THE YALE JOURNAL OF BIOLOGY AND MEDICINE 62 (1989), 379-392

Oncogene Expression *in Vivo* by Ovarian Adenocarcinomas and Mixed-Mullerian Tumors

BARRY M. KACINSKI, M.D., Ph.D.,[a,b] DARRYL CARTER, M.D.,[c]
ERNEST I. KOHORN, M.D.,[b] KHUSHBAKHAT MITTAL, M.B., B.S.,[c]
R. SHAEFFER BLOODGOOD,[a] JOHN DONAHUE, B.S.,[a]
CAROL A. KRAMER, M.D.,[a] DIANA FISCHER, Ph.D.,[d]
ROB EDWARDS, B.S.,[c] SETSUKO K. CHAMBERS, M.D.,[b]
JOSEPH T. CHAMBERS, Ph.D., M.D.,[b] AND PETER E. SCHWARTZ, M.D.[b]

Departments of [a]Therapeutic Radiology, [b]Obstetrics and Gynecology, [c]Pathology, and [d]Biostatistics, Yale University School of Medicine, New Haven, Connecticut

Received July 3, 1989

Six-micron paraffin sections of paraformaldehyde-fixed specimens of 24 ovarian benign and neoplastic specimens were assayed for tumor cell-specific oncogene expression by a sensitive, quantitative in situ hybridization technique with probes for 17 oncogenes, beta-actin, and *E. coli* beta-lactamase. In the benign, borderline, and invasive adenocarcinomas, multiple oncogenes, including *neu, fes, fms,* Ha-*ras, trk,* c-*myc, fos,* and PDGF-A chains, were expressed at significant levels relative to a housekeeping gene (beta-actin). In the mixed-Mullerian tumors, a rather different pattern of oncogene expression was observed, characterized primarily by expression of *sis* (PDGF-B chain).

For the adenocarcinomas, statistical analysis demonstrated that expression of several genes (*fms, neu,* PDGF-A) was closely linked to others (c-*fos,* c-*myc*) known to have important roles in the control of cell proliferation, but only one gene, *fms,* correlated very strongly with clinicopathologic features (high FIGO histologic grade and high FIGO clinical stage) predictive of aggressive clinical behavior and poor outcome. The authors discuss the role that tumor epithelial cell expression of the *fms* gene product might play in the auto- and paracrine control of growth and dissemination of ovarian adenocarcinomas.

In some cell culture systems, serial transfection of morphologically benign primary cell lines with viral or cellular oncogenes can confer, stepwise, phenotypic traits characteristic of malignant cells [1,2]. Such observations have inspired hypotheses that a similar incremental progression of cellular oncogene activation (mutation, aberrant over- or underexpression, and so on) occurs during the development of spontaneous neoplasms [3–7]. Ovarian epithelial neoplasms are a system well-suited for testing such hypotheses, since they encompass a broad spectrum of lesions, ranging from benign hyperproliferative serous and mucinous adenomatous cysts, serous and mucinous adenomas of borderline malignant potential to invasively malignant well-differentiated, moderately differentiated, and poorly differentiated serous, mucinous,

Abbreviations: ISH: in situ hybridization M-CSF, CSF-1: macrophage colony stimulating factor MMT: mixed-Mullerian tumor

This research was supported by ACS grant CD-262, a Swebilius Foundation Cancer Research Award, NIH research grant CA-47292, a Leukemia Society Special Fellowship, and a Bristol-Myers Cancer Research Award to BMK. Tissue retrieval and processing was funded through the NIH Core Facility Grant to the Yale Comprehensive Cancer Center.

Address reprint requests to: Barry M. Kacinski, M.D., Dept. of Therapeutic Radiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

Copyright © 1989 by The Yale Journal of Biology and Medicine, Inc.
All rights of reproduction in any form reserved.

and endometroid adenocarcinomas, and include less common histologies such as mixed-Mullerian (mixed mesodermal) tumors, and others [8–10]. Clinical presentations also vary and range from small lesions confined within the ovarian capsule to aggressively malignant neoplasms which have disseminated throughout the peritoneal cavity and metastasized to distant visceral sites. At least for the adenocarcinomas, clinical extent of disease and histologic grade at presentation correlate very strongly with outcome [8–10]. We anticipated, therefore, that studies of cellular changes of oncogene expression in ovarian neoplasia would provide us with valuable information on those relationships which exist between prognostically important clinical and pathologic characteristics and qualitative and quantitative changes in tumor cell-specific oncogene expression.

In 1989, over 30 different genes have been identified as potential oncogenes [6,7,11–13]. Most previously published reports have focused on the importance of the expression of a single oncogene or oncogene class in tumor tissue or tumor-derived cell lines [14–17]. In this paper, we report the use of a sensitive and quantitative in situ hybridization technique [18–22] to assay levels of expression of 17 different oncogenes in 24 ovarian benign and neoplastic specimens. This quantitative data was subjected to statistical analyses, revealing many interesting relationships, some of which link high-grade, high-stage presentations to genes not otherwise implicated in the biology of epithelial neoplasms.

