ARTICLE

ABCA Transporter Gene Expression and Poor Outcome in Epithelial Ovarian Cancer

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Background   ATP-binding cassette (ABC) transporters play various roles in cancer biology and drug resistance, but their association with outcomes in serous epithelial ovarian cancer (EOC) is unknown.

Methods   The relationship between clinical outcomes and ABC transporter gene expression in two independent cohorts of high-grade serous EOC tumors was assessed with real-time quantitative polymerase chain reaction, analysis of expression microarray data, and immunohistochemistry. Associations between clinical outcomes and *ABCA* transporter gene single nucleotide polymorphisms were tested in a genome-wide association study. Impact of short interfering RNA–mediated gene suppression was determined by colony forming and migration assays. Association with survival was assessed with Kaplan–Meier analysis and log-rank tests. All statistical tests were two-sided.

Results   Associations with outcome were observed with ABC transporters of the "A" subfamily, but not with multidrug transporters. High-level expression of *ABCA1*, *ABCA6*, *ABCA8*, and *ABCA9* in primary tumors was statistically significantly associated with reduced survival in serous ovarian cancer patients. Low levels of *ABCA5* and the C-allele of rs536009 were associated with shorter overall survival (hazard ratio for death = 1.50; 95% confidence interval [CI] =1.26 to 1.79; *P* = 6.5e–6). The combined expression pattern of *ABCA1*, *ABCA5*, and either *ABCA8* or *ABCA9* was associated with particularly poor outcome (mean overall survival in group with adverse ABCA1, ABCA5 and ABCA9 gene expression = 33.2 months, 95% CI = 26.4 to 40.1; vs 55.3 months in the group with favorable ABCA gene expression, 95% CI = 49.8 to 60.8; *P* = .001), independently of tumor stage or surgical debulking status. Suppression of cholesterol transporter ABCA1 inhibited ovarian cancer cell growth and migration in vitro, and statin treatment reduced ovarian cancer cell migration.

Conclusions   Expression of ABCA transporters was associated with poor outcome in serous ovarian cancer, implicating lipid trafficking as a potentially important process in EOC.

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Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer mortality in women, and survival rates have not improved over recent decades (1). A better understanding of the contributing factors will help to optimize chemotherapy drug selection and improve treatment outcome for individual patients.

The ABC transporter superfamily consists of 48 transmembrane proteins characterized by their ability to mediate ATP-dependent transport of diverse exogenous and endogenous substances across the lipid bilayer. The superfamily is further divided into seven subfamilies from ABCA to ABCG based on the sequence of the amino acids in the ATP-binding domain (2). Various members of the ABCB, ABCC, and ABCG subfamilies are well known for roles in drug resistance in cancer cells in vitro. However, their impact on clinical outcome is still controversial (3). In addition to roles in cytotoxic drug efflux, ABC transporters actively transport endogenous substrates that contribute to tumor biology, which are also likely to influence tumor phenotype

and treatment response (4,5). Indeed, we previously demonstrated that several members of the ABCC/MRP subfamily are independently associated with survival in children with neuroblastoma, that the combined expression pattern of these transporters was a powerful predictor of clinical outcome, and remarkably, that these transporters influence neuroblastoma biology independently of their more established roles in chemotherapeutic drug efflux (6).

ABC transporters are responsible for the transport of a variety of inflammatory mediators and lipids that have direct relevance to tumor progression in ovarian cancer (7–10) and thus could contribute to clinical outcome and be potential therapeutic targets in this disease. Therefore we examined the expression of the entire ABC transporter gene family in a cohort of primary serous EOC specimens and investigated the relationship between gene expression and clinical outcome. This represents the largest study of its kind, with accurate determination of gene expression matched to comprehensive clinical follow-up data.

Methods

Patient Cohorts

RNA samples from 143 serous EOC tumors were obtained from the Australian Ovarian Cancer Study (AOCS), a population-based, case–control study undertaken between 2002 and 2006 (Supplementary Tables 1 and 2, available online) (11,12). Sample size was determined to have 80% power to detect the difference between two equally sized groups, with a constant hazard ratio (HR) of 2 at a .05 two-sided significance level.

Outcome and expression data generated by the Agilent microarray platform for 407 patients, most with high-grade, advanced-stage disease were extracted from The Cancer Genome Atlas (TCGA) interface (http://tcga-data.nci.nih.gov/) (13).

For the genome-wide association study (GWAS), 1430 case patients were available through 10 Ovarian Cancer Association Consortium sites, as described elsewhere (14). Patient selection was limited to those receiving only paclitaxel and carboplatin as first-line chemotherapy (Supplementary Table 1, available online).

The cohort for immunohistochemical analysis was comprised of 91 patients with serous EOC diagnosed between 1987 and 2007 and treated at Westmead Hospital, Sydney, Australia (Supplementary Methods and Supplementary Tables 1 and 3, available online).

Institutional review board approval was obtained by each cohort study, and this project was approved by the University of New South Wales Human Research Ethics Committee.

ABC Transporter Expression Analysis

RNA was prepared from tumor specimens using the RNeasy extraction kit (QIAGEN, Melbourne, Australia), reverse transcribed with Moloney Murine Leukemia Virus reverse-transcriptase (Life Technologies, Melbourne, Australia) as described (15,16) and applied to custom-made Taqman low-density array cards (Applied Biosystems, Sydney, Australia) as described in the Supplementary Methods (available online). Details of tissue microarray sample preparation, staining, and analysis are presented in the Supplementary Methods (available online).

GWAS Analysis

We conducted a two-stage GWAS to identify germline single nucleotide polymorphisms (SNPs) associated with progression-free survival (PFS) and overall survival (OS) in serous EOC patients. We first examined the association between SNPs in *ABCA1*, *ABCA5*, *ABCA6*, *ABCA8*, and *ABCA9* from 385 strictly selected samples to maximize detection of informative SNPs (discovery stage). We then expanded the analysis to a total of 1430 case patients from nine sites within the Ovarian Cancer Association Consortium and genotyped the leading SNP at each locus (except *ABCA9* because no SNPs at this locus showed association) (replication stage).

