An arrow indicates the approximate positions of these genes in relation to the markers of their respective chromosomes.

Figure 6A: Agarose gel showing the products of semi-quantitative RT-PCR resolved on a 1.6% agarose gel. Sample numbers are indicated on top of the figure. M: 100 bp ladder, The top band is the product of amplification with 8q Novel Gene primers. Bottom band is the product of amplification with GAPDH primers F and R. **6B:** Autoradiograph of LOH results of corresponding tumor samples with intron 2 microsatellite marker. For each panel, the tumor # is shown above. N: Normal DNA, T: tumor DNA. Arrow indicates loss of the allele in the tumor.

TABLE LEGENDS

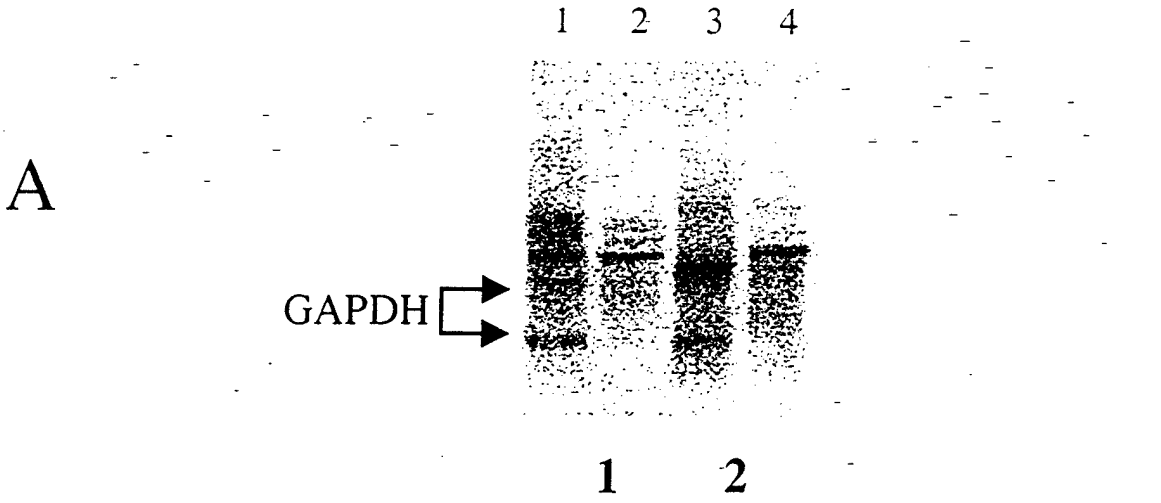
Table 1:Tumor cohort. + : Tumors in which the specific analysis was performed. Cl Cell; Clear Cell, Endo: Endometrioid.

Table 2: Common down regulated genes in all four libraries. The unigene cluster IDs and gene descriptions are included.

Table 3: Chromosomal localization of down regulated genes from SSH libraries.

Table 4: Results of semi-quantitative RT-PCR analysis of down regulated genes in ovarian cancer cell lines. +: Presence of a product, -: Absence of a product, weak: Presence of a weak product, ND: Not determined.