MATERIALS AND METHODS

Tissue Specimen Accrual, Preparation, and in Situ Hybridization (ISH)

All tissue specimens were obtained from patients of the Hunter Radiation Therapy and Ob/Gyn clinics of the Yale University School of Medicine, in accordance with Yale HIC protocol 3303. Small biopsies (maximum, ~3 mm thick) of ovarian neoplastic or benign tissues were obtained during therapeutic or diagnostic procedures (by PES, EIK, SKC, and JTC) and placed into freshly prepared PGP fixative (4 percent paraformaldehyde, 0.5 percent glutaraldehyde, 0.1 M Na-phosphate [pH 7.5]) within one to two minutes of harvest. Fixation was continued for four to six hours. Specimens were processed for embedding in paraffin, six-micron sections cut, prepared for, and carried through in situ hybridization and nuclear track emulsion autoradiography, as has been described elsewhere [18–24]. Probes for ISH were prepared by appropriate restriction digest of chimeric plasmids with cloned oncogenes, labeled with ^{35}S-dCTP by random primer extension [25], using alpha-^{35}S-labeled dCTP to give specific activities averaging 5×10^8 dpm/mcg DNA [26], and were complementary to coding sequences of beta-actin [27] (PstI), and enterobacterial beta lactamase [28] (EcoRI, PstI), c-*myc* [29] (third exon; ClaI, EcoRI), N-*myc* [30] (third exon; AccI, AvaI), L-*myc* [31] (second and third exon; SmaI, EcoRI), c-*fos* [32] (NcoI, XhoI), *myb* [33] (KpnI, XbaI), p53 [34] (EcoRI, BglII), Ha-*ras* [35] (SstI, PstI), Ki-*ras* [36] (EcoRI), N-*ras* [37] (SalI, NcoI), *sis* [38] (PstI, XbaI), PDGF-A chain [39] (SstI, HindIII), *erbB* [40] (BamHI), *neu* [41] (BamHI), *fes* [42] (PstI), *fms* [43] (PstI), *ros* [44] (EcoRI, PvuII), and *trk* [45] (NcoI, EcoRI). Sections of confluent monolayers of BeWo cells [46] (grown in Weymouth's + 10 percent fetal calf serum, 37°C, 5 percent CO_2) were processed with each experimental run as positive controls. In our experiments, BeWo cells show consistently elevated levels of expression of c-*myc*, *fms*, and *fos* complementary mRNAs and were useful positive controls (see Fig. 1) [47,48]. Non-neoplastic ovarian tissues are included as negative controls (e.g., cases 1 and 2).

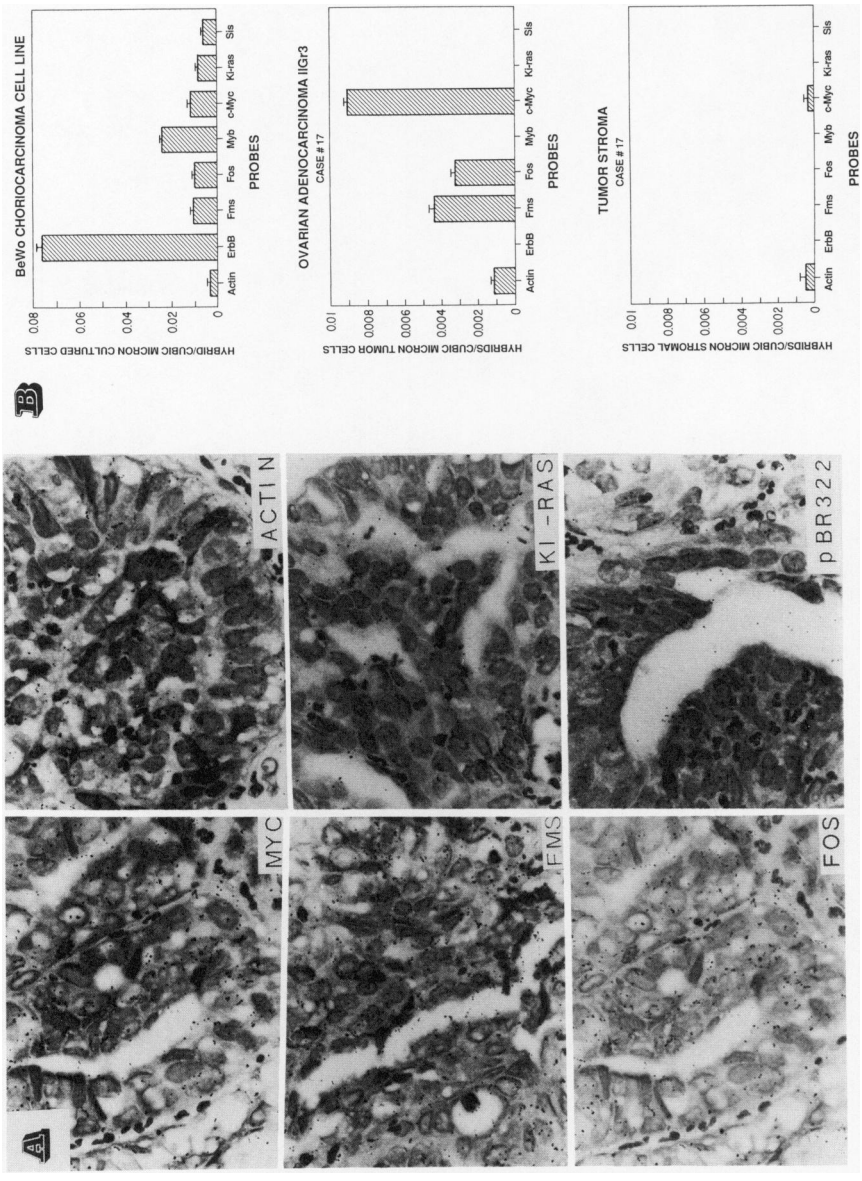

FIG. 1. In situ hybridization technique for tumor and stroma of specimen 17. **A.** In situ hybridization is carried out as described in the text with four oncogene, actin, and beta-lactamase (pBR322) probes. Sections are photographed at 100× (oil immersion) to demonstrate clearly the difference in grain counts for the negative control beta-lactamase probe and the other genes. **B.** Hybrids per micron^3 for beta-actin and seven oncogene probes are presented for BeWo choriocarcinoma cells (our positive control) and case 17 tumor and stroma. Tissue preparation, sectioning, processing of sections, in situ hybridization, autoradiography, and staining are carried out as described in Methods.

