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ORIGINAL REPORT

Tumor Vascular Proteins As Biomarkers in Ovarian Cancer

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ABSTRACT

Purpose
This study aimed to identify novel ovarian cancer biomarkers and potential therapeutic targets through molecular analysis of tumor vascular cells.

Methods
Immunohistochemistry-guided laser-capture microdissection and genome-wide transcriptional profiling were used to identify genes that were differentially expressed between vascular cells from human epithelial ovarian cancer and healthy ovaries. Tumor vascular markers (TVMs) were validated through quantitative real-time polymerase chain reaction (qRT-PCR) of immunopurified tumor endothelial cells, in situ hybridization, immunohistochemistry, and Western blot analysis. TVM expression in tumors and noncancerous tissues was assessed by qRT-PCR and was profiled using gene expression data.

Results
We identified a tumor vascular cell profile of ovarian cancer that was distinct from the vascular profile of normal ovary and other tumors. We validated 12 novel ovarian TVMs. These were expressed by immunopurified tumor endothelial cells and localized to tumor vasculature. Select TVMs were found to be specifically expressed in ovarian cancer and were absent in all normal tissues tested, including female reproductive tissues with physiologic angiogenesis. Many ovarian TVMs were expressed by a variety of other solid tumors. Finally, overexpression of any one of three ovarian TVMs by vascular cells was associated with decreased disease-free interval (all $P < .005$).

Conclusion
We have identified for the first time the molecular profile of ovarian tumor vasculature. We demonstrate that TVMs may serve as potential biomarkers and molecular targets for ovarian cancer and a variety of other solid tumors.

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INTRODUCTION

Gene expression profiling is a convenient method to uncover tumor-specific genes that may function as biomarkers or therapeutic targets. So far, important efforts have been made regarding the cancer cell as the main source of biomarkers.[1,2] However, emerging studies argue that tumor components other than tumor cells may contribute significantly to cancer molecular signatures. In landmark studies, tumor neovessels have been shown to be different from vessels of normal tissues,[3] expressing unique genes.[4,5] Cellular and molecular changes characterizing the angiogenic switch are essential for tumor growth beyond a few millimeters.[6] Furthermore, specialization of the vasculature may even precede tumor establishment at metastatic sites.[7] These observations suggest that molecular changes in tumor vasculature might provide sensitive markers of tumor initiation or metastasis. Similarly, it is expected that genes expressed uniquely by the vasculature of tumors may provide important therapeutic targets.

Ovarian cancer critically depends on blood supply for expansive growth, and increased angiogenesis is associated with rapid tumor recurrence and decreased survival.[8,9] Unique among solid tumors, targeting tumor angiogenesis by blocking vascular endothelial growth factor (VEGF) as monotherapy has produced tangible clinical results in ovarian cancer,[10-14] suggesting that vascular development is central to ovarian cancer biology. Thus, we hypothesized that ovarian cancer is an appropriate tumor to evaluate for vasculature-specific genes to uncover novel tumor biomarkers.

To date, the expression profile of vascular cells in ovarian cancer remains unreported. We used immunohistochemistry-guided laser-capture microdissection (immuno-LCM) and transcriptional profiling[15] to characterize tumor vascular cells (TVCs) from 21 epithelial ovarian cancers and four

normal ovaries. From the cancer vascular profile, we validated 12 novel tumor vascular markers (TVMs) and demonstrated that three TVMs have prognostic value. Several of these could function as tumor biomarkers or therapeutic targets; they were expressed at high levels in ovarian cancer and were either absent or expressed at significantly lower levels in normal tissues.

METHODS

Patient Tissues

Stage III epithelial ovarian cancer (n = 46 categorized as either serous, mucinous, endometrioid [n = 33], or clear-cell/undifferentiated [n = 13] and stage II/III invasive ductal breast cancer [n = 5]) frozen specimens from untreated patients were provided by the University of Turin (Turin, Italy). Fresh tumors stage II/III lung adenocarcinoma (n = 5) and metastatic melanomas (n = 5) for cell purification and/or molecular studies were collected at the University of Pennsylvania (Philadelphia, PA). Normal human tissues, including ovary, were provided by the Cooperative Human Tissue Network, for which clinical information is unavailable. All specimens were processed in compliance with institutional review board and Health Insurance Portability and Accountability Act requirements.

Immuno-LCM and RNA Isolation and Amplification

Anti-hCD31 (BD Pharmingen, San Diego, CA) mixed with anti-CD146 (Chemicon, Temecula, CA), and secondary antibody (Biotin anti-mouse secondary antibody; Vector, Burlingame, CA) were used (1:10) in phosphate-buffered saline (PBS) containing RNA Protector (1:10; Roche, Basel, Switzerland). Immuno-LCM[15-18] was performed using Microcut (MMI, Glattbrugg, Switzerland) to isolate up to 2×10^3 immunopositive cells per specimen. RNA was isolated using the TRIzol microprotocol, and RNA was amplified using Messageamp (Ambion, Austin, TX), as detailed elsewhere.[15] After two rounds of amplification, cRNA was biotin-labeled (12 to 24 hours, ENZO [Farmingdale, NY] RNA labeling kit) and purified using RNA cleanup (Qiagen, Valencia, CA).

Gene Expression

Biotin-cRNA was fragmented, hybridized to HG-U133 A/B arrays (Affymetrix, Santa Clara, CA) and scanned as recommended in the Affymetrix GeneChip protocol (www.affymetrix.com). Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed as detailed elsewhere.[15] Primers are provided in Table A1 (online only). All transcripts were confirmed using 3% agarose gel electrophoresis. In all cases, expression was normalized to beta-actin (β-actin). Experiments comparing tumors, normal tissues, and angiogenic tissues were also normalized to either *CD31* or *VE-cadherin*, to control for vascular density. In situ hybridization (ISH) as performed using a protocol provided by K.W. Kinzler (Johns Hopkins, Annapolis, MD).[4] Digoxigenin (DIG) -labeled antisense RNA probes were transcribed using DIG RNA labeling reagents and T7 RNA polymerase (Roche) from 500- to 600–base pair PCR templates containing T7 promoters in the antisense primers. Primers are detailed in Table A2 (online only). We used two rounds of amplification and detected hybridized probe with streptavidin-Cy3 conjugate (Arcturus, Mountain View, CA; 1:200, 5 minutes). Slides were washed and mounted with fluorescent mounting media containing 4′,6-Diamidino-2-phenylindole (DAPI; Vector). Double immunofluorescence and immunohistochemistry was performed as previously described.[19] Primary antibodies against CD31, STC2 (Genway, San Diego, CA), F2RL1, and DR6 were incubated for 2 hours. Western blot was performed using 300 μg of protein from patient sera and probed with goat antihuman *DR6* antibody. All antibodies used are detailed in Table A3 (online only).

