

***FKBP10*/FKBP65 expression in high-grade ovarian serous carcinoma and its association with patient outcome**

MICHAEL C.J. QUINN^{1*}, PAULINA M. WOJNAROWICZ^{2*}, AMY PICKETT³, DIANE M. PROVENCHER^{1,4,5}, ANNE-MARIE MES-MASSON^{1,6}, ELAINE C. DAVIS³ and PATRICIA N. TONIN^{2,7,8}

¹Research Centre of the University of Montreal Hospital Centre/Montreal Cancer Institute;
²Department of Human Genetics, McGill University; ³Faculty of Medicine, Department of Anatomy and Cell Biology, McGill University; ⁴Division of Gynecologic Oncology, University of Montreal;
 Departments of ⁵Obstetrics and Gynecology and ⁶Medicine, University of Montreal;
⁷The Research Institute of the McGill University Health Centre; ⁸Department of Medicine, McGill University, Montreal, QC, Canada

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Abstract. The frequent loss of chromosome 17 in epithelial ovarian carcinomas (EOC), particularly high-grade serous carcinomas (HGSC), has been attributed to the disruption of *TP53* (at 17p13.1) and other chromosome 17 genes suspected to play a role in tumour suppressor pathways. In a transcriptome analysis of HGSC, we showed underexpression of a number of chromosome 17 genes, which included *FKBP10* (at 17q21.1) and *collagen I α 1* (*COL1A1*; at 17q21.33). *FKBP10* codes for the immunophilin FKBP65 and is suspected to act as a chaperone for *COL1A1*. We have investigated *FKBP10* (gene) and FKBP65 (protein) expression in HGSC samples and EOC cell lines that differ in their tumourigenic potential. *COL1A1* expression was also investigated given the purported function of FKBP65. RT-PCR analysis verified underexpression of *FKBP10* and *COL1A1* in HGSCs (n=14) and six tumourigenic EOC cell lines, relative to normal ovarian surface epithelial cells and a non-tumourigenic EOC cell line. Immunohistochemistry analyses of 196 HGSC samples using tissue microarrays revealed variable staining intensities in the epithelial tumour component where only 7.8% and 1.0% of samples stained intensely for FKBP65 and *COL1A1*, respectively. Variable staining intensities were also observed for the stromal component where 23.6% and 24.1% stained intensely for FKBP65 and *COL1A1*, respec-

tively. There was no significant correlation of staining intensity of either protein with disease stage. Staining of FKBP65 was clearly visible in normal epithelial cells of the ovarian surface and fallopian tube. There was a significant correlation between absence of FKBP65 staining in the epithelial cell component of the tumour and prolonged overall survival (p<0.001). Our results suggest that underexpression of FKBP65 protein is characteristic of HGSCs and that this expression profile may be linked to molecular pathways associated with an unfavourable outcome in cancer patients.

Introduction

Loss of heterozygosity of chromosome 17 is a frequent occurrence in epithelial ovarian cancer (EOC) and particularly in high-grade serous carcinomas (HGSC), which is one of the most common histotypes of EOCs (1-6). This has been largely attributed to the inactivation of the tumour suppressor gene *TP53*, which is located at 17p13.1, as it is the most frequently mutated gene in HGSCs, however, other genes involved in tumour suppressor pathways have been proposed (1-4,7-14). For example, our group recently identified 158 underexpressed chromosome 17 genes in a transcriptome analysis of HGSCs, which included *FKBP10*, a gene that maps to 17q21.1. Interestingly, *FKBP10* was among the genes upregulated in a genetically modified EOC cell line rendered non-tumourigenic as a consequence of a unique gene complementation assay involving chromosome transfer, and thus may be one of a number of genes transcriptionally reprogrammed as a consequence of tumour suppression (15,16).

FKBP10 encodes FKBP65, a 65 kDa FK506 binding protein that is a member of the FKBP-type peptidyl-prolyl cis/trans isomerase family (17,18). This protein localizes to the endoplasmic reticulum and acts as a molecular chaperone and binding partner of type I collagen (19-21). The interaction with type I collagen is interesting as *COL1A1*, which is a gene that maps to 17q21.33, was also found significantly underexpressed in our chromosome 17 transcriptome analyses of HGSCs (14). FKBP65

Correspondence to: Dr Patricia N. Tonin, Medical Genetics, Room L10-132, Montreal General Hospital, 1650 Cedar Avenue, Montreal, QC H3G 1A4, Canada
 E-mail: patricia.tonin@mcgill.ca

*Contributed equally

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is expressed in developing tissues and re-expressed in adult tissues following injury (19,20). In the mouse, the only tissues that continue to express FKBP65 are reproductive tissues (ovary, uterus and mammary glands) during phases of growth and remodelling (E.C. Davis, unpublished data). Although somatic mutations inactivating *FKBP10* have not been identified, such as in recent reports of genome-wide exomic sequencing analyses of HGSCs by The Cancer Genome Atlas Research Network (5) and other cancer types (22), germline *FKBP10* mutations have been described in association with autosomal-recessive osteogenesis imperfecta and Bruck syndrome, which are connective tissue disorders characterized by defects in type I collagen (23,24). Though epithelial malignancies in patients with osteogenesis imperfecta are rare, there is at least one case report of a 32-year-old who developed a low-grade serous ovarian carcinoma with stage IIIb disease (25).