TABLE 1
In Situ Hybridization Data (hybrids/cu micron × 1,000,000)

No.	Grade	Stage	Actin	*erb-B*	*fes*	*ros*	*trk*	P53	*fos*	*myb*	c-*myc*	L-*myc*	N-*myc*	Ha-*ras*	Kl-*ras*	N-*ras*	PDGF-A	*sis*
								Benign and Borderline										
1	0	0	290	0	0	630	5,097	0	0	0	508	0	124	0	0	0	0	0
2	0	0	110	0	474	0	297	87	89	0	339	0	0	0	0	167	0	0
3	0.5	II	359	1,083	1,490	0	0	146	349	238	512	305	148	1,279	112	356	437	296
4	0.5	I	514	2,903	2,264	0	0	0	0	0	517	0	0	0	1,399	268	291	0
5	0.5	III	773	3,719	2,946	0	0	0	295	0	2,995	170	0	4,246	0	0	0	0
								Grade 1 and 2 Adenocarcinomas										
6	1	III	87	3,670	815	217	212	95	453	558	347	141	355	1,814	0	0	327	0
7	2	II	717	787	2,214	0	0	286	516	0	2,489	0	260	2,643	0	119	2,468	0
8	2	III	85	1,786	724	0	98	229	363	163	123	0	164	262	0	297	634	132
9	2	I	249	0	0	0	0	1,153	626	0	1,392	0	773	1,083	2,482	0	1,350	159
10	2	III	148	2,285	22,285	1,097	328	0	548	2,657	1,961	0	170	1,835	0	0	1,040	165
11	2	III	193	2,545	3,023	4,162	12,513	138	1,139	0	4,527	190	0	3,037	0	0	1,195	0
12	2	III	412	0	2,517	0	1,167	181	1,800	0	1,357	89	352	4,281	401	1,160	1,097	0

								Grade 3 Adenocarcinomas									
13	3	III	294	695	1,111	0	0	136	169	703	166	344	70	0	214	1,933	661	0
14	3	III	186	0	3,473	459	0	170	580	1,378	1,329	0	306	297	1,272	0	2,721	745
15	3	III	672	0	5,701	465	5,385	342	1,406	0	2,510	93	752	7,944	0	0	602	0
16	3	III	766	13,216	0	306	6,172	476	2,369	0	3,909	0	0	0	3,895	0	949	1,223
17	3	II	1,132	0	4,633	1,779	3,642	520	3,234	0	9,069	254	253	1,062	0	3,635	2,109	0
18	3	III	676	43,586	3,824	2,320	414	400	4,879	0	6,918	103	527	5,990	0	787	3,185	218
19	3	III	333	0	0	2,615	6,039	99	1,476	1,104	3,438	91	0	473	2,172	226	1,645	201
								Mixed-Mullerian Tumors									
20	MMT	III	146	456	673	256	0	456	316	163	123	0	164	262	0	297	634	132
21	MMT	III	446	6,988	2,840	0	0	160	1,012	702	1,188	200	198	9,209	17,856	541	1,772	0
22	MMT	III	123	724	0	0	921	0	1,378	2,011	1,793	0	0	0	406	605	0	1,385
23	MMT	III	316	1,413	1,877	151	977	0	484	641	738	148	227	0	221	0	152	731
24	MMT	III	204	949	4,654	303	2,243	0	914	0	1,434	0	0	321	375	0	194	2,158

Specimen number and histology, grade, stage, cell volume, and hybrids per micron^3 for actin and 14 oncogene probes.

In situ hybridization data expressed as hybrids per micron^3 and cell volume in micron^3 is presented for two benign (cases 1, 2), three borderline (cases 3–5), one grade 1 (case 6), six grade 2 (cases 7–12), seven grade 3 (cases 13–19) ovarian adenocarcinomas, and five anaplastic mixed-Mullerian tumors (MMT) (cases 20–24) with admixed carcinomatous and sarcomatous histologies. Histological designation and tumor grade and histology (grade 1: well-differentiated; grade 2: moderately well-differentiated; grade 3: poorly differentiated) were determined by our pathologists (DC and KM) by the conventions accepted by the Federation Internationale de Gynecologie et Obstetriques (FIGO) [65]. Likewise, clinical stage was determined by review of the operative findings by the involved clinicians (BMK, EIK, PES, SKC, and JTC) and quantified according to the FIGO designations [49] (stage I: confined to the ovary; stage II: spread to pelvic organs; stage III: intra-abdominal spread; stage IV: distant metastases). Data on individual cases for *fms* and *neu* are not included here since they will be submitted for publication elsewhere.

Computer-Assisted Grain Count Acquisition and Data Analysis

The hematoxylin- and eosin-stained ISH autoradiograms are analyzed by light microscopy and grain counts quantitated with the aid of the Olympus Corporation Cue 2 VISION Image Analysis System. Randomly chosen fields of epithelium or stroma are visualized and grain counts quantified with the Cue 2 Image Analysis System, which automatically resolves black silver grains from cell features to size and count total silver grains for each ~7,750-micron^2 (100×) oil immersion field. Ideally, enough fields are viewed and silver grains counted for a specific histologic feature (e.g., tumor epithelium, tumor stroma, and adjacent normal tissue) to yield a total of 500–1,000 grains. Grain counts per field are converted to hybrids per micron^3 by multiplicative factors[1] which take into account section thickness, size and specific activity of probes, exposure duration, and microscope field size. Pearson's correlation test was applied to the tabulated data for each probe with the aid of the PRODAS Professional Database Analysis System, Version 3.2 (Conceptual Software, Houston, TX) to yield R-values and *p*-values of the pairwise comparisons of oncogene and actin mRNA hybrids per micron^3 with each other, FIGO grade (0–3) and FIGO stage (0–IV). Borderline lesions were assigned a nominal grade of 0.5 to reflect their histologic status intermediate between benign (grade 0) and well-differentiated, invasive (grade 1) adenocarcinomas [8,49].