Cell Culture and Transfection

Human ovarian cancer cell lines 27/87 (17), A2780 (T. Hamilton, Fox-Chase Cancer Center, Philadelphia, PA) and SKOV-3 (ATCC, Manassas, VA) were cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The cell lines were authenticated by short tandem repeat (STR) DNA profiling in November 2011 (Cell ID System, Promega, Sydney, Australia). Lipofectamine 2000 reagent (Invitrogen) was used to deliver short interfering RNA (siRNA) targeted toward *ABCA*1 (5 nM, sense: 5′ GGAGAUGGUUAUACAAUAGUUUU) or siCONTROL nontargeting siRNA (Dharmacon, Lafayette, CO). Subsequent cell-based assays are described in the Supplementary Methods (available online).

Statistical Analyses

For real-time quantitative PCR (RT-qPCR) expression data, values were categorized high or low by the median value. Clinical outcome between groups was compared using Kaplan–Meier analysis followed by two-sided log-rank tests (SPSS version 22, IBM Software, Sydney, Australia). Treatment of TCGA data is detailed in the Supplementary Methods (available online). Cox proportional hazards modelling (SPSS) was used to generate hazards ratios and 95% confidence intervals (CIs). Assumption of proportionality for each covariable was checked by visual inspection of plots of log (-log(S(t))) over time. In analyses where associations between gene expression and outcome in the AOCS dataset were subsequently examined for cross-validation in TCGA, no multiple testing adjustment was used. For all other gene expression survival analyses, *P* values were derived after Bonferroni adjustment for multiple testing.

For cell-based assays, differences in phenotypic traits between groups were assessed by two-sided Student *t* tests, unless indicated otherwise. For comparisons involving more than two groups, one-way analysis of variance was performed, followed by pair-wise *t* tests with Bonferroni correction. Significance was assessed at the level of *P* less than .05.

Results

ABC Transporter Gene Expression and Clinical Outcome in EOC

For the 143 tumors analyzed by RT-qPCR, the only genes whose high expression was associated with outcome belong to the *ABCA* subfamily, with expression of *ABCA6*, *ABCA8*, and *ABCA9* associated with shorter PFS or OS (Table 1; Figure 1, A–C). The effect of *ABCA6* expression on PFS and *ABCA6* and *ABCA9* expression on OS remained statistically significant after adjustment for tumor stage and residual disease (Table 1). In contrast, low levels of the MRP/

Table 1. Cox regression analysis of factors prognostic for outcome in serous ovarian cancer*

Factor†	Progression-free survival		Overall survival	
	Relative hazard (95% confidence interval)	P‡	Relative hazard (95% confidence interval)	P‡
Overall cohort (n = 143) Univariate analysis				
High *ABCA6* expression	1.87 (1.29 to 2.72)	.001	2.0 (1.28 to 3.14)	.002
High *ABCA8* expression	1.37 (0.95 to 1.97)	.09	1.66 (1.07 to 2.56)	.02
High *ABCA9* expression	1.55 (1.07 to 2.2)	.02	1.8 (1.20 to 2.80)	.009
Low *ABCC11* expression	1.56 (1.08 to 2.27)	.02	1.43 (0.93 to 2.22)	.11
Multivariable models				
1) High *ABCA6* expression	1.61 (1.12 to 2.32)	.01	1.74 (1.11 to 2.73)	.02
Advanced tumor stage	4.22 (1.69 to 10.53)	.002	8.33 (1.13 to 58.82)	.04
Residual disease >1cm	2.09 (1.44 to 3.13)	.0001	2.26 (1.45 to 3.52)	.0003
2) High *ABCA9* expression	1.43 (0.99 to 2.06)	.06	1.63 (1.04 to 2.56)	.03
Advanced tumor stage	4.37 (1.75 to 10.87)	.002	8.85 (1.22 to 62.5)	.03
Residual disease >1cm	2.03 (1.39 to 2.97)	.0002	2.18 (1.40 to 3.39)	.0006
Patients with ≤1 cm residual disease (n = 89)				
Univariate analysis				
High *ABCA1* expression	1.45 (0.89 to 2.36)	.13	1.95 (1.04 to 3.70)	.04
Low *ABCA5* expression	1.20 (0.75 to 1.97)	.44	1.88 (1.01 to 3.51)	.04
High *ABCA6* expression	2.09 (1.27 to 3.44)	.003	2.14 (1.15 to 4.00)	.02
High *ABCA8* expression	1.49 (0.91 to 2.42)	.11	2.14 (1.15 to 3.97)	.02
High *ABCA9* expression	1.93 (1.18 to 3.14)	.009	2.36 (1.25 to 4.45)	.008
Multivariable models: overall survival				
1) High *ABCA1* expression	1.82 (0.95 to 3.49)	.07		
Low *ABCA5* expression	1.96 (1.05 to 3.69)	.04		
High *ABCA6* expression	1.72 (0.91 to 3.28)	.10		
Advanced tumor stage	9.52 (1.30 to 71.4)	.03		
2) High *ABCA1* expression	2.10 (1.11 to 3.96)	.02		
Low *ABCA5* expression	2.04 (1.08 to 3.82)	.03		
High *ABCA8* expression	2.28 (1.22 to 4.26)	.01		
Advanced tumor stage	11.36 (1.55 to 83.3)	.02		
3) High *ABCA1* expression	1.94 (1.04 to 3.70)	.04		
Low *ABCA5* expression	2.14 (1.14 to 4.03)	.02		
High *ABCA9* expression	2.31 (1.21 to 4.40)	.01		
Advanced tumor stage	9.26 (1.29 to 71.4)	.03		

* Relative hazards were calculated as the antilogs of the regression coefficients in the proportional hazards regression. Cox proportional hazards analysis indicated that stage and debulking status (defined as ≤ or >1 cm) were independent predictors of progression-free and overall survival (Supplementary Table 2, available online), and these factors were included in multivariable models. All statistical tests were two-sided.