Cells

$CD146^+$, VE-$cadherin^+$, $CD45^-$ tumor endothelial cells (TECs) were isolated by fluorescence-associated cell sorting (FACS), after Ficol gradient centrifugation, from mechanically dispersed fresh human epithelial ovarian cancer, lung adenocarcinoma or melanoma specimens using fluorescein isothiocyanate (FITC) anti–VE-cadherin antibody (Medsystems Diagnostics, Vienna, Austria), biotinylated anti-CD146 coupled with streptavidin phycoerythrin (PE) Cy7, and APC-anti-CD45 (BD Pharmingen).[19,20] Normal murine serum (10%; Sigma, St Louis, MO) and 25 mg/mL anti-mouse Fc receptor (BD Pharmingen) were added before incubation to avoid nonspecific binding. FACS-isolated TECs were more than 95% pure (data not shown). TECs were analyzed immediately by qRT-PCR. Human umbilical-vein endothelial cells (HUVECs) were cultured in endothelial basal medium with 10% bovine serum and 100 ng/mL VEGF. Peripheral-blood mononuclear cells (PBMCs) were obtained freshly through leukapheresis from healthy donors and analyzed immediately.

Bioinformatics and Statistical Analyses

Present/absent indicators (gene expressed/not expressed) were produced with MAS5.0 Suite (Affymetrix). All samples were normalized with the median defined as 1.0. Analysis of expression profiles used Genespring software (Agilent, Santa Clara, CA). Statistical significance for mRNA expression differences between tissue types was determined using a two-tailed *t* test, and differences were characterized by fold changes defined as the ratio of the normalized mean expression of the tumor to the normal vascular samples. A heat map condition tree was developed using the Genespring hierarchical clustering algorithm. Descriptive statistics were performed with SPSS statistics software (SPSS Inc, Chicago, IL). Means are presented with SEs. Nonparametric analysis based on the ranks of the expression level for tumor and normal samples was developed in SAS 9.1.[21] The log-rank statistic was used to compare the Kaplan-Meier disease-free interval (DFI) distribution of six tumors with the highest TVM expression values (highest tertile) to the remaining 11 tumors using SAS 9.1.[21]

Archived Gene Expression Datasets

Data used for expression of the TVM in normal and tumor tissue samples are available via the Gene Expression Omnibus (GEO; National Center for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/geo) with series numbers GSE3526 and GSE2109, respectively. All CEL files were similarly processed using the rate monotonic algorithm.[22]

RESULTS

Identification of TVMs

We used optimized immuno-LCM coupled with RNA amplification and genome-wide gene expression profiling[15] to profile tumor vasculature cells from 21 stage III ovarian cancers and four normal ovaries. Unsupervised hierarchical clustering was used to determine whether the transcriptional profile of microdissected TVCs differed from that of normal vascular cells. We included 17,920 genes after elimination of genes for which the difference between tumor and normal mean expression level was less than its SE. TVCs were accurately distinguished from normal vascular cells (Fig 1A).

Seeking to identify TVMs, genes that fulfilled at least one of the following three criteria were identified (Fig 1B): the gene was present in at least 15 of 21 TVC samples and absent in at least three of four normal vascular samples; or was present in at least 18 of 21 TVC samples and was at least three-fold overexpressed in TVC relative to normal vascular samples; or was expressed at the highest values in the TVC samples (ie, at least 20 of 21 TVC samples had higher expression values relative to normal samples). Seventy genes emerged from this analysis (Fig 1B).

Consistent with our hypothesis that the tumor vascular profile of ovarian cancer would be distinct, several of the identified markers had not been previously reported as TVMs. We selected 12 new putative TVMs for further validation: *adlican* (matrix-remodeling associated 5,

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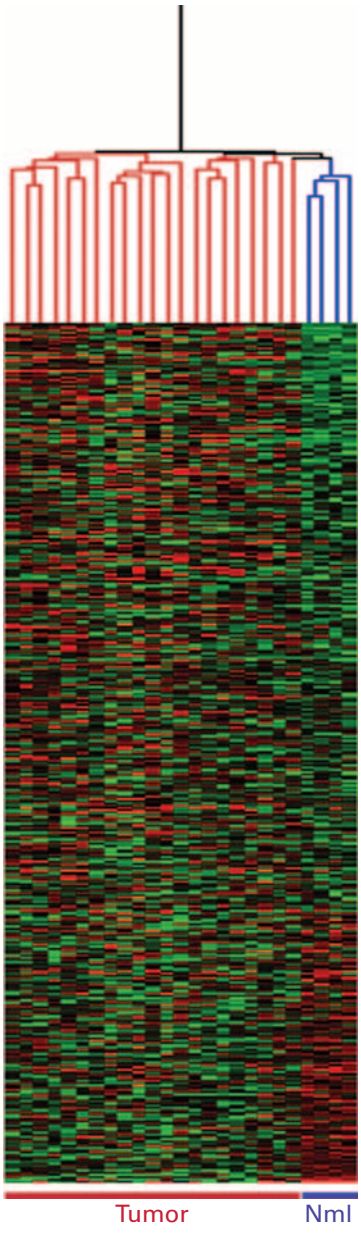