The localization of *FKBP10* to chromosome 17 and its interesting expression profile in the murine adult normal ovary, HGSCs and our genetically modified EOC cell line, prompted our further investigation of this gene in ovarian cancer samples. In this study, we investigated the expression profile of *FKBP10* in HGSCs, and its expression in a set of well-characterized EOC cell lines that differ in their growth characteristics and tumorigenic potential. We also investigated protein expression by immunohistochemistry analysis of a tissue microarray and related the expression profile to patient outcome. *COL1A1* gene and protein expression was also investigated given its purported interaction with FKBP65.

Materials and methods

Tissue specimens, cell lines, and clinical information. The HGSCs, primary cultures of normal ovarian surface epithelial cells (NOSE), and EOC cell lines (OV90, TOV112D, TOV81D, TOV21G, TOV1946, OV1946 and TOV2223) examined for gene expression have been described previously (1,14,26). Briefly, the HGSC samples were from chemotherapy naïve patients and the cell lines were derived from long-term passages of malignant ovarian ascites from an undifferentiated adenocarcinoma (OV-90), high-grade endometrioid adenocarcinoma (TOV112D), serous carcinomas (TOV81D, TOV1946, OV1946 and TOV2223) and a clear cell carcinoma (TOV21G), where TOV1946 and OV1946 were derived from malignant ovarian ascites (OV1946) or tumour (TOV1946) from the same patient.

The HGSC (n=196) cases represented in the tissue array were derived from archival blocks of paraffin-embedded tissues samples as previously described (27). The tumour grade and disease stage (Table I) were designated according to the International Federation of Gynaecology and Obstetrics. Disease-free interval, defined as time to doubling of the upper normal limit of the serum cancer antigen marker CA-125 or the detection of a new lesion by ultrasound or CT-scan imaging, and overall survival, defined according to the Response Evaluation Criteria in Solid Tumors (28) were extracted from the Système d'Archivage des Données en Oncologie (Table I). Normal ovary and fallopian tube tissues used in immunohistochemistry analyses were retrieved from archival paraffin-embedded samples. Normal ovarian tissues were obtained from ovariectomy (age 40) and hysterectomy (age 45) cases due to a benign uterine tumour. The fallopian tube tissues were adjacent normal tissues collected

from women diagnosed with serous ovarian cancer. All samples and related clinical information were obtained with informed written consent at the Centre Hospitalier de l' Université de Montreal (CHUM) - Hôpital Notre Dame.

Gene expression analyses. Gene expression was assessed by semi-quantitative RT-PCR analysis using cDNA synthesized from total RNA prepared as previously described (3,13,15,29). Primers were designed using Primer3 software (30) based on genomic structures of *FKBP10* and *COL1A1* available from the March 2006 human reference sequence (NCBI Build 36.1/hg18) assembly (31) and alignment of reference sequences of each gene, NM_021939 and NM_000088.3, respectively. The *FKBP10* forward primer is 5'-GTGGAACAAGGAAGACACC-3', and the reverse primer is 5'-CTTCCTTCTCTCTCCAGGAC-3', yielding a 238 base pair product. The *COL1A1* forward primer is 5'-GTGCTCCTGTATTGCTG-3', and the reverse primer is 5'-CTCGCTTTCCTTCCTCTC-3', yielding a 207 base pair product. The RT-PCR-based assays were performed essentially as previously described (32). RT-PCR of *18S* RNA was performed to assess RNA quality. Primer sequences for *18S* were reported previously (3).