† Expression dichotomized according to median expression of cohort.

‡ Genes with a statistically significant uncorrected *P* value (<.05) were subsequently validated in The Cancer Genome Atlas (Supplementary Table 5, available online).

ABCC subfamily member *ABCC11* were associated with shorter PFS (Table 1; Figure 1D). When these genes were examined in an independent dataset available through TCGA, high levels of *ABCA6* and *ABCA8* were associated with reduced PFS or OS (Figure 1, E and F; Supplementary Table 5, available online), whereas high *ABCA9* levels were associated with reduced PFS or OS in patients without macroscopic disease after surgery (Figure 1G; Supplementary Table 5, available online). Each of these associations remained statistically significant in multivariable analysis of TCGA data (Supplementary Table 5, available online). The association between low-level *ABCC11* and shorter PFS was not validated in this cohort.

The extent of residual tumor remaining after surgery is a critical determinant of clinical outcome (Table 1). To identify genes whose expression may be associated with progression in relatively favorable disease, analysis was carried out in AOCS patients with less than 1 cm of residual disease after surgery. In addition to *ABCA6*, *ABCA8*, and *ABCA9*, high expression of the cholesterol transporter, *ABCA1*, was statistically significantly associated with reduced OS in this group (Figure 1H; Table 1). Thus, the cumulative 5-year survival in patients expressing high levels of *ABCA1* was 42.2%, compared with 68% for those expressing low levels of *ABCA1* (log-rank *P* = .03). In contrast, low levels of *ABCA5* expression were associated with shorter OS (*P* = .04) (Figure 1I; Table 1). High *ABCA1* expression or low *ABCA5* expression was also associated with reduced overall survival after adjustment for tumor stage (Supplementary Table 6, available online). Validation in TCGA patients indicated that *ABCA1* gene expression was associated with PFS and OS (Figure 1J; Supplementary Table 5, available online), which remained unchanged upon adjustment for tumor stage and surgical debulking status (Supplementary Table 5, available online). Multivariable Cox proportional hazards modelling was carried out in the subset of patients with less than 1 cm of residual disease to establish which of these *ABCA* genes might have prognostic value independently of each other and of tumor stage. Stepwise inclusion of each of the five genes in the model indicated that expression of *ABCA1* and *ABCA5*, together with either *ABCA8* or *ABCA9*, but not *ABCA6*, was independently associated with OS (Table 1).
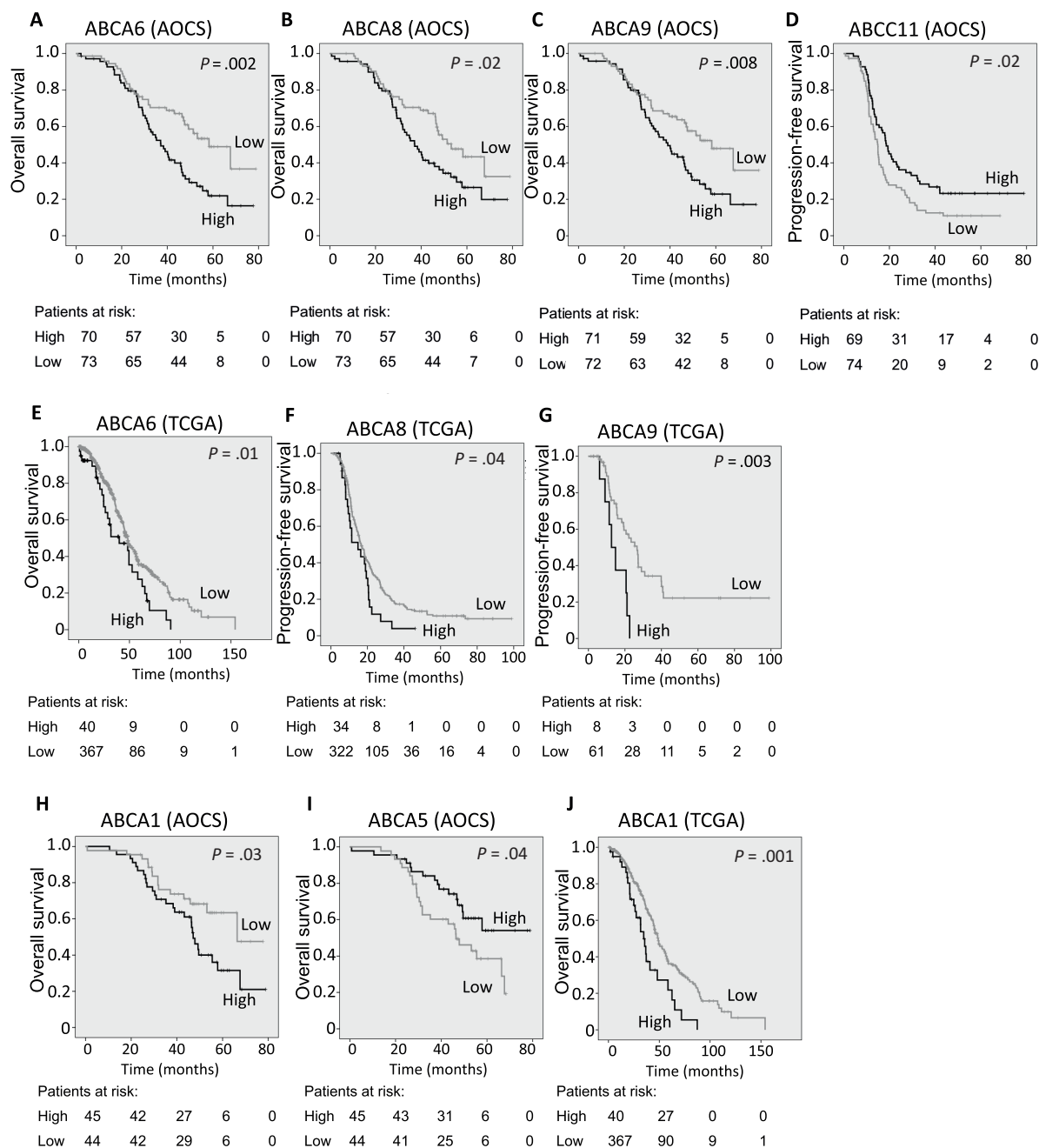
Figure 1. Kaplan–Meier survival analysis of ABCA gene expression in serous ovarian cancer. In the Australian Ovarian Cancer Study (AOCS) cohort, high expression of *ABCA6* (**A**), *ABCA8* (**B**) or *ABCA9* (**C**) or low expression of *ABCC11* (**D**) is associated with overall survival (OS) (**A**–**C**) or progression-free survival (PFS) (**D**) in high-grade serous ovarian cancer. In The Cancer Genome Atlas (TCGA) cohort, high expression of *ABCA6* (**E**) *ABCA8* (**F**), or *ABCA9* (**G**) is associated with OS (**E**) or PFS (**F** and **G**). In AOCS patients with 1 cm or less of residual disease, high expression of *ABCA1* (**H**) or low expression of *ABCA5* (**I**) is associated with reduced OS, as is high expression of *ABCA1* in the overall TCGA cohort (**J**). Kaplan–Meier curves were generated after dichotomization of gene expression to high or low expression with respect to the median for real-time quantitative PCR (RT-qPCR) data (**A**–**D**, **H**, **I**) or upper decile for microarray data (**E**–**G**, **J**) of expression among tumors, as described in the Methods. *P* values were calculated by two-sided log-rank tests.