B

Common Gene Name or Genebank No.	Fold Difference Tumor *v* Normal	No. Present in		Rank of Normal Specimens
		Tumor (21)	Normal (4)	
ADAM12	5.1	13	0	1,2,4,6
Adlican	5.4	19	2	1,2,5,8
AI798118	7.7	15	0	1,2,3,5
AK000168	3.0	17	1	4,6,7,15
AML-1	30	12	0	1,2,3,5
ANKRD9	3.2	16	1	1,4,7,16
Av758342	4.0	17	2	1,2,9,10
AW242409	4.9	17	2	1,3,5,9
Aw510657	3.9	18	4	4,5,6,7
AW517716	7.4	12	0	1,2,3,13
BDKRB2	3.7	15	1	2,3,5,11
C6orf37	5.2	15	1	2,3,5,7
C6orf55	2.4	18	1	1,2,3,8
C6orf69	2.0	19	3	1,2,4,7
C7orf32	3.4	16	1	1,2,4,7
C11orf8	4.08	13	1	1,2,5,17
CD24	3.7	16	1	2,6,7,8
CDS1	3.7	15	2	1,2,3,8
CENTA2	2.1	16	3	1,3,4,5
CLDN1	2.4	16	1	1,2,3,13
COL11A1	10.1	15	0	1,2,7,8
COL15A1	4.8	21	4	1,3,8,12
COL22A1	3.6	11	0	1,2,3,5
CSPG2	5.8	21	4	1,2,3,6
DEFB1	2.4	11	0	1,2,4,13
DSG2	4.2	11	1	2,3,15,17
EGFL6	4.6	19	4	1,2,6,7
EIF2AK4	2.8	18	1	1,2,4,9
EBP41L3	4.0	16	2	1,4,5,13
ESM1	3.7	20	1	1,4,5,7
FBXO32	2.2	16	1	1,2,5,8
FJX1	11.3	16	0	1,2,3,5
FOLH1	8.8	13	1	1,2,4,6
FLJ22875	2.3	16	0	2,3,6,7
FLJ46072	3.8	20	1	1,2,3,5
Fz2RL1	3.6	13	0	1,7,10,12
FZD10	2.0	15	0	2,3,6,10
GPM6B	14.63	16	0	1,2,5,6
GPR105	3.3	11	0	1,2,6,10
HOXB7	2.8	16	1	1,2,7,14
IGSF4	6.0	18	1	1,4,5,6
IVNS1ABP	3.3	21	4	1,2,3,5
KCNK5	2.5	16	1	1,3,5,9
LZTS1	4.3	19	1	1,2,6,7
MCM4	2.4	13	0	1,2,4,6
NID2	4.1	16	1	1,4,8,15
OlfML2B	2.4	18	1	1,2,4,11
OSBPL3	4.1	17	1	1,3,4,20
PCDH17	2.9	21	4	1,3,4,7
PLCE1	3.8	13	1	1,3,4,16
PLXDC1 (TEM7)	4.0	21	3	1,3,5,8
PRDM1	2.4	15	0	1,3,4,5
RGC32	9.0	16	2	1,3,6,8
RGS1	8.0	12	1	2,3,5,16
RRM2	4.7	16	1	2,4,5,21
SCNN1A	15.8	16	0	1,2,3,6
SCO2	4.42	20	2	1,2,6,10
SlamF8 (BLAME)	3.5	13	0	2,3,6,8
SLIT2	2.0	16	1	3,4,6,9
SPON1	2.5	16	1	1,6,7,17
SPRY4	2.8	20	2	1,2,3,5
STC2	9.0	11	0	1,2,4,5
STCH	4.0	15	2	1,6,7,9
TACSTD1	11.6	16	2	1,2,7,10
TARS	2.8	16	1	2,5,9,11
TBX2	2.2	14	1	1,5,6,24
THY1	3.1	19	3	1,2,4,8
TNFAIP6	5.6	17	0	1,2,3,6
TNFSFR21 (DR6)	8.2	11	0	1,3,4,7
UPP1	4.97	15	1	1,3,5,20

Fig 1. Analysis of tumor vascular cell arrays. (A) Unsupervised hierarchical clustering of ovarian tumor vascular cell and normal (Nml) vascular cell profiles. (B) List of tumor vascular markers identified using three independent criteria. Genes in bold font have previously been identified in vascular cells. Genes highlighted in blue are further analyzed in this article.

MXRA5); *C11orf8* (metallophosphoesterase domain containing 2, *MPPED2*); collagen type-XI alpha-1 (*COL11A1*); death receptor-6 (*DR6*; TNF receptor superfamily member-21, *TNFRSF21*); epidermal growth factor-like-domain multiple-6 (*EGFL6*); four jointed box-1 (*FJX1*); coagulation factor-II (thrombin) receptor-like 1 (*F2RL1*); frizzled homolog-10 (*FZD10*); glycoprotein M6B (*GPM6B*); leucine zipper putative tumor suppressor-1 (*LZTS1*); olfactomedin-like 2B (*OLFML2B*); and stanniocalcin-2 (*STC2*).

Validation and Localization of TVMs

To confirm TVM expression by TVCs we immunopurified VE-cadherin$^+$, CD146$^+$, CD45$^-$ TECs by FACS from five fresh ovarian cancer specimens. TVM mRNA expression was evaluated by qRT-PCR in TECs as well as in HUVECs and PBMCs. All TVM were detected in purified TEC, confirming the vascular origin of the genes (Fig 2A). *Adlican*, *C11orf8*, *EGFL6*, *FZD10*, and *LZTS1* were absent in HUVECs, whereas the remaining TVMs were expressed at similar or lower levels in HUVECs than in TECs. The known tumor vascular marker *TEM1*, tested as control, was expressed in TECs but not in HUVECs. Most TVMs were absent or expressed at the lowest level of detection in PBMCs.

ISH was used to confirm the vascular localization of those TVMs that showed relatively high expression in immunopurified TECs. *Adlican*, *C11orf8*, *DR6*, *EGFL6*, *FZD10*, *FJX1*, and *GPM6B* (Fig 2B; data not shown) were detected in vascular structures by ISH in five independent ovarian cancer samples. Expression was limited to small vessels except for *FZD10*, which was also found in larger vessels. TVMs were not detected in three independent normal ovary specimens, with the exception of *FJX1*, which was detected at very low levels in normal ovarian vasculature (Fig 2B; not shown).

For several of the TVMs for which commercial antibodies are available, we performed immunofluorescence or immunohistochemistry to confirm protein expression within vascular structures. Immunofluorescence demonstrated colocalization of STC-2 with CD31 (endothelial localization; Fig 3A). Immunohistochemistry demonstrated expression of F2RL1 in a periendothelial location and in stromal cells in tumors, whereas staining in normal ovary was rare or absent. DR6 protein was diffusely expressed in tumor vascular structures and faintly in tumor stroma. DR6 protein was expressed in the vasculature of normal ovaries, although at significantly lower levels (Fig 3B).

Lastly, because the DR6 protein is predicted to be secreted, we performed Western blot analysis on serum from untreated patients with stage III ovarian cancer and normal controls. DR6 protein was easily detected in serum from all patients, with 3.5-fold higher levels were present in the serum of cancer patients (Fig 3C).