Immunohistochemistry analysis of tissue arrays. FKBP65 and COL1A1 protein expression was assessed by immunohistochemistry analysis using a tissue array containing 0.6 mm cores derived from paraffin-embedded tissue blocks representing 196 HGSC cases selected based on a review of hematoxylin and eosin-stained slides prepared as described previously (27). The array also contained 11 normal fallopian tube samples. Immunohistochemistry analysis was performed on the tissue array and sections prepared from two normal ovary paraffin-embedded tissue blocks. Five-micron sections were mounted onto frosted plus slides, deparaffinized in Citrisolv (Fisher Scientific) for 90 min and then rehydrated in an ethanol gradient. Before primary antibody incubation, antigen retrieval was performed using 0.05% Tween-20 in 10 mM sodium citrate (pH 6.0) at 90°C for 20 min. The slides were washed with 0.1% Triton X-100 in Tris-buffered saline (TBS) [0.5 M Tris, 1.5 M NaCl, (pH 7.4)]. The slides were then washed and blocked with TBS containing 0.1% bovine serum albumin (TBS/BSA) for 3x10 min and then incubated with Ultra V Block (LabVision Corp., Fremont, CA, USA) for 7 min. After washing again in TBS/BSA, the slides were incubated overnight at 4°C with either a polyclonal FKBP65 antibody, raised against a synthetic peptide to the C-terminus of the mouse FKBP65 (33), or a polyclonal type I collagen antibody (Calbiochem), diluted at 1:300 and 1:1000, respectively. Slides were incubated in Value Primary Antibody Enhancer (LabVision Corp.) for 20 min before incubation in AP Value Polymer (LabVision Corp.) for 30 min at room temperature. Staining was visualized using FastRed (LabVision Corp.) and slides were counterstained with hematoxylin for 30 sec. The tissue arrays were scanned with an Aperio ScanScope XT Digital Slide Scanner and images were viewed at high resolution using Aperio ImageScope Software version 11.02. Two observers examined the images independently and scored them based on staining intensity ranked as absent, low, moderate or high, for both the epithelial and stromal tissue components of each core. The inter-observer correlation coefficients were 0.792 for FKBP65 and 0.747 for

Table I. Description of HGSC tissue array.

Patient characteristic	All cases	G2	G3
No. of cases	196	30	166
Mean age of diagnosis, years (range)	62 (34-89)	60 (42-82)	62 (34-89)
Stage I	15	2	13
Stage II	19	2	17
Stage III	139	23	116
Stage IV	23	3	20
Mean disease free interval, months (range)	22 (0-134)	15 (0-57)	23 (0-134)
No. of censored patients	52	6	46
No. of non-censored patients	144	24	120
Mean overall survival, months (range)	35 (0-134)	28 (0-102)	36 (0-134)
No. of censored patients	110	19	91
No. of non-censored patients	86	11	75

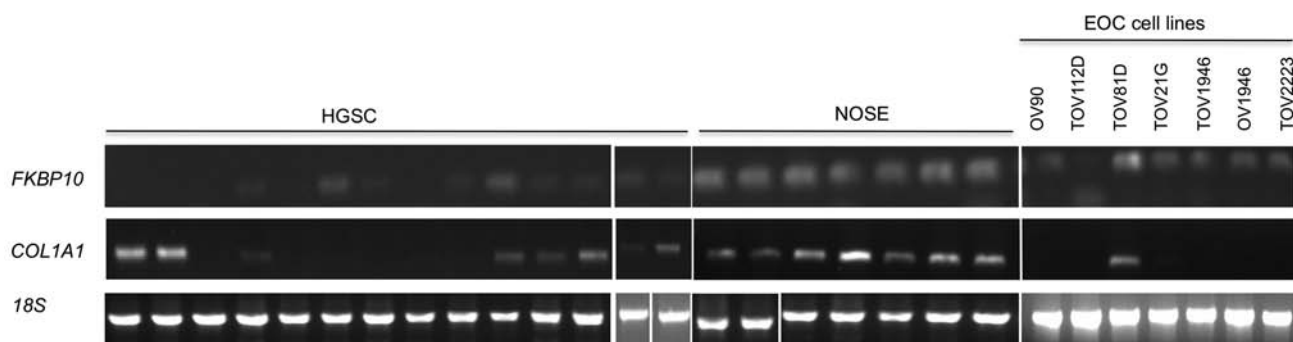


Figure 1. Semi-quantitative RT-PCR analyses of *FKBP10* and *COL1A1*. RT-PCR analyses of *FKBP10* and *COL1A1* in high-grade serous ovarian cancer samples (HGSC), normal ovarian surface epithelial cells (NOSE) and epithelial ovarian cancer (EOC) cell lines. The RT-PCR expression analysis of 18S is also shown for RNA quality.

COL1A1. The inter-observer correlation coefficient was calculated using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA), where the minimum threshold was 0.7.

Statistical analyses. Spearman analysis was used to evaluate the correlation of FKBP65 and *COL1A1* staining intensities. The association between tumour grade and staining intensity was evaluated using an independent sample t-test. The association between disease stage and staining intensity was evaluated using a one-way ANOVA. The relationship between staining intensity and disease-free interval or overall survival was evaluated using the log-rank test and visualized by Kaplan-Meier survival curve analysis. All statistical analyses were performed with SPSS software version 16.0 (SPSS Inc.), p-values <0.05 were considered significant.