Combined Expression of *ABCA* Genes and Clinical Outcome

Because expression of *ABCA1*, *ABCA5*, and either *ABCA8* or *ABCA9* was independently associated with survival when analyzed together in a multivariable model (Table 1), these were considered to be the most robustly associated genes and to account for the majority of the predictive value. Thus, the effect of combined *ABCA1*, *ABCA5*, and either *ABCA8* or *ABCA9* gene expression on patient outcome was examined using a similar approach to that reported previously (6). Tumors were divided according to eight possible combinations based on high or low expression of each set of three genes: for example, high expression of all three genes, high expression of two genes but low expression of the third, and so on. These eight combinations were compared with Kaplan–Meier curves, and clusters were recategorized into groups such that curves not statistically significantly
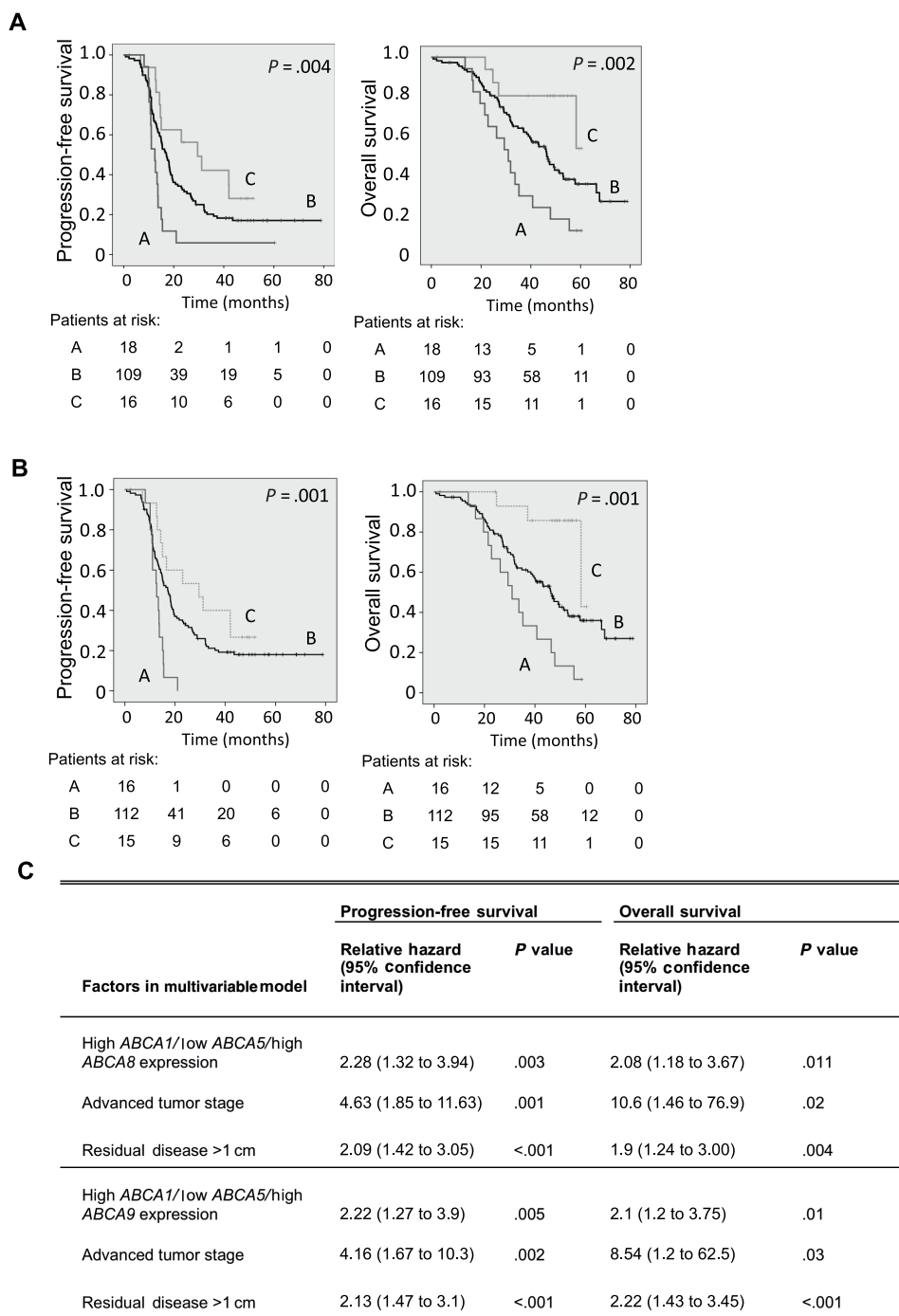
Figure 2. Survival analysis by combined expression of the *ABCA1*, *ABCA5*, and *ABCA8* or *ABCA9* genes in Australian Ovarian Cancer Study (AOCS) patients. Patients were categorized into eight clusters on the basis of their combined *ABCA1*, *ABCA5*, and *ABCA8* or *ABCA9* expression pattern. Kaplan–Meier survival analysis of these clusters revealed three statistically distinct groupings (groups A, B, and C) that were associated with the risk of relapse associated with individual *ABCA* gene expression. **A**) Group A included only those patients whose tumors displayed high levels of *ABCA1* and *ABCA8* and low levels of *ABCA5*, reflecting unfavorable *ABCA* gene expression. Group C included only those patients whose tumors displayed low levels of *ABCA1* and *ABCA8* and high levels of *ABCA5*, reflecting favorable *ABCA* gene expression. Group B was comprised of all other patients. Kaplan–Meier survival graph shows progression-free and overall survival according to combined expression groupings in all patients. **B**) Group A included only those patients whose tumors displayed high levels of *ABCA1* and *ABCA9* and low levels of *ABCA5*. Group C included only those patients whose tumors displayed low levels of *ABCA1* and *ABCA9* and high levels of *ABCA5*. Group B was comprised of all other patients. Kaplan–Meier survival graph shows progression-free and overall survival according to combined expression groupings in all patients. *P* values for Kaplan–Meier curves in (**A**) and (**B**) were calculated according to two-sided log-rank tests. In each case, gene expression was classified as high or low with respect to the median. **C**) Multivariable Cox regression analysis of factors prognostic for outcome in serous ovarian cancers (all tumors). For the factor of combined ABCA gene expression, the comparison was made for group A vs all other patients. Relative hazards were calculated as the antilogs of the regression coefficients in the proportional hazards regression. Multivariable analysis was performed after inclusion of all listed prognostic factors into the Cox regression model.