Ovarian TVMs Are Novel Tumor Biomarkers

Because TVMs are expressed specifically by tumors harboring TECs, we hypothesized that TVMs could potentially function as tumor biomarkers. An important prerequisite of tumor biomarkers is that they be overexpressed in tumor relative to normal tissue from the same organ. All 12 TVMs tested were overexpressed in ovarian cancers ($n = 25$, stage III, independent set) relative to normal ovaries ($n = 5$; $P < .05$ for all TVMs) by qRT-PCR. In fact, most TVMs were expressed at the lowest limits of detection in normal ovaries (Fig 4A).

An ideal ovarian tumor biomarker would discriminate between tumor and physiologic angiogenesis occurring in female reproductive tissues. *Adlican*, *COL11A1*, *F2RL1*, *GPM6B*, and *STC2* were expressed at levels 10- to 350-fold higher in tumor versus corpus luteum, proliferative endometrium, or placenta (Fig 4B). However, *C11orf8*, *EGFL6*, *FJX1*, *LZTS1*, and *OLFML2B*, as well as *TEM1*, were expressed at similar levels in one or more reproductive tissues with physiologic angiogenesis and in ovarian tumors. Thus, some of the TVMs appear specific to ovarian tumor angiogenesis versus physiologic angiogenesis.

More generally, tumor biomarkers are characterized by their overexpression in tumor tissue relative to other normal tissues. Several TVMs, including *adlican*, *COL11A1*, *EGFL6*, *F2RL1*, *FZD10*, *LZTS1*, and *OlfML2B* showed limited or no expression by qRT-PCR in normal tissues, but were expressed at high levels in ovarian cancer. In contrast, *C11orf8*, *DR6*, *FJX1*, *GPM6B*, and *STC2* were expressed in at least one normal tissue (Fig 4C).

As an independent analysis, we examined the expression profile of TVMs in normal tissues in a publicly available gene expression profile data set that included 44 normal tissue types from five male and five female human donors. Results were concordant with the above qRT-PCR data, showing little or no TVM expression in normal ovary (Fig 5A). *C11orf8*, *CL11A1*, *EGFL6*, *FJX1*, *F2RL1*, *FZD10*, *LZTS1*, and *STC2* were also absent in almost all normal human tissues, with few exceptions. *Adlican*, *DR6*, and *GPM6B* were detected in many normal tissues including reproductive organs and the nervous system. Similar results were obtained using publicly available serial analysis of gene expression (SAGE) data sets (data not shown).

Expression Profile of TVMs in Tumors

We evaluated whether TVMs identified in ovarian cancer are also expressed in other tumors using data from a publicly available data set with 755 cancer specimens from 22 different organ sites. All TVMs were overexpressed in ovarian cancer relative to normal ovary. Select TVMs such as *adlican*, *COL11A1*, *DR6*, *F2RL1*, and *GPM6B* were overexpressed in the majority of tumors (Fig 5B). Significant variability in expression of the remaining TVMs was detected among tumors.

We independently tested expression of TVM by qRT-PCR in lung cancer, mesothelioma, breast cancer, melanoma, and ovarian cancer (Fig 6A). As expected, most TVMs were expressed at quantitatively different levels in different types of malignancy. These differences persisted when expression was normalized to *CD31* or *VE-cadherin*, to control for vascular density.

TVMs Are Expressed by TECs in Other Tumors

Given the heterogeneity of expression of TVMs among tumors, we tested whether TECs from different tumors express TVMs at different levels. We FACS purified VE-cadherin$^+$, CD146$^+$, CD45$^-$ tumor endothelial cells from melanoma ($n = 4$), lung cancer ($n = 4$), and ovarian cancer ($n = 5$) and analyzed TVMs by qRT-PCR. As expected, we observed significant quantitative differences of TVM expression among tumor types. For example, *adlican* and *EGFL6* were primarily expressed in ovarian TECs. Lung TECs expressed *COL11A1*, *DR6*, *F2RL1*, and *STC2*. Melanoma TECs expressed most TVMs, but not *adlican*, *EGFL6*, and *STC2* (Fig 6B).

TVMs Are Prognostic Markers

Finally for each TVM, the Kaplan-Meier DFI distributions were computed for patients with high TVM expression (in the highest tertile of TVM expression in microdissected TVCs) compared with those with lower TVM expression (Fig 6C). The expression of *STC2*, *EGFL6*, and *FZD10* in the highest tertile was associated with significantly decreased DFI (all $P < .005$). By contrast, the survival curves of patients with highest tertile and lower expression of the vascular markers *CD31* and *VE-cadherin* were not significantly different (not shown), suggesting that the observed effect with TVMs is not simply a result of increased content of endothelial cells.