Table 5: Markers used for LOH analysis and % LOH in early and late stage tumors



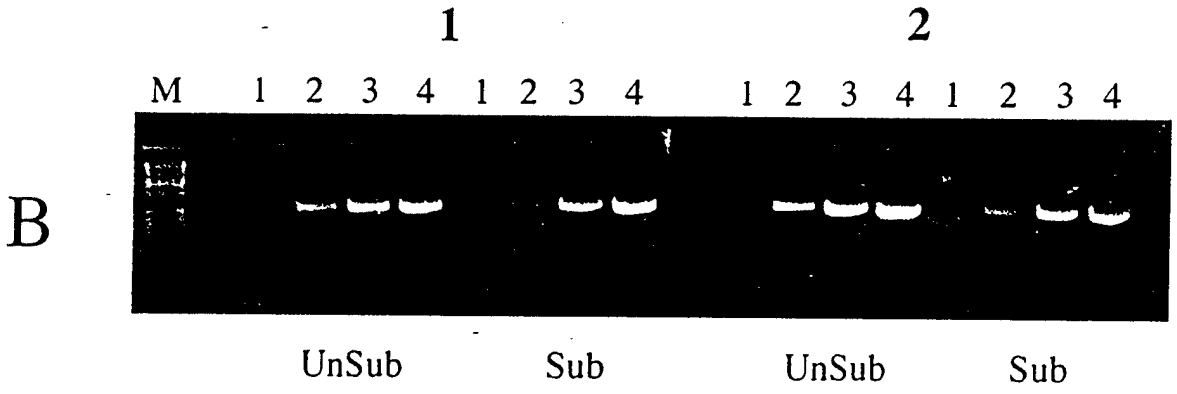
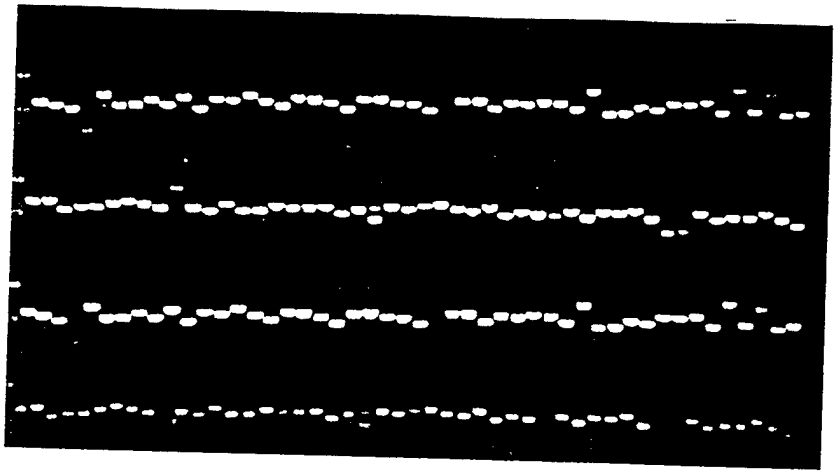