different from one another were included in the same group. These statistical groupings were found to accurately reflect risk associated with *ABCA* gene expression. Thus, for the combination including *ABCA8*, group A was statistically distinct from all other groups and included only those patients whose tumors displayed high levels of both *ABCA1* and *ABCA8* and low levels of *ABCA5*, in each case reflecting unfavorable *ABCA* gene expression. This group had the shortest PFS and OS (mean PFS in months = 15.3, 95% CI = 9.7 to 20.8; mean OS in months = 33.1, 95% CI = 26.1 to 40.1 for all cases) (Figure 2A). In contrast, group C included patients whose tumors had a favorable *ABCA* gene expression (ie, low *ABCA1* and *ABCA8* and high *ABCA5* expression), and survival was longer (mean PFS in months = 30.9, 95% CI = 22.8 to 39.1, *P* = .004; mean OS in months = 52.8, 95% CI = 45.7 to 59.9, *P* = .002; vs group A) (Figure 2A). The remaining case patients (group B) had intermediate survival, and the six subgroups did not differ statistically from one another. The combined expression status of *ABCA1*, *ABCA5*, and *ABCA9* was similarly strongly predictive of outcome (mean PFS in months: group A = 12.9, 95% CI = 11.4 to 14.5, vs group C = 30.2, 95% CI = 21.9 to 38.5, *P* = .001; mean OS in months: group A = 33.2, 95% CI = 26.4 to 40.1, vs group C = 55.3, 95% CI = 49.8 to 60.8, *P* = .001) (Figure 2B). The combined expression levels of *ABCA1/ABCA5/ABCA8* or *ABCA1/ABCA5/ABCA9* were strongly associated with PFS and OS after adjusting for tumor stage and surgical debulking status (Figure 2C).

ABCA Transporter Gene SNPs and Clinical Outcome

From the discovery-stage GWAS (see Methods), we identified SNPs in *ABCA1*, *ABCA5*, *ABCA6*, and *ABCA8* genes associated with PFS. The leading SNPs for each gene were genotyped on 1151 case patients in the replication stage. The overall associations combining case patients from both discovery and replication stages are presented in Table 2. Of these, the *ABCA5* variant, rs536009, strongly associated with both PFS and OS in serous ovarian cancer (HR = 1.33, 95% CI = 1.14 to 1.55, *P* = 3.1e–4 for PFS; HR_{death} = 1.50, 95% CI = 1.26 to 1.79, *P* = 6.5e–6 for OS) (Table 2; Supplementary Figure 1, available online). Variant rs536009 would lead to a nonsynonymous amino acid change in ABCA5; however, because of strong linkage disequilibrium at this locus (Supplementary Figure 1C), it is unclear whether this variant is causal.