Results

Gene expression analyses of *FKBP10* and *COL1A1*. A previous gene expression microarray analysis of chromosome 17 genes identified *FKBP10* and *COL1A1* among the 158 genes underexpressed in HGSCs as compared with NOSEs

(14). To verify these observations, we investigated the expression of these genes by performing RT-PCR analyses on the samples used in the microarray analyses. As shown in Fig. 1, *FKBP10* and *COL1A1* expression was clearly detectable in all NOSEs. In contrast, *FKBP10* expression in the HGSCs was undetectable or expressed at levels lower than that observed in the NOSEs. *COL1A1* expression was more variable, ranging from clearly detectable levels comparable to those observed in NOSEs in some samples, to undetectable or lower levels of expression relative to the NOSEs (Fig. 1). Gene expression of *FKBP10* and *COL1A1* was also investigated in cell lines established as long-term passages from chemotherapy naïve EOC samples (1,26). *FKBP10* expression was detectable in all EOC cell lines with the highest level of expression observed in TOV81D (Fig. 1). In contrast, *COL1A1* expression was detectable in only TOV81D (Fig. 1), which is a non-tumourigenic EOC cell line.

Immunohistochemical staining of FKBP65 and *COL1A1*. Immunohistochemistry analysis was performed to characterize FKBP65 protein expression in normal ovary and fallopian tube as the expression profile in human tissues purported to

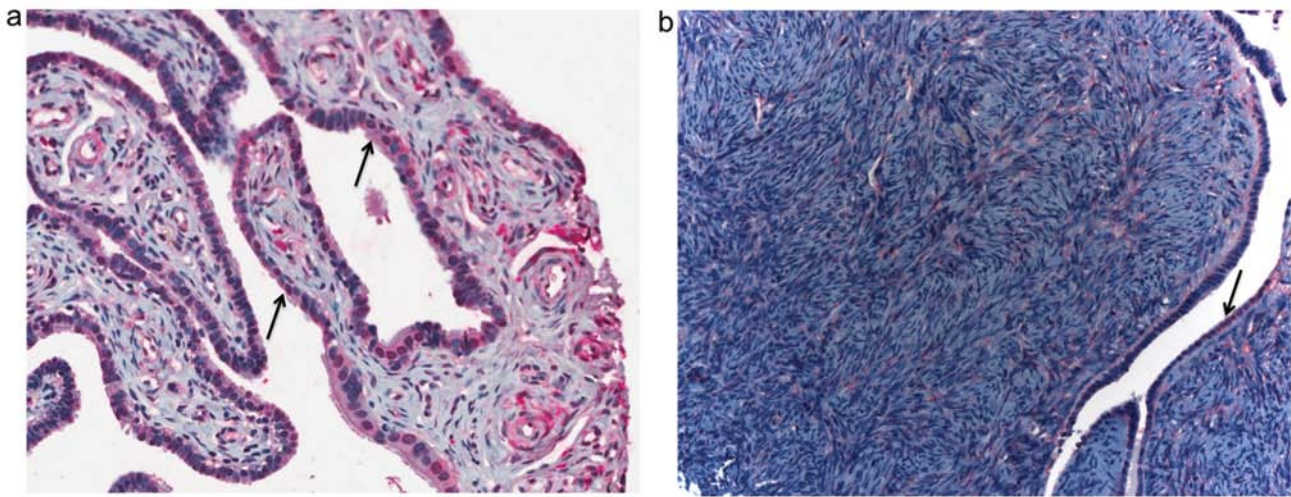


Figure 2. Example of immunostaining of FKBP65 in normal tissues. Immunostaining of FKBP65 in (a) normal fallopian tube tissue and (b) normal whole ovary tissue. Note positive immunoreactivity in epithelial cells as indicated by arrows. Image is shown at x20 magnification from the Aperio image scope scan.