Fig. 1



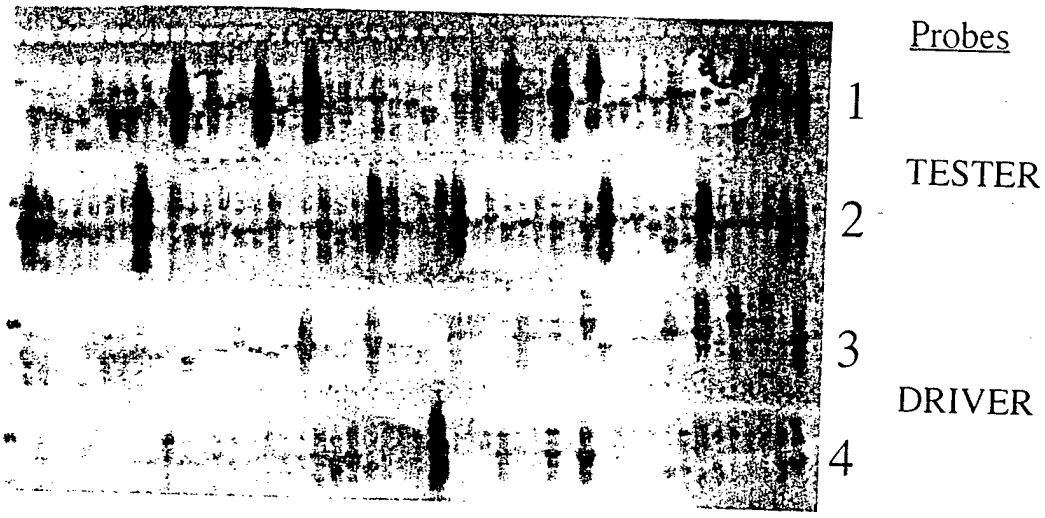
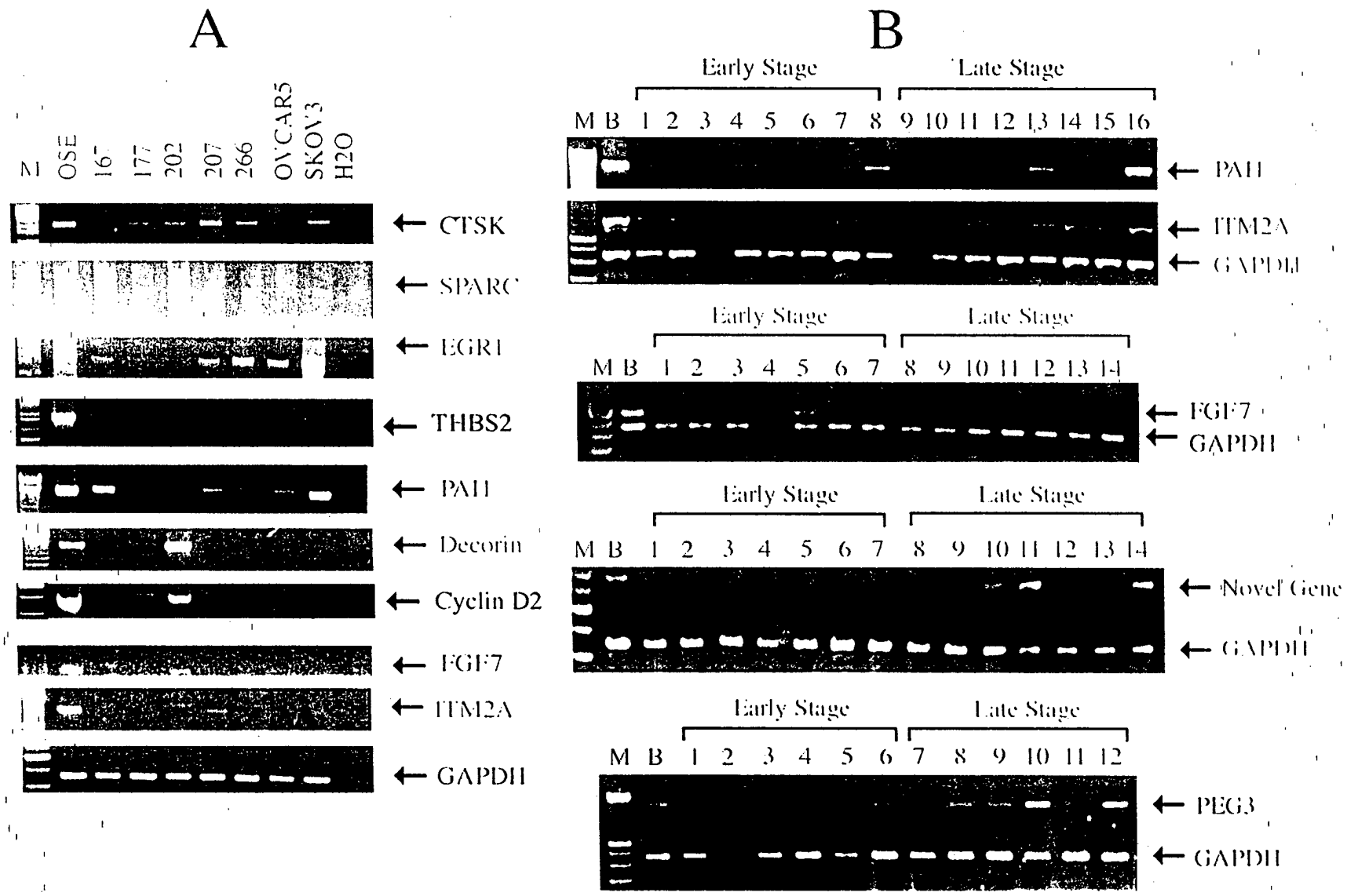