ABCA Gene Expression in Molecular Subtypes of EOC

Previously, Tothill (12) examined global gene expression profiles of 285 EOCs and found that the high-grade serous tumors (n = 215) clustered into four distinct groups based on similarities in their gene expression patterns, C1, C2, C4 and C5 (12), and these were subsequently verified in TCGA data (18). Expression data for *ABCA* genes in the Tothill dataset (12) indicated that *ABCA1*, *ABCA6*, and *ABCA8* were each more highly expressed in C1 tumors than in the other classes, and a similar trend was seen for *ABCA9* (Figure 3A; Supplementary Table 7, available online). In the TCGA dataset (n = 380 high-grade serous tumors) similarly we observed that *ABCA1*, *ABCA6*, *ABCA8*, and *ABCA9* were each more highly expressed in C1 tumors (Figure 3B; Supplementary Table 7, available online). Molecular classes within TCGA data were of sufficient size for survival analysis. Within the C1 tumors, high expression of *ABCA1* (mean OS in months: high *ABCA1* = 35.9, 95% CI = 24.0 to 47.9; vs low *ABCA1* = 61.6, 95% CI = 51.5 to 71.8; *P* = .003; *P* = .02 after Bonferroni correction) and high expression of *ABCA6* (mean OS in months: high *ABCA6* = 40.8, 95% CI = 32.7 to 48.9; vs low *ABCA6* = 64.4, 95% CI = 52.6 to 76.2; *P* = .009; *P* = .045 after Bonferroni correction) were associated with reduced OS (Figure 3C). Within C1 tumors, low expression of *ABCA5* (mean OS in months: low *ABCA5* = 36.8, 95% CI = 28.1 to 45.5; vs high *ABCA5* = 60.8, 95% CI = 50.7 to 71.0; *P* = .02) was associated with reduced OS, but this was not statistically significant after Bonferroni correction (*P* = .09).

The C1 tumor expression profile is characterized by markers of activated myofibroblasts, genes involved in extracellular matrix production, remodelling, and angiogenesis, and gene sets associated with poor prognosis in other studies (12,19). Therefore, we investigated *ABCA* gene expression in tumor vs infiltrating cells using data from Tothill and colleagues, who previously microdissected five C1 tumors and characterized the epithelial and stromal components by expression microarray (12). Each of these five *ABCA* genes was expressed in both epithelial and stromal cells, although expression of *ABCA6*, *ABCA8*, and *ABCA9* was biased toward the stromal cells (Supplementary Figure 2, available online). Immunohistochemical detection of ABCA1 protein in serous EOC tumor sections confirmed strong staining for ABCA1 protein in tumor cells in cases with high *ABCA1* mRNA levels by RT-qPCR, (Figure 4A). Furthermore, consistent with the observed associations between *ABCA1* mRNA expression and outcome, in an independent cohort of 91 serous EOC tumors, high-level staining of ABCA1 protein was associated with poor PFS and OS (mean PFS in months: high *ABCA1* = 21.9, 95% CI = 9.3 to 34.5, vs low *ABCA1* = 50.0, 95% CI = 33.1–66.9, *P* = .04; mean OS in months: high *ABCA1* = 36.0, 95% CI = 22.4 to 49.7, vs low *ABCA1* = 63.8, 95% CI = 48.5 to 79.2, *P* = .04) (Figure 4B).

Table 2. Overall association between ABCA single nucleotide polymorphisms with progression-free survival and overall survival in non-Hispanic white women with serous epithelial ovarian cancer (EOC) and in women with EOC of all histological subtypes*

			PFS				OS			
			Serous (n = 1000)		All EOC (n = 1348)		Serous (n = 1058)		All EOC (n = 1430)	
SNP	Gene	A1†	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
rs1331924	ABCA1	G	1.15 (0.97 to 1.36)	.11	1.15 (0.99 to 1.34)	.07	1.12 (0.92 to 1.37)	.26	1.08 (0.91 to 1.29)	.37
rs536009	ABCA5	C	1.33 (1.14 to 1.55)	3.1E−4	1.18 (1.03 to 1.35)	.02	1.50 (1.26 to 1.79)	6.5E−6	1.28 (1.10 to 1.49)	1.8E−3
rs6502005	ABCA6	C	1.04 (0.83 to 1.30)	.74	1.05 (0.85 to 1.29)	.65	1.10 (0.84 to 1.45)	.49	1.08 (0.84 to 1.40)	.54
rs7212686	ABCA8	C	1.09 (0.98 to 1.22)	.10	1.08 (0.98 to 1.19)	.12	1.10 (0.97 to 1.25)	.13	1.08 (0.97 to 1.21)	.17

* Hazard ratios (HR) were determined with Cox proportional hazards models. All statistical tests were two-sided. CI = confidence interval; OS = overall survival; PFS = progression-free survival.