RESULTS

Quantitative in situ hybridization analyses were carried out with specific probes for beta-actin [27], pBR322 [28], and 17 oncogenes [29–45] on two benign, three borderline, one grade 1 (mucinous), six grade 2 (serous), seven grade 3 (serous and poorly differentiated) adenocarcinomas, and five anaplastic mixed-Mullerian tumors (MMT) of the ovary. Representative data sets of hybrids per micron^3 are presented for the BeWo human choriocarcinoma cell line [46] and the tumor epithelium and stroma of a grade 3 papillary serous adenocarcinoma of the ovary (Fig. 1). For the adenocarcinoma, we demonstrate the localization of c-*myc*, *fos*, and *fms* mRNA to tumor epithelium but not stroma.

Similar data for actin and 17 oncogene probes are presented for all 24 specimens in Tables 1 and 2. *T*-test comparison of hybrid values for the 14 adenocarcinomas to the five benign and borderline malignant neoplasms identify only *fms* and PDGF-A hybrid levels as significantly higher in the invasive adenocarcinomas, although several other genes (*neu*, *fes*, Ha-*ras*, *trk*, c-*myc*, as well as *fms* and PDGF-A) are expressed at higher levels in the neoplastic epithelial cells than a housekeeping gene, beta-actin. For the small collection of MMTs, *fos* and *myc* were both expressed at higher levels than actin, while higher expression of *fos* transcripts differentiated the MMTs from the benign and borderline neoplasms.

[1]For example, for a 1 kb probe labeled to 5×10^8 dpm/μg specific activity, one hybrid emits ~1.4 ^{35}S beta particles after a 48-hour (our standard) exposure to yield one silver grain in a photoemulsion whose detection efficiency was 100 percent. Our estimates of hybrids per micron^2 and hybrids per 100 × field are presented in terms of such an ideal emulsion, since the absolute efficiency of NTB-2 emulsion for isotopes such as ^{35}S is not precisely known. Basic physical dosimetric constraints, however, limit photoemulsion detection efficiency for ^{35}S-beta particles to ~10 percent (and conceivably much less), and, hence, one grain could represent the beta emissions of seven or more radiolabeled hybrids and the hybrids per cubic micron values which we derive actually underestimate the true number of radioactive hybrids present.

TABLE 2
T-Test Comparison of Hybrids Per Micron^3 ($\times 10^6$) Values of 14 Adenocarcinomas, Five Benign and Borderline, and Five Mixed-Mullerian Ovarian Tumors

	14 Adenocarcinomas		5 Benign and Borderline		5 Mixed-Mullerian	
Probe	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean
Actin	425	84	409	112	247	60
p53	299	75	46	30	123	89
fms[a,b,c]	2,662	522	384	222	935	595
neu[a,b]	2,955	1,104	985	499	1,085	465
erb-B	4,897	3,117	1,540	760	2,105	1,231
fes[a,b]	3,594	1,519	1,434	546	2,008	822
ros	959	344	126	126	142	63
trk[a,b]	2,568	1,001	1,079	1,006	828	412
PDGF-A[a,b,c]	1,428	234	145	92	550	323
sis	202	95	59	59	880[d]	403
Ki-*ras*	744	331	302	275	3,780	3,530
Ha-*ras*[a,b]	2,193	643	1,106	824	1,959	1,814
N-*ras*	582	280	158	71	289	129
fos[a,b]	1,397	355	146	74	820[a,b,c]	190
myb	470	210	48	48	703	353
C-*myc*[a,b]	2,824	696	973	505	1,054[a,b]	289
L-*myc*	93	29	95	62	70	43
N-*myc*	284	68	54	34	118	49

T-test comparisons were carried out on the means and standard errors (SE) of the mean of the hybrids per micron^3 ($\times 10^6$) values for the 14 adenocarcinomas, five benign and borderline, and five mixed-Mullerian tumors relative to each other as well as to a housekeeping gene (actin) and a cell proliferation gene (p53). Statistically significant comparisons (p-value $< .05$) are indicated by symbols [a,b,c] and [d] as defined in the key.

[a]Significantly greater than actin
[b]Significantly greater than p53
[c]Significantly greater than same gene in benign and borderline tumors
[d]Significantly greater than in adenocarcinomas

In Table 3, we present the statistically significant pairwise correlations observed for the data summarized in Tables 1 and 2 for the five benign and borderline and 14 ovarian adenocarcinoma specimens. Of particular interest are the strong correlations seen between FIGO histologic grade with levels of *fms*, PDGF-A, *fos*, and c-*myc* hybrids; the significant correlation of stage with levels of *fms* hybrids; and the strong correlations of *fms* and PDGF-A levels with each other and with *fos* and c-*myc* hybrid levels. Other less obvious but significant correlations between different oncogene probes are also revealed by this analysis and summarized in Table 3. When the five mixed-Mullerian tumors are compared as a group to the five benign and borderline neoplasms and the 14 ovarian adenocarcinomas, levels of *sis* (PDGF-B chain) expression were found to correlate significantly with the presence of MMT histologic features (R-value, 0.50801; p-value, 0.02638) while *fms* (R-value, −0.40290; p-value, 0.08721) and PDGF-A (R-value, −0.43548; p-value, 0.06328) expression correlated nearly significantly with their absence.

Ras oncogene mRNA expression was also noted in the tumor epithelium of most of the specimens but did not significantly correlate with either tumor grade or stage, nor