Fig. 2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177
202
207
266
OVCAR5
SKOV3
H2O
CTSK
SPARC
EGR1
THBS2
PAI1
Decorin
Cyclin D2
FGF7
ITM2A
GAPDH
B
Early Stage
Late Stage
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PAI1
ITM2A
GAPDH
M B 1 2 3 4 5 6 7 8 9 10 11 12 13 14
FGF7
GAPDH
Novel Gene
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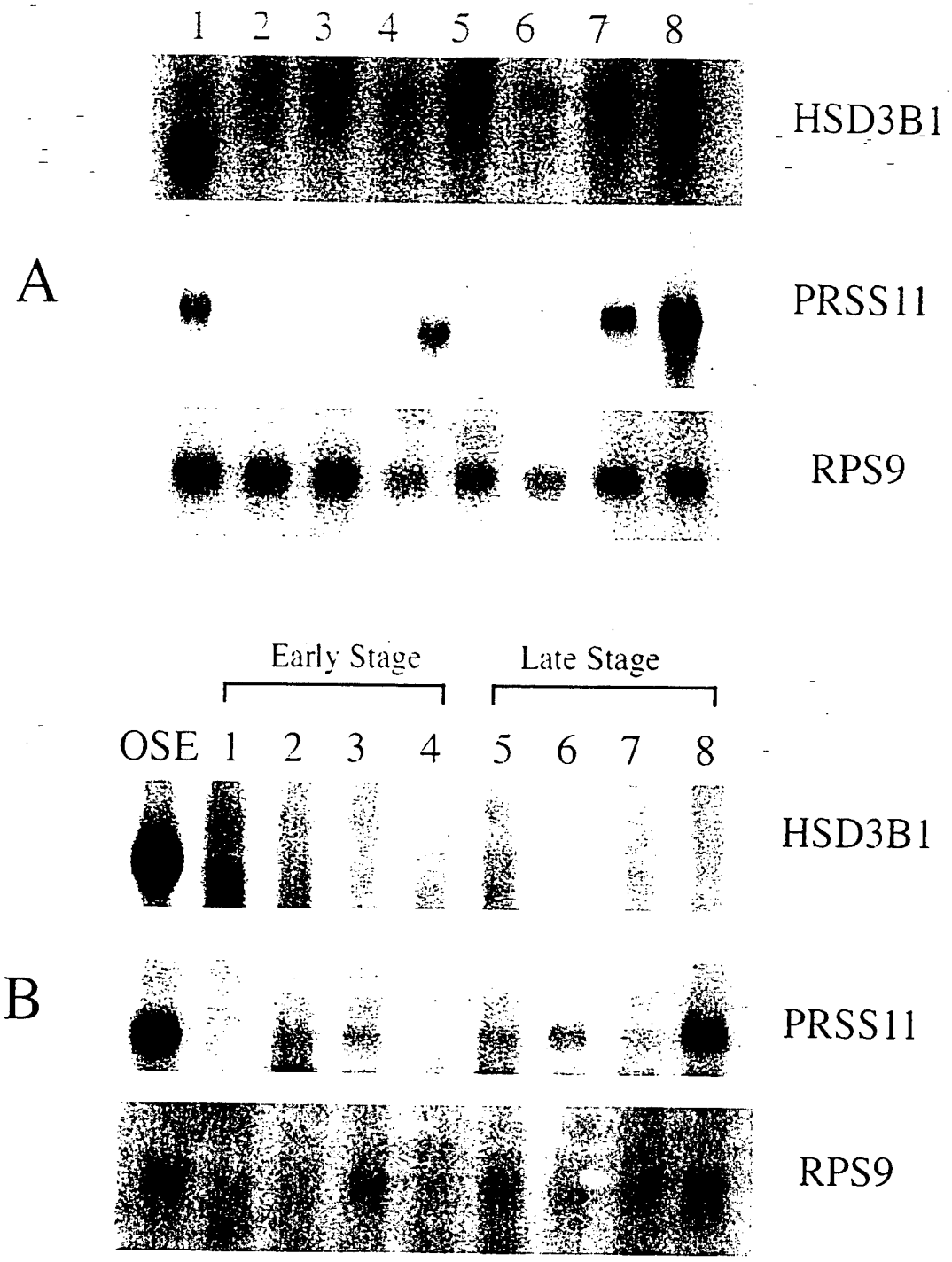
M B 1 2 3 4 5 6 7 8 9 10 11 12
PEG3