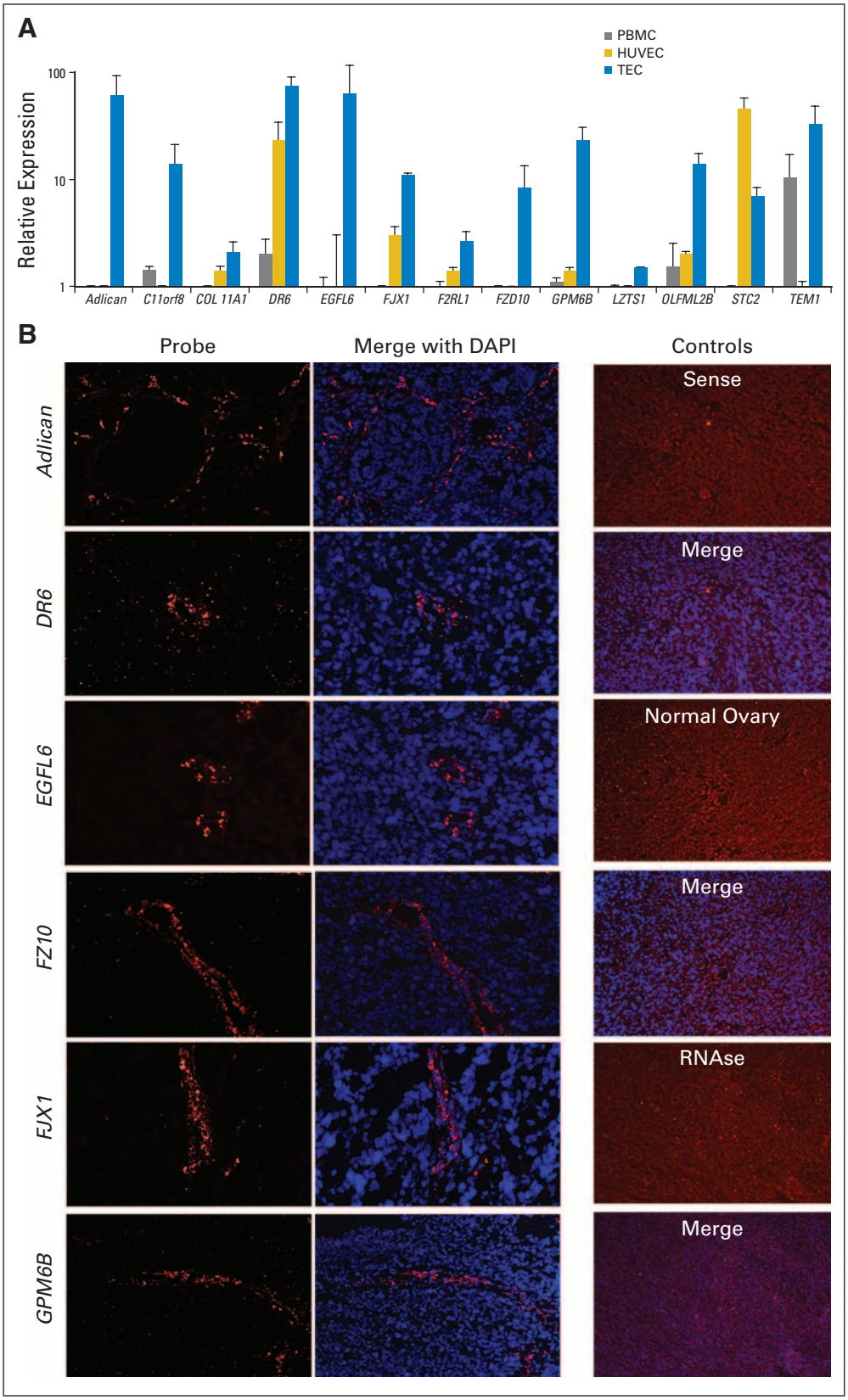

Fig 2. (A) Expression of tumor vascular markers (TVMs) by quantitative real-time polymerase chain reaction (qRT-PCR) in purified tumor endothelial cells (TECs), human umbilical-vein endothelial cells (HUVECs) and peripheral-blood mononuclear cells (PBMCs). (B) TVM expression in ovarian tumor vasculature (in situ hybridization). Controls: sense-strand RNA (Sense); normal ovary, antisense probe (Nml Ov); and RNAse-pretreated tumor (RNAse). DAPI, 4′,6-Diamidino-2-phenylindole.

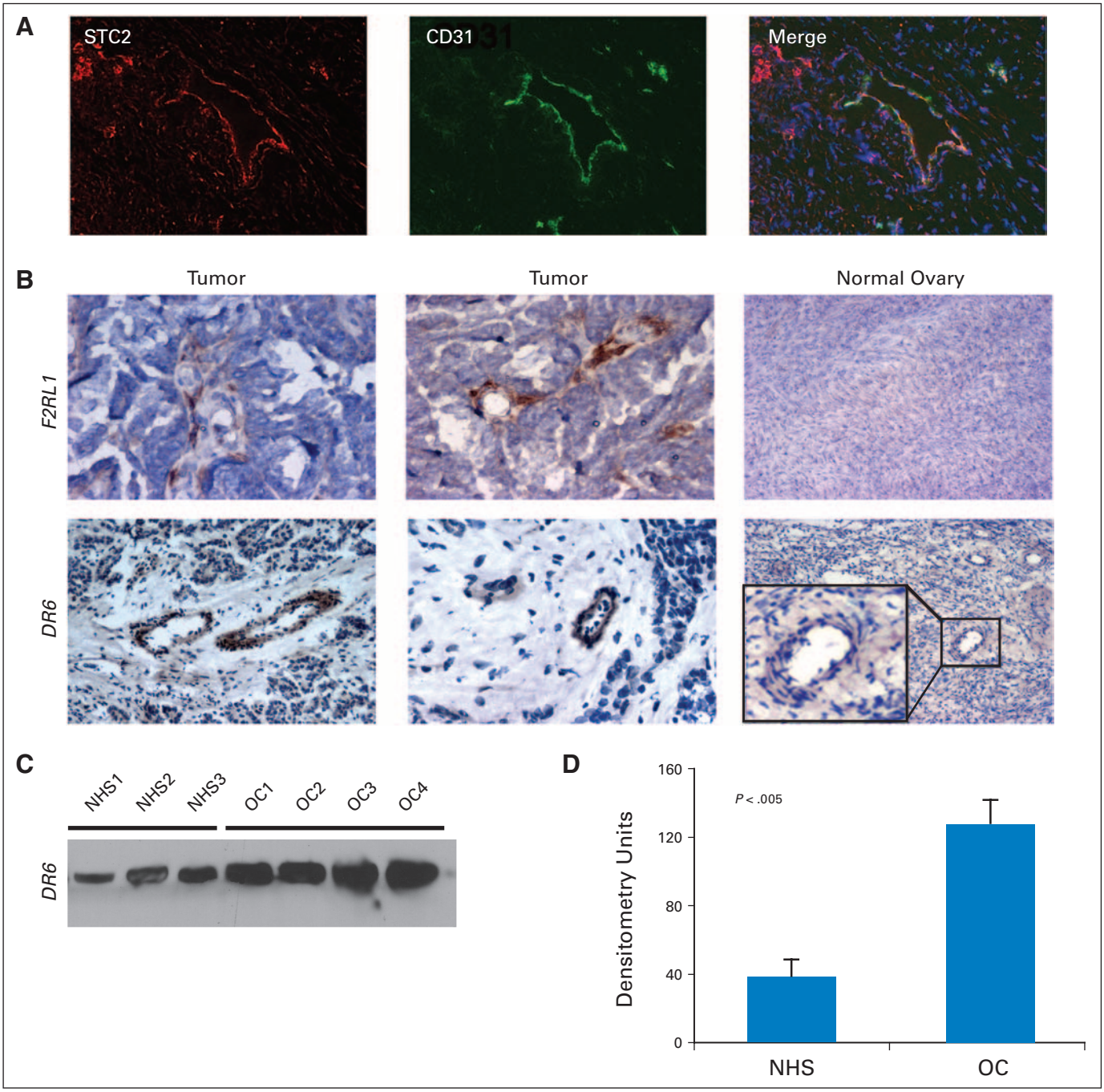

Fig 3. Expression of tumor vascular marker (TVM) protein. (A) Immunofluorescent colocalization of STC2 and CD31 in ovarian tumors. Merge includes 4′,6-Diamidino-2-phenylindole (DAPI) nuclear staining. (B) Immunohistochemical localization of F2RL1 and DR6 protein. (C, D) Western blot of DR6 protein in control normal human sera (NHS) and sera from ovarian cancer patients (OC), with densitometric quantitation of expression.