TABLE 3
Statistically Robust Correlations of Oncogene Expression Data: Two Benign, Three Borderline, and 14 Adenocarcinoma Specimens[a]

var1	var2	R-value	*p*-value	var1	var2	R-value	*p*-value
Grade	*fms*	0.70728	0.00071	PDGF-A	Grade	0.69096	0.00105
Grade	PDGF-A	0.69096	0.00105	PDGF-A	*fos*	064764	0.00272
Grade	stage	0.64948	0.00262	PDGF-A	c-*myc*	0.60063	0.00654
Grade	*fos*	0.59813	0.00683	PDGF-A	*fms*	0.58521	0.00848
Grade	c-*myc*	0.51557	0.02386	PDGF-A	*neu*	0.52628	0.02063
Stage	*fms*	0.49204	0.03236	*sis*	Ki-*ras*	0.72985	0.00039
				fos	c-*myc*	0.84154	0.00001
p53	n-*myc*	0.65866	0.00217	*fos*	*fms*	0.80647	0.00003
				fos	*neu*	0.78503	0.00007
erbB	*fos*	0.74699	0.00024	*fos*	erbB	0.74699	0.00024
erbB	*neu*	0.50042	0.0291	*fos*	PDGF-A	0.64764	0.00272
erbB	c-*myc*	0.47308	0.04079	*fos*	Grade	0.59813	0.00683
erbB	*fms*	0.46754	0.04354	*fos*	*ros*	0.49048	0.033
neu	*fos*	0.78503	0.00007				
neu	c-*myc*	0.71614	0.00056	*myb*	*fes*	0.73312	0.00036
neu	N-*ras*	0.61469	0.0051				
neu	PDGF-A	0.52628	0.02063	c-*myc*	*fos*	0.84154	0.00001
neu	*fms*	0.50506	0.02741	c-*myc*	*fms*	0.73817	0.00031
neu	erbB	0.50042	0.0291	c-*myc*	*neu*	0.71614	0.00056
neu	n-*myc*	0.47535	0.0397	c-*myc*	*ros*	0.64257	0.00301
				c-*myc*	PDGF-A	0.60063	0.00654
fes	*myb*	0.73103	0.00038	c-*myc*	N-*ras*	0.5227	0.02167
				c-*myc*	Grade	0.51557	0.02386
fms	*fos*	0.80647	0.00003	c-*myc*	erbB	0.47308	0.04079
fms	c-*myc*	0.73817	0.00031				
fms	grade	0.70728	0.00071	l-*myc*	n-*ras*	0.57144	0.01059
fms	*ros*	0.58685	0.00826				
fms	PDGF-A	0.58521	0.00848	n-*myc*	p53	0.65866	0.00217
fms	*neu*	0.50506	0.02741	n-*myc*	Ha-*ras*	0.56135	0.01239
fms	stage	0.49204	0.03236	n-*myc*	*neu*	0.47535	0.0397
fms	erbB	0.46754	0.04354				
				Ha-*ras*	n-*myc*	0.56135	0.01239
ros	*trk*	0.73312	0.00036				
ros	c-*myc*	0.64257	0.00301	Ki-*ras*	*sis*	0.72985	0.00039
ros	*fms*	0.58685	0.00826				
ros	*fos*	0.49048	0.033	N-*ras*	*neu*	0.61469	0.0051
				N-*ras*	l-*myc*	0.57144	0.01059
trk	*ros*	0.73312	0.00036	N-*ras*	c-*myc*	0.5227	0.02167

[a]Summary of statistically "robust" correlations on the data from Table 1

were any significant pairwise correlations noted between hybrid levels complementary to the three *ras* gene probes.

DISCUSSION

By the careful analysis of a collection of 24 human ovarian specimens specially fixed and processed for in situ hybridization, we have obtained quantitative data on tumor cell-specific expression of actin and 17 oncogene transcripts. Overall, ovarian neoplas-

tic epithelial cells appear to express significant level (relative to actin) of many different oncogene transcripts, including *neu, fes, fms, trk,* c-*myc,* and PDGF-A. Of the 17 oncogenes studied, however, only *fms* hybrid levels correlate strongly with both high FIGO clinical stage and/or high histologic grade, which are the two clinicopathologic features of ovarian adenocarcinomas most strongly predictive of aggressive behavior and poor outcome [8–10]. *Sis* expression correlated with the presence of mixed-Mullerian as opposed to adenocarcinoma histologic features, an observation which is not wholly unexpected, since the *sis* gene product (PDGF-B chain) has been implicated by others as an autocrine mitogen in sarcomatous neoplasms [50]. Levels of *fos* transcripts (a gene expressed at higher levels in many types of rapidly proliferating cells) distinguished these aggressive, but rare, neoplasms from benign or borderline specimens.

Many statistically significant correlations were observed between levels of expression of different mRNAs (Table 3), and many are not surprising in the context of what is now known about the physiology of the genes involved. Thus, strong correlations should be and were observed between levels of *fos* and c-*myc* hybrids even though the probes themselves have no homology, since both genes are known to be expressed together in rapidly proliferating cells. Likewise, the correlations between the *src* family oncogenes *erbB, neu, ros, fms* with *fos* and c-*myc* are reasonable if the *erbB, neu, ros,* and *fms* protein kinases and their ligands play some role in the control of ovarian epithelial cell proliferation [51,52]. Indeed, the presence of such biologically reasonable correlations helps to provide valuable internal confirmation of the consistency and validity of our in situ hybridization data and its statistical analyses.

Other correlations, such as those found for different *src* family oncogenes with each other, may be a consequence of low-level homology and cross-hybridization between the probes for one oncogene and the mRNA of another; however, no evidence of significant correlation or cross-hybridization was even observed for the related (Ha-, Ki-, and N-*ras* and c-, L-, and N-*myc*) gene probes to suggest that the hybridization conditions used in our experiments were not adequately stringent. Hence, the strong correlations observed for pairs of different *src* family oncogene probes may indicate coordinate expression of multiple growth factor receptors (*erbB, neu, fms, ros, trk*) by the tumor epithelial cells of our specimens, an interpretation consistent with the strong correlations which we observed between expression of some of these *src* family genes and expression of c-*myc* and *fos* (refer to Table 3).

The interpretation of some of the correlations (such as that of *fes* with *myb, sis* with Ki-*ras,* c-*myc* with N-*ras,* n-*myc* with Ha-*ras,* n-*myc* with p53 and Ha-*ras,* and N-*ras* with *neu*) is not, however, apparent. They suggest possible coordinate expression of otherwise unrelated genes and may help to identify possible pathways of signal transduction in ovarian carcinoma cells involving co-expressed growth factor receptors, *ras*-encoded GTP-binding proteins, and nuclear protein oncogenes.