Fig. 3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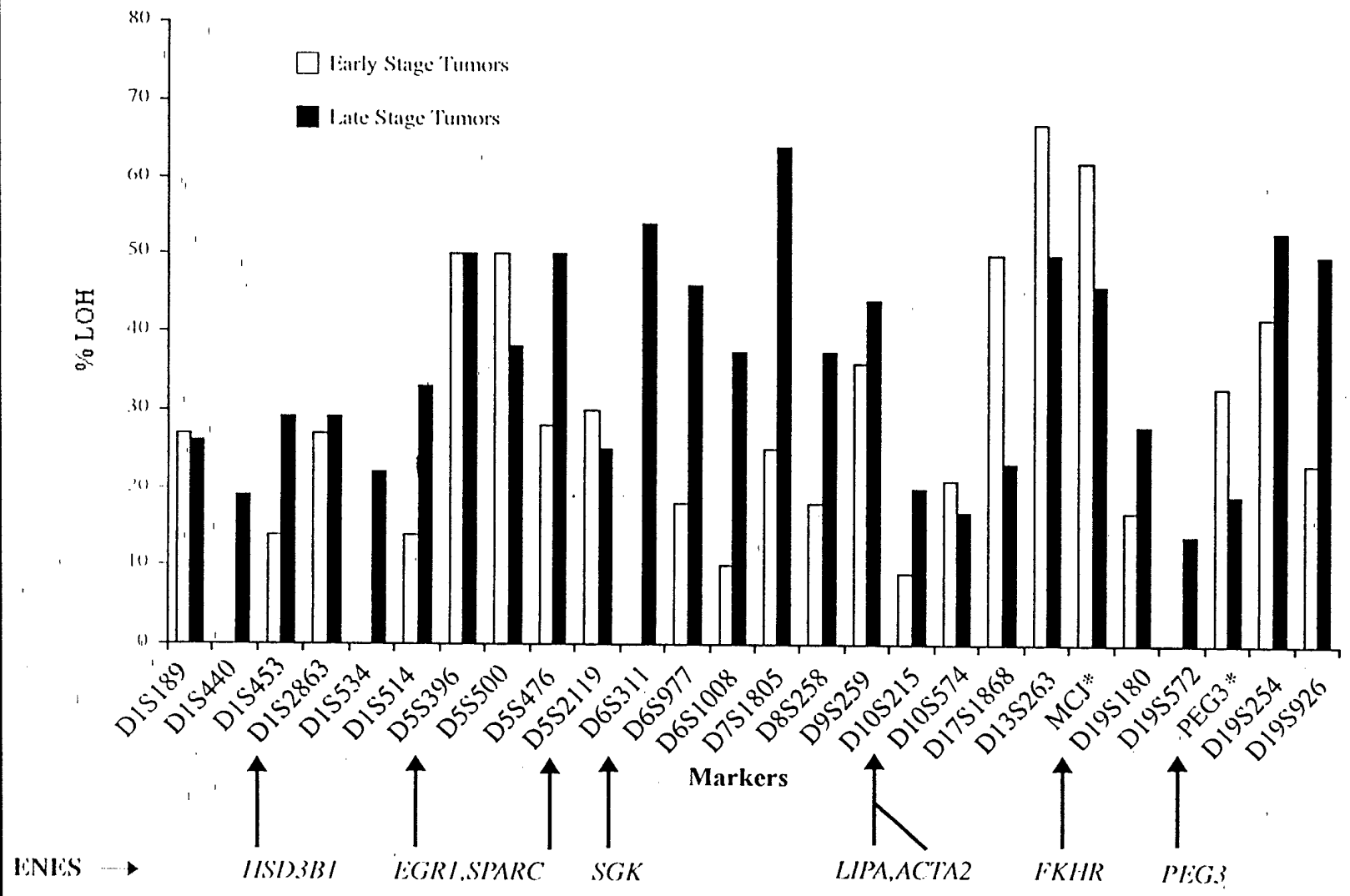
A
PRSS11
RPS9
Early Stage
Late Stage
OSE 1 2 3 4 5 6 7 8
HSD3B1
B
PRSS11
RPS9