† Reference allele of the single nucleotide polymorphism (SNP). The SNPs were selected as the leading SNP at each locus in the initial genome-wide association study.
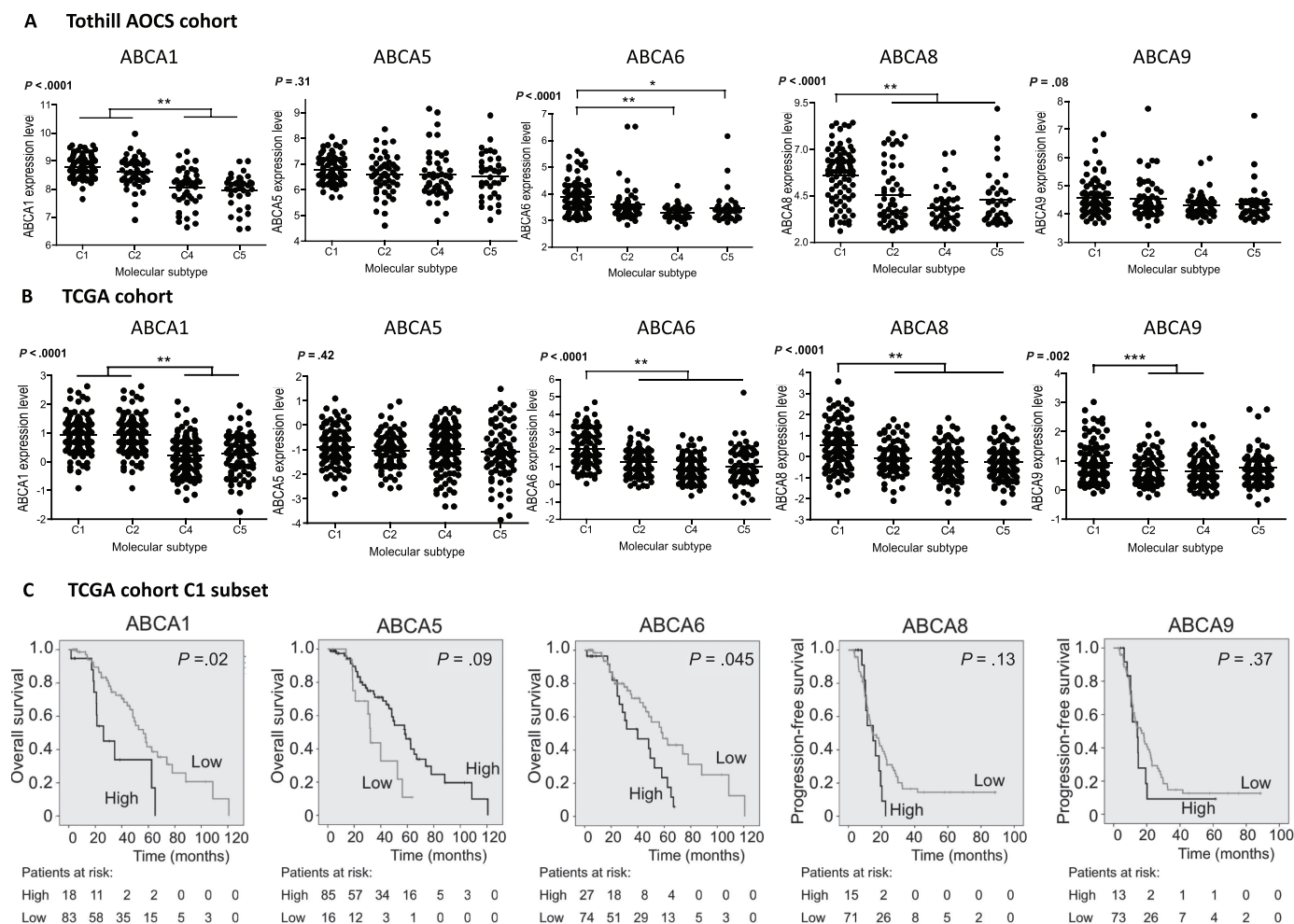
Figure 3. Expression of *ABCA1, ABCA5, ABCA6, ABCA8*, and *ABCA9* genes in serous carcinoma samples and clinical outcome according to molecular subtype classification. **A)** Expression data are derived from microarray-based gene expression profiling of 215 high-grade serous Australian Ovarian Cancer Study (AOCS) tumors (11). **B)** Expression data for 380 serous ovarian cancers were obtained through The Cancer Genome Atlas (TCGA) and were generated by University of North Carolina using the Agilent 244K Custom Gene Expression Array. *P* values displayed in (**A**) and (**B**) are from one-way analysis of variance.

Horizontal lines indicate mean values. **Asterisks** indicate statistically significant differences in mean expression between C1 type and other types after Bonferroni correction: *P = .01; **P < .001; ***P < .05. **C)** Kaplan–Meier survival analysis of ABCA gene expression in C1 molecular subtype of serous ovarian cancer (TCGA expression array data). *P* values were calculated according to two-sided log-rank tests and Bonferroni adjustment for multiple testing. Gene expression was classified as high or low with respect to the upper decile (*ABCA1, ABCA6, ABCA8, ABCA9*) or lower quartile (*ABCA5*) of expression.

ABCA1 Expression and Ovarian Cancer Cell Biology

In light of the high-level expression of *ABCA1* in tumors of aggressive phenotype and predominant expression in tumor rather than stromal cells, we investigated the role of ABCA1 in ovarian cancer biology. The 27/87 cells depleted of ABCA1 by siRNA transfection displayed reduced colony-forming ability (mean colony number ± standard deviation [SD]: ABCA1 = 26.04±7.86; Control = 60.04±10.69; P = .002, Student t test) (Figure 5A), and similar observations were made with A2780 cells (mean percentage of control ± SD = 58.2±7.76; P = .01, one-sample t test) (Figure 5B). Furthermore, in 27/87 cells, suppression of ABCA1 markedly reduced cell motility, as measured by closure of an artificial wound (mean area covered ± SD: ABCA1 = 60480±18371; control = 138500±14532; P = .004, Student t test) (Figure 5C). ABCA1 suppression also impaired migration of SKOV3 ovarian cancer cells (mean number of migrated cells ± SD: ABCA1 = 27.9±12.10; control = 74.46±13.87; P = .01, Student t test) (Figure 5D).

The importance of ABCA1 in cancer cell migration may be linked to its role in cholesterol homeostasis because cellular cholesterol and lipid content can influence morphology and mobility of the plasma membrane (20,21). In support of this possibility, when SKOV3 cells were exposed to subtoxic doses of the cholesterol-lowering drugs lovastatin or simvastatin, cell migratory ability was impaired (mean relative migration vs control ± SD: lovastatin = 0.64±0.018, P < .001, Student t test; simvastatin = 0.65 ± .15, P = .06, Student t test) (Figure 5E). Thus, in addition to their known effects on cancer cell survival, statins may be useful as inhibitors of ovarian cancer cell migration.