The observed correlation between *fms* with both grade and stage for ovarian adenocarcinomas warrants further discussion. The *fms* oncogene, first characterized in a feline retrovirus, is now known to code for the receptor for macrophage colony stimulating factor M-CSF or CSF-1, a mitogen, chemoattractant, and phenotypic activator of tissue macrophages [53,54] and trophoblast (which expresses high levels of the c-*fms* gene) with important roles in wound healing, immune response, and the implantation and development of the human placenta. Two other *fms*-related genes have also been identified, c-*kit* and PDGF-receptor, both of which are homologous to

fms primarily in the 3′ protein domain with much less homology in 5′ extracellular sequences. If the *fms* gene expressed in ovarian neoplasms is mutant or rearranged with a constitutively active protein kinase (as is the v-*fms* protein) [55,56], other physiologic changes may not be needed to produce uncontrolled cell growth and a malignant cell phenotype. Southern blot hybridization with probes derived from the 3′ and 5′ halves of the human c-*fms* gene for three benign and 23 malignant ovarian specimens failed to disclose any significant rearrangements of c-*fms* genomic structure (data not shown), while Northern blot, cDNA PCR, immunohistochemical studies, and immunological studies (to be submitted elsewhere) suggest the expression of a normal or near-normal c-*fms* mRNA and protein by ovarian adenocarcinoma cells both *in vivo* and *in vitro*.

Claims for importance for *fms* (or a closely related gene) in non-hematopoietic neoplasms are not totally without precedent. Walker et al. [57] have recently reported that overexpressed length *fms*-complementary transcripts were observed in tumorigenic cell lines derived from MNNG- or gamma ray-mutagenized primary tracheal epithelial cells. Similarly, Feldman and Eisenbach [58] have reported the association of the expression of a *fms*-complementary transcript with metastatic phenotype in several mouse carcinoma cell lines, while we have reported *in vivo fms* transcript expression in many endometrial and breast carcinomas. If tumor cells do indeed express a normal or near-normal c-*fms* gene product, then a source of CSF-1 would be necessary to allow this receptor to exert phenotypic consequences on tumor cells which express it. CSF-1 (M-CSF) [53,54,59] is present in many tissues where it is synthesized by proliferating fibroblasts, activated macrophages, and other mesenchymal cells, and it is possible that the ubiquitous low levels of this mitogen are adequate to stimulate the proliferation of cells with high levels of M-CSF receptor. Such stromal cell production of CSF-1 could be facilitated by tumor cell synthesis of stroma mitogens such as PDGF-A, whose expression is strongly linked to tumor grade and expression of *fms* in our ovarian adenocarcinoma specimens (Table 1); however, stromal CSF-1 production is not the only available source for this cytokine in ovarian carcinoma patients. We, and others, have reported ovarian tumor cell line expression of CSF-1 *in vitro* and have observed markedly elevated plasma CSF-1 levels in ovarian carcinoma patients with active disease [60]. Such high levels of circulating cytokine may facilitate tumor growth and spread to metastatic site, a possibility under active investigation. Less complete information in breast, lung, and endometrial adenocarcinoma supports the hypothesis that similar CSF-1/CSF-1 receptor para- and autocrine interactions may be important in the development and progression of aggressive epithelial malignancies at other sites. This mechanism, in and of itself, does not exclude or diminish potentially important roles for the *neu, erbB, ros,* or *ras* oncogenes in ovarian adenocarcinomas; it merely suggests that their expression is not closely linked to those high-grade, high-stage presentations prognostic of poor clinical outcome.

One particular gene, *neu,* has been the focus of much controversy concerning its role in determining the prognosis of breast and, perhaps, ovarian carcinoma patients [61,62] and is worthy of further discussion here. We have observed that levels of *neu* expression (like PDGF-A and *fms*) strongly correlate with c-*fos* and *myc* expression (Table 2) in ovarian (as well as breast [21]) carcinomas even though levels of *neu* expression in benign and borderline lesions are not significantly different from those observed in invasively malignant neoplasms (Table 2). In addition, we observed

statistically significant correlations between levels of *neu* and *fms* expression in both ovarian and breast neoplasms. Such observations have led us to predict that the *neu* gene product is in some way involved in the control of epithelial cell proliferation in the ovary and breast and that it is at least co-expressed (and may interact) with the *fms* gene product. We are, however, still wary of any attempts [61] to relate levels of *neu* gene expression with prognosis in ovarian cancer, since, in our analysis, *neu* expression levels did not correlate significantly with either tumor grade or stage—both of which are extremely strong prognosticators of short- and long-term outcome in ovarian carcinoma patients treated with either standard chemotherapy or radiotherapy [8–10].

Likewise, in many human tumors, expression, and often overexpression, of a mutated *ras* oncogene is recognized to be an important step in the development of malignant neoplasms [14–16]. In fact, nearly all of our borderline and invasively malignant specimens show significant levels of *ras*-complementary hybrids (refer to Table 1), and several of our anaplastic ovarian MMT specimens even show significant hybridization to more than one *ras* probe. Our in situ hybridization techniques are not able to discriminate mutant from wild-type *ras* gene expression in our specimens, but we hope that further refinements of in situ hybridization and in situ transcription and PCR techniques [63] and the recent development of antibodies able to discriminate mutant from wild-type *ras* proteins [64] will help us to elucidate the role these overexpressed *ras* oncogenes play in determining the malignant phenotypes of ovarian adenocarcinoma cells.