DISCUSSION

This study identified for the first time the expression profile of ovarian cancer vasculature in situ, and showed that high-throughput genome-wide discovery efforts addressing tumor vasculature can unveil interesting candidate genes for further testing as tumor biomarkers or therapeutic targets. The immuno-LCM isolation used in this study has the advantage of capturing TECs in their natural microenvironment, and, on the basis of the use of frozen samples, allowed us to analyze the largest number of samples to date in tumor vasculature profiling. Our protocol also allows for the inclusion of pericytes, and our work suggests that pericytes are likely to be one source of TVMs. Seventy genes were identified as TVMs including several known TVMs or TVM family members. We validated 12 novel TVMs; these were expressed by immunopurified TECs, and expression in vivo was localized to vascular structures. Thus, the approach undertaken was robust and can provide a detailed molecular map of tumor vasculature.

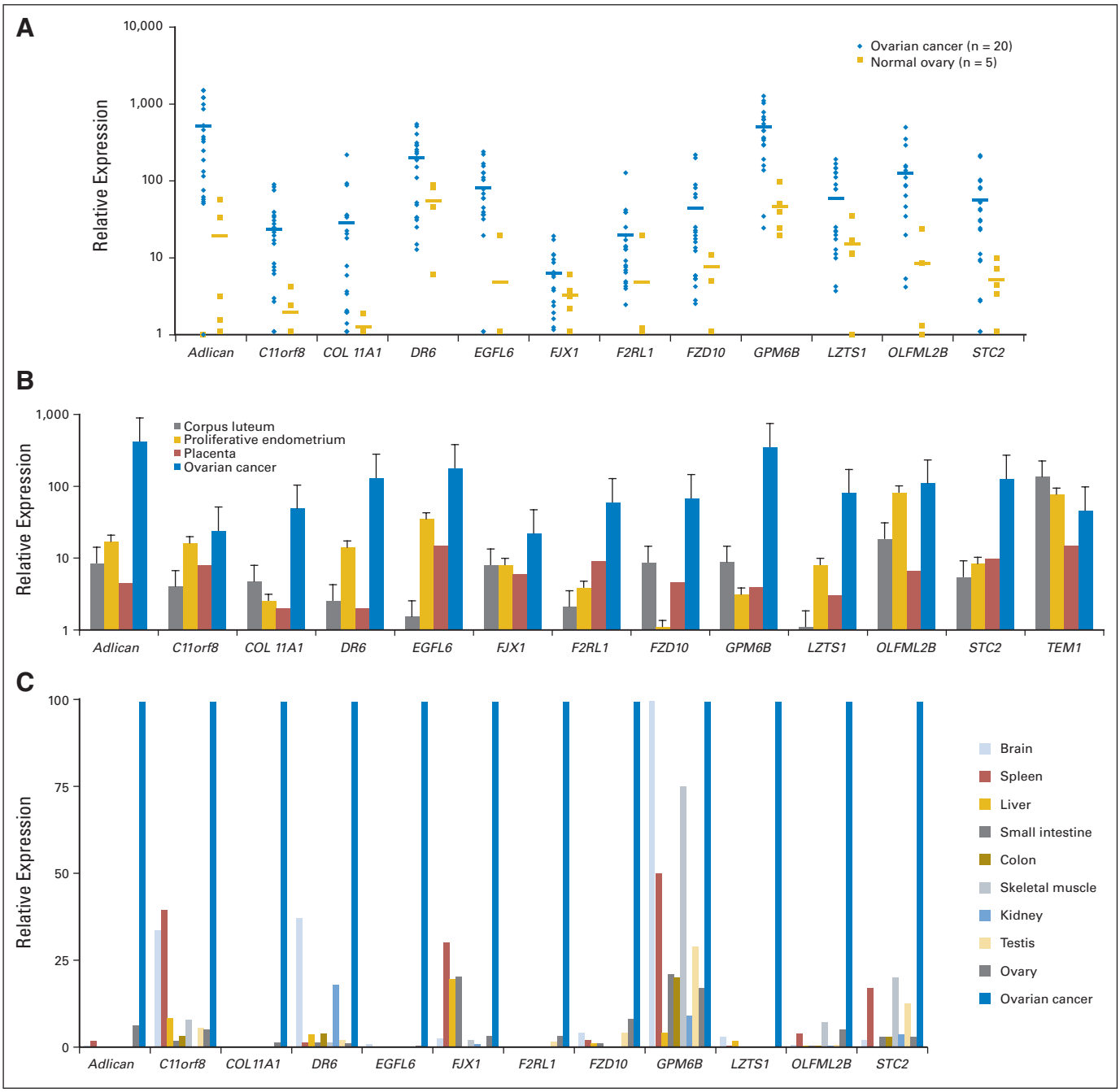

Fig 4. Quantitative real-time polymerase chain reaction analysis of tumor vascular marker expression in (A) ovarian cancer and normal ovaries, (B) ovarian cancer and tissues with physiologic angiogenesis, and (C) ovarian cancer and a panel of normal human tissues. Expression for each normal tissue was normalized against ovarian cancer, which was defined as 100% for each gene.

Although ovarian vascular cells expressed numerous genes identified in other tumor vascular profiles, including several collagen molecules, *nidogen 2*, *Thy-1*, *FolH1*, and *TEM7*, the majority of the TVMs identified have not been reported in vascular profiling studies from colon cancer, glioblastoma multiforme, or breast ductal carcinoma in situ.[4,23,24] This could be due in part to differences in the techniques utilized, the number of samples analyzed, or true heterogeneity of vascular endothelium among tumors. Analysis of TVM expression from immunopurified TVCs from ovarian cancer, lung cancer, and melanoma suggested significant quantitative differences among TVCs from the three tumor types. These results argue that the distinct molecular profiles observed to date are due in part to TEC specialization in different tumor types.