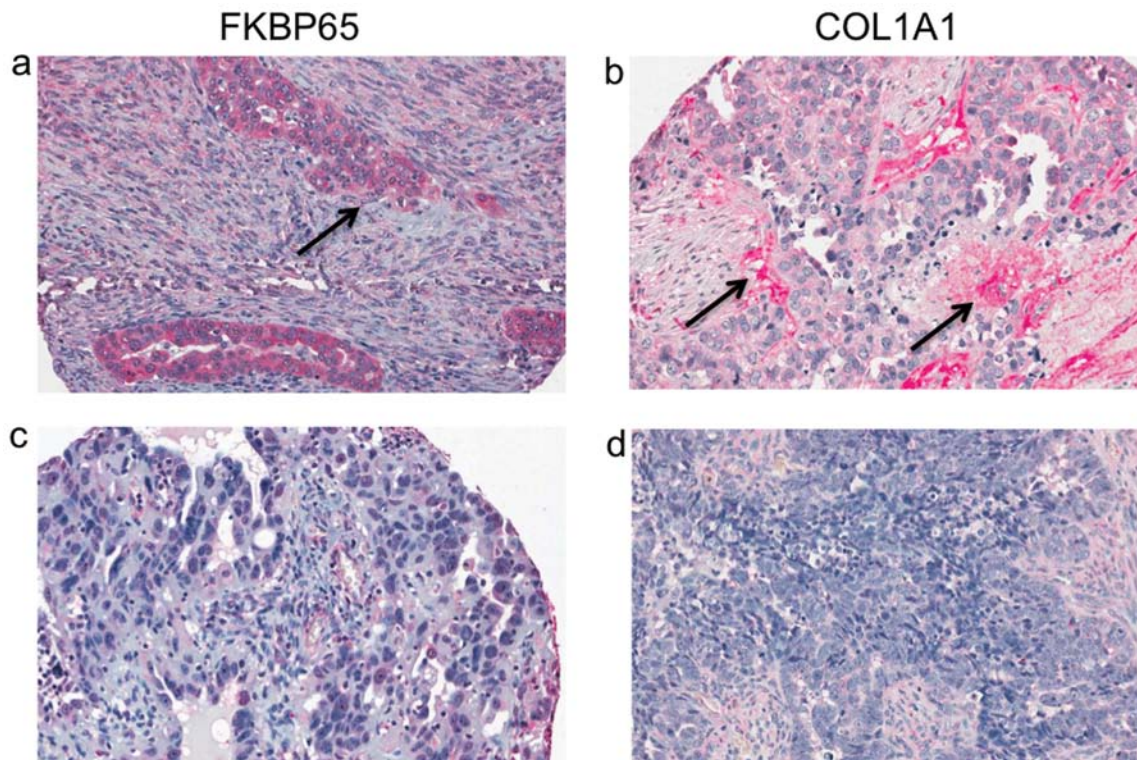


Figure 3. Examples of immunostaining patterns of FKBP65 and COL1A1. Matched HGSC patient cores are (a) and (b), and (c) and (d). (a) High epithelial staining for FKBP65, and (c) no detectable staining of FKBP65. COL1A1 staining is shown in (b) and (d). Arrows indicate positively staining epithelial cells in (a) and positively staining stromal cells in (b). All images are shown at x20 magnification from the Aperio image scope scan.

be the origins of HGSCs have not previously been described. Staining was evident in both the epithelial and stromal cells of the tissues (Fig. 2).

FKBP65 and COL1A1 protein expression in HGSCs was investigated by immunohistochemistry analysis using a tissue array containing 196 cores from tumour samples (Table I). Staining was localized to the cytoplasm for both FKBP65 and COL1A1 and was observed in both the epithelial and

stromal cell components of the tumour samples (Fig. 3). To characterize the expression pattern, intensity of staining was scored as absent, low, moderate or high for both the epithelial and stromal cell components where possible, as scoring was not possible for some of the samples due to the type of cells present or the quality of core (Fig. 4). The majority of the epithelial cell components of the tumour samples scored as either low or moderate for FKBP65 expression, and less than