REFERENCES

1. Lee W, Schwab M, Westaway D, Varmus HE: Augmented expression of normal c-*myc* is sufficient for cotransformation of rat embryo fibroblast with a mutant *ras* gene. Mol Cell Biol 5:3345–3346, 1985
2. Land H, Parada LF, Weinberg RA, Wolf D, Rotter V: Cooperation between the genes encoding p53 tumor antigen and *ras* in cellular transformation. Nature 312:649–651, 1984
3. Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596–602, 1983
4. Land H, Parada LF, Weinberg RA: Cellular oncogenes and multistep carcinogenesis. Science 222:771–778, 1983
5. Foulds L: The experimental study of tumor progression: A review. Cancer Res 14:327–339, 1954
6. Vogt PV: Spontaneous segregation of non-transforming viruses from cloned sarcoma viruses. Virology 46:939–946, 1971
7. Bishop JM: Cellular oncogenes and retroviruses. Ann Rev Biochem 52:301–354, 1983
8. Scully RE: Ovarian tumors. Am J Pathol 87:686–720, 1977
9. Ozols RF, Garvin AJ, Costa J: Advanced ovarian cancers: Correlation of histologic grade with response to therapy and survival. Cancer 45:572–581, 1980
10. Bargmann F: Carcinoma of the ovary, a clinicopathological study of 86 autopsied cases with special reference to mode of spread. Act Gynecol Scand 45:211–231, 1966
11. Seemayer TA, Cavanee WK: Molecular mechanisms of oncogenesis. Lab Invest 60:585–599, 1989
12. Kraus MH, Pierce JH, Fleming TP, Robbins KC, et al: Mechanisms by which genes encoding growth factors and growth factor receptors contribute to malignant transformation. Ann NY Acad Sci 551:320–335, 1988
13. Serunian LA, Cantley LC: Growth factor and oncogene influences on cell growth regulation. Ann NY Acad Sci 551:309–319, 1988
14. Thor A, Hand PH, Wunderlich D, Caruso A, Muraro R, Schlom J: Monoclonal antibodies defined differential *ras* gene expression in malignant and benign colon disease states. Nature 311:562, 1984
15. Viola MV, Fremwerk F, Oravez S, Deb S, Funkel G, Lunde J, Hand PH, Thor A, Schlom J: Expression of the *ras* oncogene p21 in prostate cancer. N Engl J Med 314:133–137, 1986

16. Ohuchi N, Hand PH, Merlo G, Fujita J, Constantini-Mariani R, Thor A, Nose M, Callahan R, Schlom J: Enhanced expression of c-Ha-*ras* p21 in stomach adenocarcinomas defined by immunoassays using monoclonal antibodies and *in situ* hybridization. Cancer Res 47:1413–1420, 1987
17. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177–182, 1986
18. Lawrence JE, Singer RH: Quantitative analysis of *in situ* hybridization methods for the detection of actin gene expression. Nucleic Acids Res 13:1777–1799, 1985
19. Angerer LM, Angerer RC: Detection of poly-A RNA in sea urchin eggs and embryos by quantitative *in situ* hybridization. Nucleic Acids Res 9:2819–2840, 1981
20. Kacinski BM, Carter D, Mittal K, Kohorn EI, Bloodgood RS, Donahue J, Donofrio L, Edwards R, Schwartz PE, Chambers JT, Chambers SK: High level expression of *fms* proto-oncogene mRNA is observed in clinically aggressive human endometrial adenocarcinomas. Int J Rad Onc Biol Phys 15:823–829, 1988
21. Yee L, Kacinski BM, Carter DC: Oncogene structure, function and expression in breast cancer. Sem Diag Path 6:110–125, 1989
22. Kacinski BM, Yee LD, Carter D: Quantitation of tumor cell expression of the p-glycoprotein (MDR1) gene in human breast carcinoma clinical specimens. Cancer Bulletin 41:44–48, 1989
23. Hayashi S, Gillam JC, Delaney AD, Tener GM: Acetylation of chromosome squashes of Drosophila melanogaster decreases the background of ^{125}I-labeled RNA. J Histochem Cytochem 26:677–679, 1978
24. Godard CM, Jones KW: Detection of AKR MuLV-specific RNA in AKR mouse cell by *in situ* hybridization. Nucleic Acids Res 6:2849–2861, 1979
25. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Press, 1982, pp 75–96, 129–132
26. Bartocci A, Pollard JW, Stanley ER: Regulation of colony stimulating factor 1 during pregnancy. J Exp Med 164:956–961, 1986
27. Cleveland DW, Lopata MA, MacDonald RJ, Cowan NJ, Rutter WJ, Kirschner WJ: Number and evolutionary conservation of alpha and beta tubulin and cytoplasmic beta and gamma actin using specific cloned cDNA probes. Cell 20:95–105, 1984
28. Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyeden HL, Boyer HL: Construction and characterization of new cloning vehicles II. A multipurpose cloning system. Gene 26:197–203, 1977
29. Battey J, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G, Leder P: The human c-*myc* oncogene: Structural consequences of translocation into the IgH locus in Burkitt's lymphoma. Cell 34:779–782, 1983
30. Kohl NE, Legouy E, DePinho RA, Nisen PD, Smith RK, Gee CE, Alt FW: Human N-myc is closely related in organization and nucleotide sequence to c-myc. Nature 319:73–77, 1986
31. Nau MM, Brooks BJ, Battey J, Sauesville E, Gazdar A, Kirsch IR, McBride J, Bartness V, Hollis GF, Minna JD: L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318:69–73, 1985
32. Curran T, MacCoumell WP, vanStraaten F, Verma IM: Structure of the FBJ osteosarcoma virus genome. Molecular cloning of its helper virus and the cellular homologues of the fos gene from mouse and human cells. Mol Cell Biol 3:914–921, 1984
33. Klempnauer KH, Gonda TJ, Bishop JM: Nucleotide sequence of the retroviral leukemia gene v-*myb* and its cellular homologue c-*myb;* the architecture of a transduced oncogene. Cell 31:453–462, 1982
34. Jenkins JR, Rudge K, Redmond S, Wade-Evans A: Cloning and expression of a full length mouse cDNA sequence encoded in the transformation associated protein p53. Nucleic Acids Res 12:5609–5626, 1984
35. Ellis RW, DeFeo D, Furth MF, Scolnick EM: Mouse cells contain two distinct ras gene mRNA species that can be translated to a p21 protein. Mol Cell Biol 2:1339–1345, 1982
36. Shimizu K, Goldfarb DM, Suard Y, Perucho M, Li Y, Kamata T, Ferramisco J, Stavnezer E, Fogh J, Wigler M: Three transforming genes are related to the viral ras oncogenes. Proc Natl Acad Sci USA 80:2112–2116, 1983
37. Shimizu K, Goldfarb M, Perucho M, Wigler M: Isolation and preliminary characterization of the transforming gene of a human neuroblastoma. Proc Natl Acad Sci USA 80:383–387, 1983
38. Robbins K, Devare SG, Aaronson SA: Molecular cloning of an integrated simian sarcoma virus: Genome integration and organization of infectious DNA clones. Proc Natl Acad Sci USA 78:2918–2922, 1981