Many of the TVMs identified were markedly overexpressed in ovarian cancer relative not only to normal ovary but also to a comprehensive panel of normal organs. By highly sensitive qRT-PCR, *adlican*, *COL11A1*, *EGFL6*, *F2RL1*, *FZD10*, *LZTS1*, and *OlfML2B* were detected at the lowest limits, or not at all, in normal tissues tested. These

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Normal Tissue	N	*Adlican*	*C11orf8*	*COL 11A1*	*DR6*	*EGFL6*	*FJX1*	*F2RL1*	*FZD10*	*GPM6B*	*LZTS1*	*OLFML2B*	*STC2*
Adipose_tissue	10	333	11	14	267	69	34	16	19	54	25	122	16
Adrenal gland cortex	4	358	20	20	60	13	42	10	14	87	25	56	14
Bone marrow	5	70	10	16	84	19	22	9	12	31	28	35	14
Bronchus	3	256	18	63	387	24	35	33	21	84	22	73	18
Cerebellum	9	33	19	17	143	16	61	7	16	1,406	21	30	13
Cerebral cortex	9	37	40	40	335	17	71	7	12	2,517	47	36	12
Cerebrum	43	39	41	39	340	18	76	9	14	3,027	46	34	14
Cervix	4	593	57	13	110	26	51	12	42	244	20	153	18
Colon cecum	3	445	14	15	147	15	23	62	16	324	21	46	15
Coronary artery	3	162	14	15	184	15	33	8	13	263	25	130	20
Endometrium	4	231	48	26	204	52	141	16	39	121	24	294	14
Esophagus	4	194	12	13	315	28	28	39	155	111	27	38	13
Heart atrium	4	159	14	16	117	15	23	12	11	163	23	60	24
Heart ventricle	3	170	26	16	109	16	29	8	12	97	25	36	18
Kidney cortex	4	107	74	13	390	14	32	94	10	35	22	30	22
Kidney medulla	4	197	122	14	313	16	33	121	12	117	21	31	16
Liver	4	76	8	14	73	16	25	12	12	16	21	26	12
Lung	3	179	6	12	178	151	31	12	14	157	23	98	20
Lymph nodes	4	111	13	11	282	16	26	10	17	71	26	63	13
Mammary gland	3	340	20	14	221	298	36	17	16	116	33	96	47
Medulla	9	40	62	36	536	24	63	8	14	3,154	24	36	13
Midbrain	107	47	53	41	405	20	69	8	15	2,792	39	48	14
Myometrium	5	209	25	12	138	16	32	7	15	60	22	95	13
Nipple cross section	4	505	7	21	137	18	79	165	57	229	29	73	53
Oral mucosa	4	370	9	14	506	25	45	59	218	60	30	81	13
Ovary	4	116	9	29	89	16	84	17	14	34	23	48	16
Pharyngeal mucosa	4	375	9	16	556	31	51	39	247	73	29	62	15
Pituitary gland	8	63	86	36	116	24	29	11	17	118	28	29	19
Prostate gland	3	188	202	14	207	20	38	35	46	234	21	37	15
Salivary gland	4	216	79	20	97	18	26	31	20	111	21	40	13
Saphenous vein	3	365	9	16	103	19	39	22	21	67	26	281	27
Skeletal muscle	5	61	33	18	67	17	29	8	17	25	31	50	29
Spinal cord	8	50	28	38	792	27	100	10	17	5,203	22	30	19
Spleen	4	175	11	15	75	15	69	13	11	203	20	25	25
Stomach	11	510	24	15	326	20	25	45	17	195	24	49	15
Testes	3	42	18	30	77	15	21	7	12	16	37	163	14
Thyroid gland	4	415	377	13	69	15	22	33	19	58	20	153	21
Tongue	8	178	12	17	324	21	40	23	63	52	24	51	14
Tonsil	3	176	10	14	278	24	36	12	38	28	21	69	14
Trachea	3	372	24	47	595	31	38	40	27	86	21	90	14
Trigeminal ganglia	8	130	78	77	305	87	35	8	19	1,871	34	173	13
Urethra	3	912	106	17	445	33	23	31	63	297	23	304	24
Vagina	4	915	121	14	189	37	45	31	75	259	20	93	15
Vulva	4	1,309	15	16	226	38	33	70	108	126	32	69	16

B

Tumor Tissue	N	*Adlican*	*C11orf8*	*COL 11A1*	*DR6*	*EGFL6*	*FJX1*	*F2RL1*	*FZD10*	*GPM6B*	*LZTS1*	*OLFML2B*	*STC2*
Adrenal gland	2	370	22	103	924	31	143	744	12	54	53	639	127
Bladder	14	418	21	220	1,513	100	86	240	18	66	22	122	45
Brain	2	123	14	216	280	100	173	108	32	5,616	28	60	37
Breast	183	1,281	29	671	356	99	115	105	20	158	28	325	588
Cervix	9	830	38	805	584	130	92	251	119	116	20	208	47
Colon	125	614	16	309	590	47	68	709	49	68	24	178	78
Corpus Uteri	7	649	124	488	396	1,042	157	324	352	757	39	574	104
Endometrium	42	544	152	220	575	172	184	289	131	209	26	188	138
Esophagus	2	630	7	141	306	24	44	146	8	22	9	95	39
Kidney	91	183	30	35	562	19	109	355	11	60	29	194	269
Liver	15	903	21	389	712	76	93	453	25	63	27	213	98
Lung	61	867	26	512	874	273	80	238	79	144	28	244	100
Omentum	30	1,309	101	1,210	399	326	209	229	134	218	25	294	114
Ovary	91	592	123	298	535	198	196	378	125	169	28	142	129
Pancreas	4	885	19	463	846	35	78	312	12	1,045	23	377	93
Prostate	17	173	180	17	289	19	54	268	18	232	22	54	43
Rectosigmoid	32	761	22	443	591	68	77	759	42	82	26	259	82
Small Intestine	8	226	36	153	347	52	70	272	47	217	20	85	55
Stomach	6	742	85	35	584	28	79	561	26	524	53	102	32
Testis	1	86	11	16	555	17	85	17	10	381	26	214	46
Thyroid	10	352	141	68	207	41	48	198	18	114	19	83	45
Vulva	3	1,047	12	1,349	435	96	149	506	65	318	21	228	49

Legend

0-75	151-300	501-1,000	2,001-3,500
76-150	301-500	1,001-2,000	> 3,500

Fig 5. Heat map of tumor vascular marker expression in (A) normal human tissues and (B) different solid tumor types. Average expression is reported for each gene/tissue in the corresponding box. N indicates the number of different samples available for the specified tissue. Data represent 355 samples from five female and five male donors.