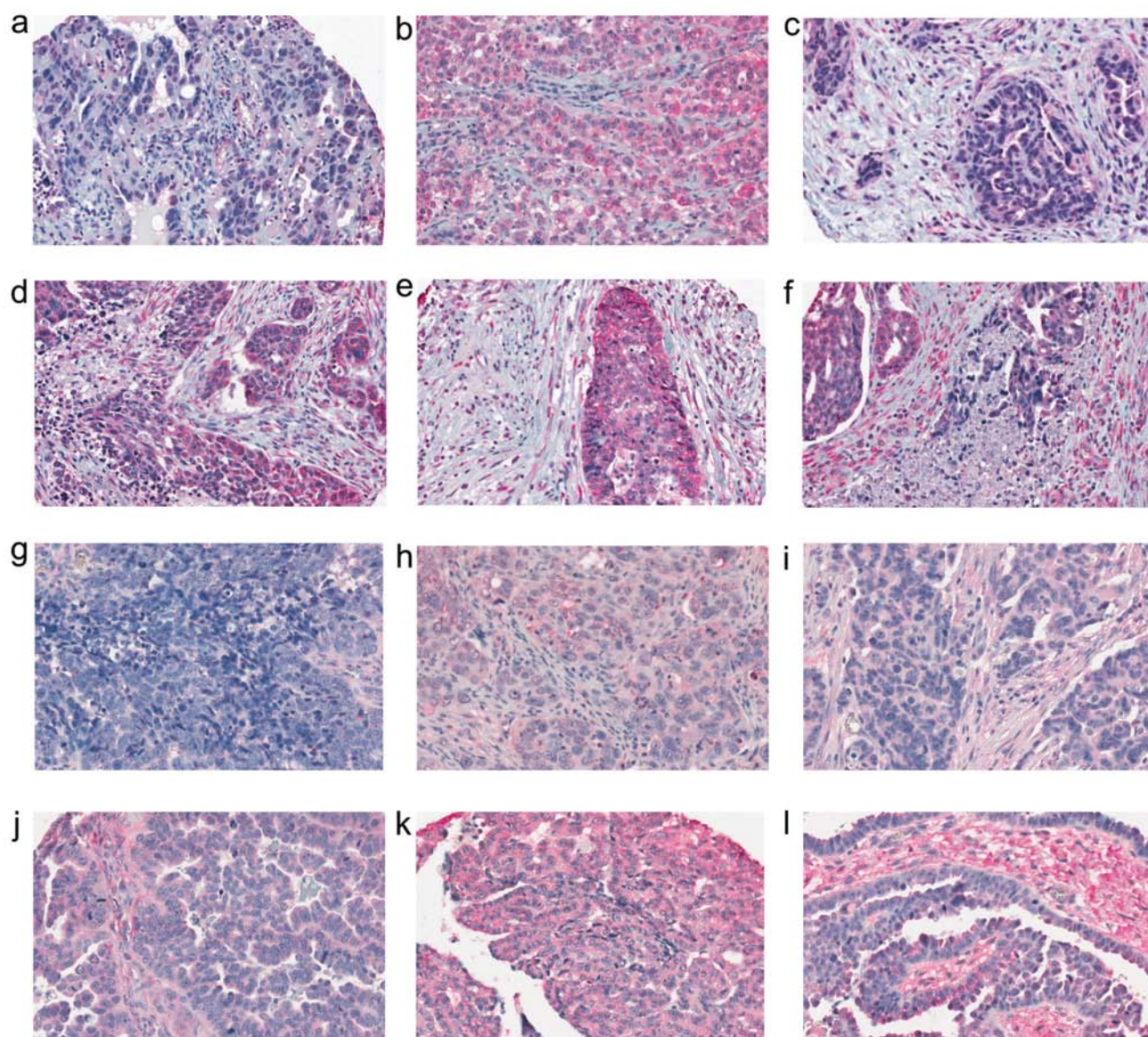


Figure 4. Examples of FKBP65 and COL1A1 staining intensity categories. Examples of FKBP65 staining intensity patterns for (a) absence of epithelial staining, (b) absence of stromal staining, (c) low epithelial and stromal staining, (d) moderate epithelial and stromal staining, (e) high epithelial staining and (f) high stromal staining. Examples of COL1A1 staining intensity patterns for (g) absence of epithelial staining, (h) absence of stromal staining, (i) low epithelial and stromal staining, (j) moderate epithelial and stromal staining, (k) high epithelial staining and (l) high stromal staining. All images are shown at x20 magnification from the Aperio image scope scan.

10% of samples scored as either absent or high (Table II). A somewhat similar staining intensity pattern for the epithelial component of the tumours was also observed with COL1A1, although there were more cases that scored with low intensity levels (Table II). In contrast, FKBP65 expression patterns for the stromal components of the tumour were almost equally distributed among all staining intensity categories, with the largest number of samples (32.3%) exhibiting moderate staining intensity (Table II). Although the largest number of samples (44.5%) also exhibited moderate staining of COL1A1 in the stromal component of the tumour, there were fewer samples that had an absent intensity score (Table II). There was a significant correlation between FKBP65 and COL1A1 staining intensity in the epithelial cell component ($p < 0.001$), but not in the stromal component ($p = 0.101$) of the tumour samples analysed.

Protein expression and disease stage, disease-free interval or overall survival. The staining intensity patterns of FKBP65 and COL1A1 were characterized with respect to disease stage, though the majority of samples (83%) were from cases with advanced stage III/IV disease (Table I). There were no statistically significant differences (data not shown) in the distribution of staining intensity patterns for the proteins assayed for either the epithelial or stromal cell components of the tumour samples and disease stage (Table III).

The staining intensities for FKBP65 and COL1A1 were also evaluated with respect to disease-free interval and overall survival. No significant relationships were observed with staining intensities of COL1A1 for the epithelial or stromal components of the tumour samples and either of these clinical parameters (data not shown). Significant relationships were also not found with staining intensities of FKBP65 for the epithelial

Table II. Distribution of staining intensities for FKBP65 and COL1A1 in HGSC samples.

Protein	Cell type	Total no. of samples scored	Staining intensity score (%)			
			Absent	Low	Moderate	High
FKBP65	Epithelial	193	2 (1.0)	107 (55.4)	69 (35.8)	15 (7.8)
	Stromal	195	46 (23.6)	40 (20.5)	63 (32.3)	46 (23.6)
COL1A1	Epithelial	191	2 (1.0)	134 (70.2)	53 (27.7)	2 (1.0)
	Stromal	191	9 (4.7)	51 (26.7)	85 (44.5)	46 (24.1)

Table III. Distribution of staining intensities for FKBP65 and COL1A1 by stage.