39. Betsholtz C, Johnson A, Helder C-H, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL, Knott TJ, Scott J: CDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. Nature 320:695–700, 1986
40. Vennstrom B, Fanshier L, Moscovici C, Bishop JM: Molecular cloning of the avian cytoblast virus genome and recovery of oncogene viruses by transfection of chicken cells. J Virology 36:575–585, 1980
41. Bargmann C, Hung MC, Weinberg RA: The neu oncogene encodes a growth factor receptor related protein. Nature 319:230–233, 1986
42. Franchini G, Evans J, Sherr CJ, Wong-Stahl F: Onc sequences (v-fes) of Snyder-Theilen feline sarcoma virus are derived from noncontiguous regions of a cat cellular gene c-fes. Nature 290:154, 1984
43. Hampe A, Gohet M, Sherr CJ, Galibert F: Nucleotide sequence of the feline retroviral oncogene v-*fms* shows unexpected homology with oncogenes controlling tyrosine-specific protein kinases. Proc Natl Acad Sci USA 81:85–89, 1984
44. Matshushima H, Wang LH, Shibuya M: Human c-*ros*-1 gene homologous to the v-*ros* sequence of the UR2 sarcoma virus encodes a transmembrane receptor molecule. Mol Cell Biol 7:3000–3004, 1986
45. Martin-Zanca D, Hughes SH, Barbacid M: A human oncogene formed by the fusion of truncated tropomysin and protein tyrosine kinase sequences. Nature 319:743–748, 1986
46. Pattilo RA, Gey AO: The establishment of a cell line of human hormone trophoblastic cells *in vitro*. Cancer Res 28:1231–1236, 1968
47. Rettenmier CW, Sacca R, Turmman WL, Roussel MF, Holt JT, Nienhuis AW, Stanley ER, Sherr CJ: Expression of the human c-*fms* proto-oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and choriocarcinoma cell lines. J Clin Invest 77:1740–1746, 1986
48. Mueller R, Tremblay JM, Adamson ED, Verma IM: Tissue and cell type-specific expression of two human oncogenes. Nature 304:454–456, 1983
49. FIGO: Annual Report on the Results of Treatment in Gynecological Cancer. Edited by F Pettersson. Stockholm, Panorama Press AB, 1986, p 111
50. Ross R, Raines E, Bowen-Pope DF: The biology of platelet-derived growth factor. Cell 46:155–169, 1986
51. Muller R, Bravo R, Burckhardt J, Curran T: Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature 312:716–720, 1984
52. Macara IG: Oncogenes, ions, and phospholipids. Am J Physiol 248:C3–C11, 1985
53. Stanley ER: The action of the colony stimulating factor CSF-1. In Biochemistry of Macrophages. Ciba Foundation Symposium 118. Edited by D Evered, J Nugent, M O'Connor. London, Pitman, 1986, pp 29–41
54. Clark SC, Kamen R: The human hematopoietic colony stimulating factors. Science 236:1229–1237, 1987
55. Coussens L, Van Beveren CV, Smith D, Chen E, Mitchell RL, Isacke CM, Verma IM, Ullrich A: Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. Nature 320:277–280, 1986
56. Roussel MR, Dull TJ, Rettenmeier CW, Ralph PW, Ullrich A, Sherr CJ: Transforming potential of the c-fms proto-oncogene (CSF-1 receptor). Nature 325:549–552, 1987
57. Walker C, Nettesheim P, Barrett JC, Gilmer TM: Expression of a *fms*-related oncogene in carcinogen-induced neoplastic epithelial cells. Proc Natl Acad Sci USA 84:1804–1808, 1987
58. Feldman M, Eisenbach L: What makes a tumor cell metastatic? Scientific American (November):60–85, 1988
59. Rajavashisth TB, Eng R, Shadduck RK, Waheed A, Ben-Avram CM, Shively JE, Lusis AJ: Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. Proc Natl Acad Sci USA 84:1157–1161, 1987
60. Kacinski BM, Stanley ER, Carter D, Chambers JT, Chambers SK, Kohorn EI, Schwartz PE: Circulating levels of CSF-1 (M-CSF), a lymphohematopoietic cytokine, may be a useful marker of disease status in patients with malignant ovarian neoplasms. Int J Rad Onc Biol Phys 17:159–164, 1989
61. Van de Vijver MJ, Peterse JL, Mooi WJ, Wisman P, Lomans J, Dalesio O, Nusse R: Neu-protein overexpression in breast cancer: Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 319:1239–1246, 1988
62. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707–712, 1988

63. Longley J, Merchant MA, Kacinski BM: In situ transcription and detection of CD1a mRNA in epidermal cells: An alternative to standard in situ hybridization techniques. J Invest Dermatol, in press
64. Carney WP, Hamer P, Petit D, Wolfe H, Cooper G, Lefebvre M, Rabin H: A monoclonal antibody reactive with an activated *ras* protein expressing valine at position 12. J Cell Biochem 32:207–214, 1986
65. Scully RE: World Health Organization classification and nomenclature of ovarian cancer. Natl Cancer Inst Mono 42:5–7, 1975