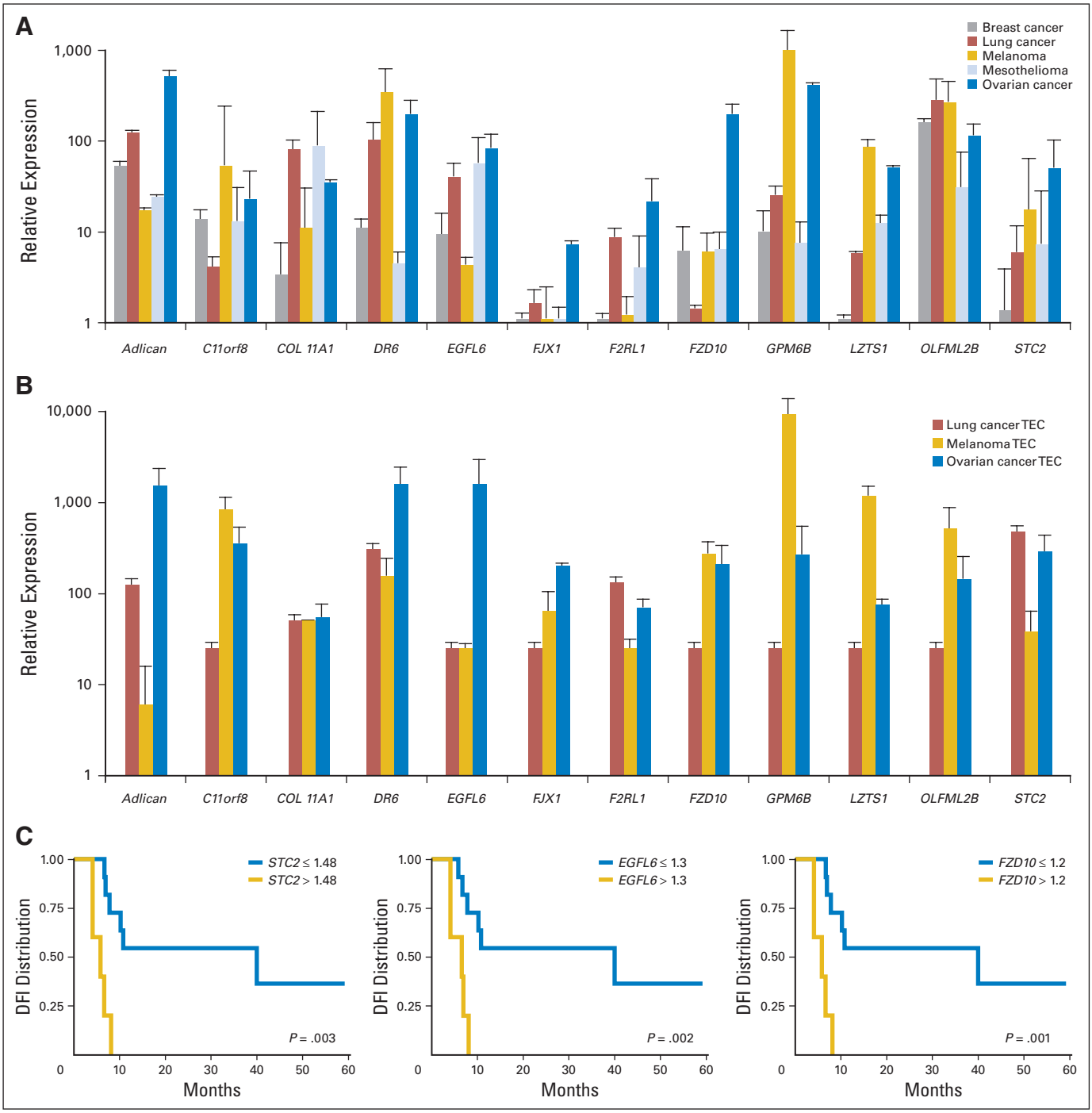

Fig 6. Tumor vascular marker (TVM) expression in different tumors. (A) Expression of TVM by quantitative real-time polymerase chain reaction (qRT-PCR) in different tumor types (whole tissue). (B) Expression of TVMs by qRT-PCR in immunopurified VE-cadherin$^+$, CD146$^+$, CD45$^-$ tumor endothelial cells from melanoma, lung cancer, and ovarian cancer. (C) Kaplan-Meier disease-free survival distributions of patients classified on the basis of TVM expression.

findings were confirmed by SAGE data and by comprehensive gene expression array data including 44 different normal tissues. Thus, some of the TVMs validated may be considered for further preclinical testing as bona fide tumor biomarkers or therapeutic targets. Our Western blot analysis suggests that secreted proteins such as DR6, adlican, and COL11A1 could provide useful serum tumor biomarkers. More sensitive/quantitative enzyme-linked immunosorbent assay analyses are necessary to test this in a larger population of patients. Sensitive antibodies could detect nascent tumors or monitor small changes in tumor status. Similarly, surface proteins expressed by TECs, such as TNFRSF21, may circumvent existing biodistribution barriers of tumors and function as useful targets for cancer antibody-based therapy or molecular imaging. Importantly, some of the TVMs identified here were not expressed by reproductive tissues that exhibit increased angiogenesis, such as placenta, corpus luteum, or proliferative endometrium. This is a critical feature to enhance specificity in

reproductive-age women requiring surveillance because of a hereditary risk of ovarian cancer.

Some of the TVMs identified in ovarian cancer were expressed in a variety of other tumors. Thus, diagnostic or therapeutic tools developed on the basis of such TVMs may be applicable to several tumor types. The use of large gene expression array databases can significantly accelerate mapping of these markers in different tumor types to select disease targets for further clinical validation. Importantly, we found that overexpression of select TVMs in purified vascular cells was significantly predictive of poor clinical outcome. Because some of these TVMs are expressed in other tumor types, their prognostic potential may be applicable to other tumors. These data confirm our hypothesis that tumor vascular proteins can provide novel biomarkers, which could aid in early tumor detection or prognosis. Additional studies will be required to determine the clinical relevance of the biomarkers identified.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest.

No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).