Protein	Cell type	Stage	Total no. of samples scored	Staining intensity score (%)			
				Absent	Low	Moderate	High
FKBP65	Epithelial	I	15	0	9 (60)	5 (33.3)	1 (6.6)
		II	18	0	12 (66.6)	5 (27.7)	1 (5.5)
		III	137	2 (1.5)	75 (54.7)	52 (38.0)	8 (5.8)
		IV	23	0	11 (97.8)	7 (30.4)	5 (21.7)
	Stromal	I	15	5 (33.3)	2 (13.3)	4 (26.6)	4 (26.6)
		II	19	0	6 (31.6)	8 (42.1)	5 (26.3)
		III	138	37 (26.8)	27 (19.6)	42 (30.4)	32 (23.2)
		IV	23	4 (17.4)	5 (21.7)	9 (39.1)	5 (21.7)
COL1A1	Epithelial	I	15	0	10 (66.6)	5 (33.3)	0
		II	19	0	15 (78.9)	4 (21.1)	0
		III	135	1 (0.7)	98 (72.6)	36 (26.7)	0
		IV	22	1 (4.5)	11 (50)	8 (36.4)	2 (9.1)
	Stromal	I	15	1 (6.6)	2 (13.3)	6 (40)	6 (40)
		II	18	0	5 (27.8)	7 (38.9)	6 (33.3)
		III	136	6 (4.4)	37 (27.2)	65 (47.8)	28 (20.6)
		IV	22	2 (9.1)	7 (31.8)	7 (31.8)	6 (27.3)

or stromal components of the tumour samples and disease-free interval (data not shown). In contrast, there was a significant association between prolonged overall survival and the presence of FKBP65 protein in the epithelial component when evaluated for each staining category ($p=0.005$) or when cases with no staining are compared with those with low, moderate and high staining combined ($p<0.001$) (Fig. 5a and c). Although there are only two samples with no detectable staining in the epithelial component of the tumour, it is interesting that the cases with the highest staining levels for FKBP65 where among the cases which had the longest overall survival (Fig. 5c). A similar analysis of FKBP65 staining found a significant association ($p=0.027$) with prolonged overall survival when the samples were analysed for the absence or presence of staining in the stromal component (Fig. 5b). Although there was no significant difference with overall survival and staining based on each staining intensity category ($p=0.087$), it is interesting that the cases scored absent for staining in the stromal component were among those with the poorest overall survival (Fig. 5d).

Discussion

In this study we have verified that *FKBP10* expression is absent or low in HGSC and the results are consistent with the low frequency of high intensity staining patterns of FKBP65 in tumour samples. FKBP65 expression in the epithelial cells of normal ovarian surface and the distal fimbriae of the fallopian tube is interesting considering that both of these tissues have been proposed as the progenitor cell type for HGSC (34). During the course of this study, decreased expression of FKBP65 in a study of 57 EOC samples of different histological subtypes, which included HGSCs, was reported independently (35). These observations suggest the possibility that FKBP65 expression is also important in the biology of the other histological subtypes of EOC. This notion is supported by the observation that *FKBP10* was underexpressed in most of our EOC cell lines, which were derived from epithelial ovarian tumour samples that differed in their histology.