Fig. 4


Early Stage Tumors
Late Stage Tumors
% LOH
80
70
60
50
40
30
20
10
0
D1S189
D1S440
D1S453
D1S2863
D1S534
D1S514
D5S396
D5S500
D5S476
D5S2119
D6S311
D6S977
D6S1008
D7S1805
D8S258
D9S259
D10S215
D10S574
D17S1868
D13S263
MCJ*
D19S180
D19S572
PEG3*
D19S254
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Markers
ENES
HSD3B1
EGR1,SPARC
SGK
LIPA,ACTA2
FKHR
PEG3

Fig. 5

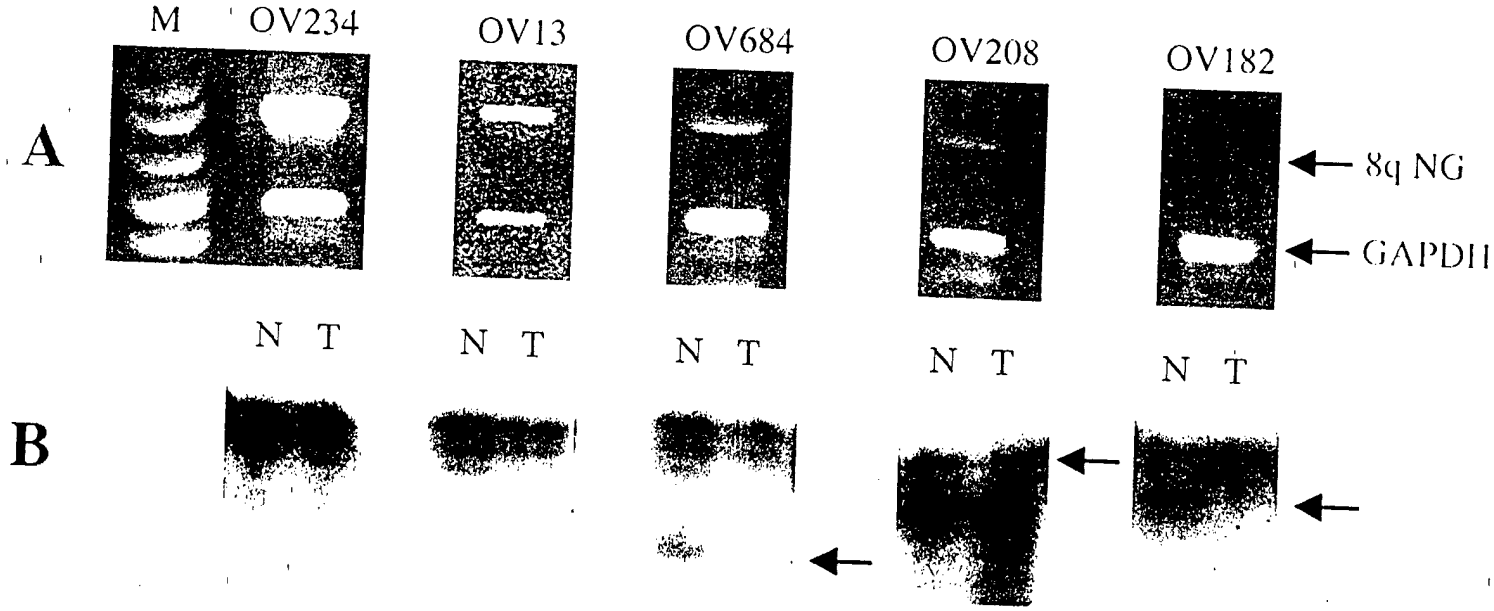

Fig. 6