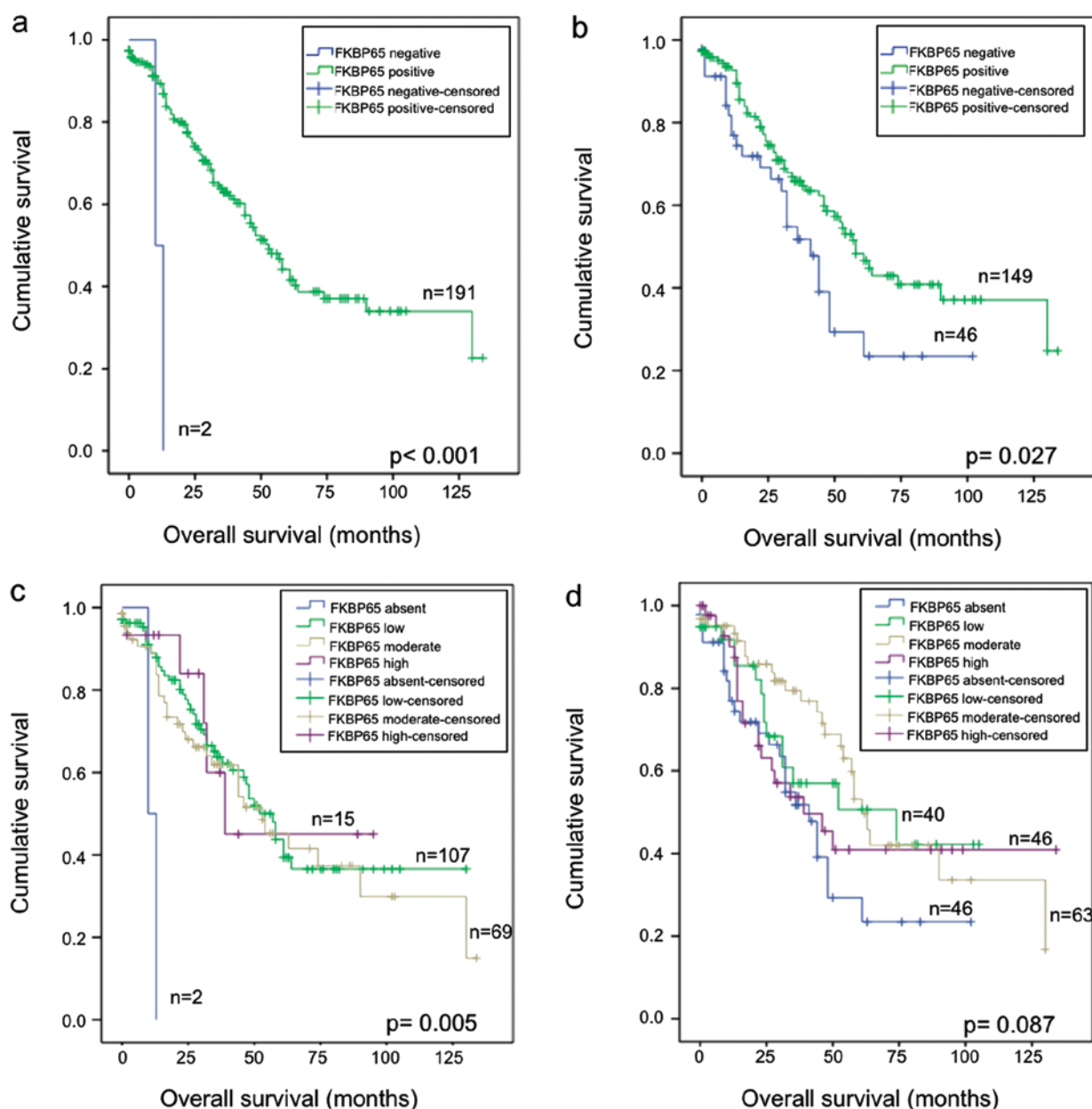


Figure 5. Analysis of FKBP65 staining intensity in HGSCs using the log ratio statistic, and visualized using Kaplan-Meier survival curves. Survival analysis was based on staining intensity grouped as present (positive) or absent (negative) in (a) epithelial and (b) stromal cell components. Survival analysis was based on staining intensity grouped as absent, low, moderate and high in (c) epithelial and (d) stromal cell components.

Our results from the *FKBP10* expression assays of the EOC cell lines also suggest that underexpression might be associated with tumorigenicity. Although our assays were semi-quantitative, the highest level of gene expression was observed in TOV81D, the only cell line in our series of EOC cell lines tested that is unable to form tumours in mouse tumour xenograft assays and lacks *in vitro* growth phenotypes characteristics of tumourigenic cell lines (26,36). Our expression results are consistent with previously published studies from our group where, by semi-quantitative RT-PCR and gene expression microarray analyses, TOV81D exhibited gene expression profiles that resemble those of NOSEs (3,14,16,37-39). Notable also is the underexpression of *FKBP10* observed in the tumourigenic OV90 cell line, as this gene was

induced in OV90 cell line hybrids that were rendered non-tumourigenic as a consequence of the transfer chromosome 3 in genetic complementation assays aimed at identifying genes implicated in tumour suppressor pathways (15,16). Thus, decreased expression of *FKBP10* may be important in tumour suppressor pathways.

We observed a significant association between FKBP65 staining intensity in the epithelial cells of HGSC samples and overall survival. An association between high FKBP65 expression and prolonged survival was also observed in the independent study of 57 EOC samples by Henriksen *et al*, although the finding was not significant (35). In their association analyses the small sample size and possibly the inclusion of different histological subtypes, which are known to exhibit

differences in outcome (40), may have affected the interpretation of results. In our analyses, though the samples with no FKBP65 staining exhibited the poorest outcome, they represented only two HGSC cases in our cohort. Notable, however, is that the samples exhibiting the highest staining intensity were among the HGSC cases with the longest overall survival. Interestingly, the staining intensities of the stromal component of the tumour samples were also associated with overall survival. There is mounting evidence that ovarian cancer progression involves reciprocal communication between malignant epithelial cells and the adjacent stromal microenvironment possibly relating to malignant epithelium-activated fibroblasts (41). Although not fully explored in the context of ovarian cancer, gene expression profiles of stromal cells have been found to be predictive of disease outcome in breast cancer (42). The expression profile of COL1A1 was similar to FKBP65 as demonstrated by the significant positive correlation of staining intensities by immunohistochemistry of HGSCs. The similarities in expression profiles have been reported previously, and this is consistent with FKBP65 being a type I collagen chaperone (19,21).

In conclusion, we found FKBP65 underexpressed in a proportion of HGSCs, and its expression correlated with that of COL1A1 in epithelial cells. We also reported on the interesting finding that absence of FKBP65 staining was associated with poorer overall survival warranting replication of our study with larger cohorts. The interesting *FKBP10* expression profiles in our EOC cell lines that differ in their tumorigenic potential could also be explored to further elucidate the molecular pathways that involve FKBP65.

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