Discussion

This is the first study to examine the entire *ABC* transporter gene superfamily in epithelial ovarian cancer in relation to clinical outcome. Expression of *ABCA1, ABCA5, ABCA6, ABCA8*, and *ABCA9* were independently associated with outcome in two discrete
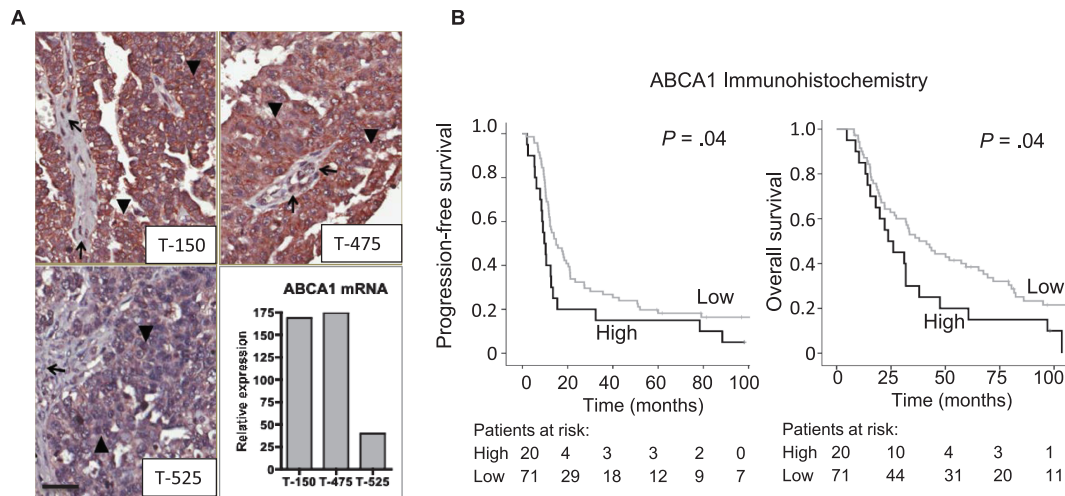
Figure 4. High ABCA1 protein levels in serous tumors are associated with poor outcome. **A**) Immunohistochemistry using ABCA1 antibody shows two tumors with intense staining in epithelial cells (**arrowheads**; **top images**) accompanied by weaker staining of stromal cells (**arrows**), whereas tumor 525 had low expression overall (**bottom left**). Scale bar = 50 μm. The highly stained tumors had relatively high expression by real-time quantitative PCR (RT-qPCR) (**bottom right**). **B**) Kaplan–Meier survival analysis of ABCA1 protein gene expression in 91 samples of a tumor tissue microarray. ABCA1 expression was classified as high or low according to intensity of staining, which was scored as described in the Supplementary Methods (available online). *P* values were calculated according to two-sided log-rank tests.
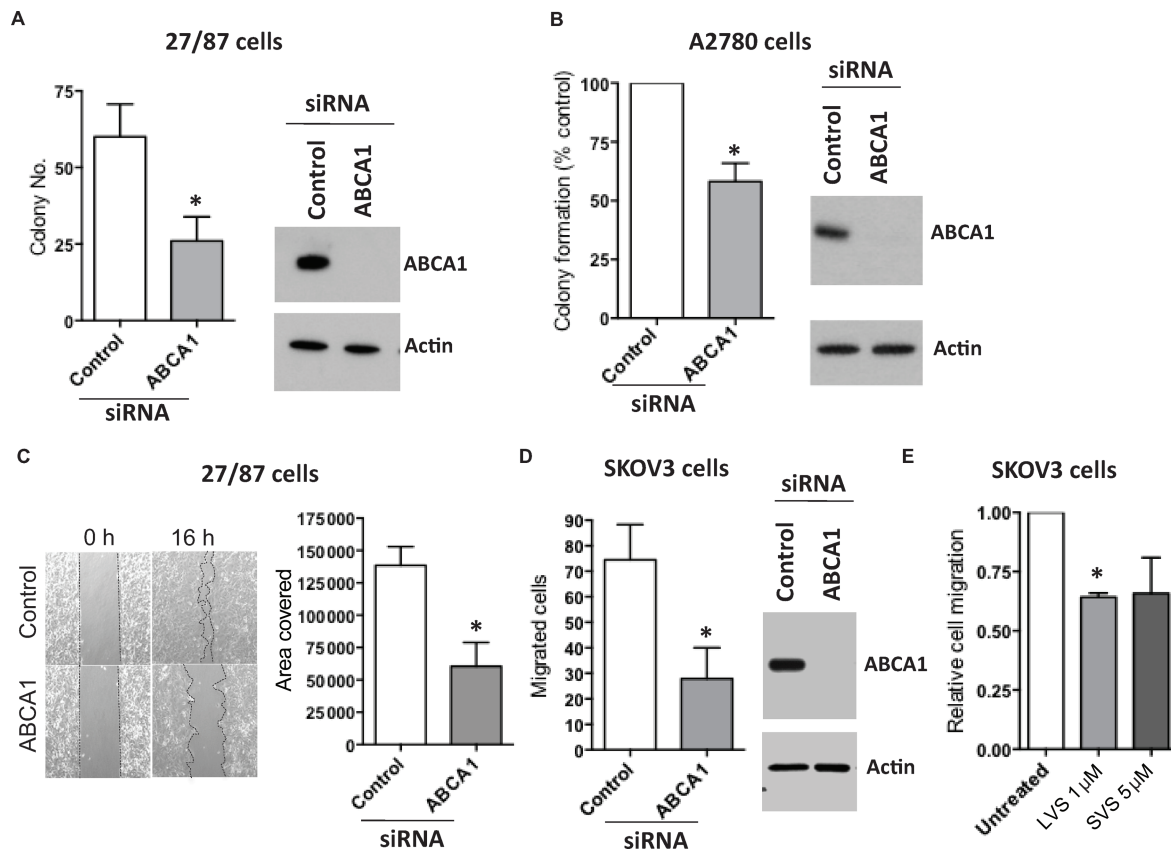
Figure 5. Impact of ABCA1 expression on epithelial ovarian cancer (EOC) cell growth and migratory capacity. **A**) Short interfering RNA (siRNA)–mediated suppression of ABCA1 in 27/87 ovarian cancer cells (5 nM siRNA; 48 hours after transfection) leads to growth inhibition, as measured by colony formation. *P* = .002. **B**) siRNA-mediated suppression of ABCA1 in A2780 ovarian cancer cells (5 nM siRNA; 48 hours after transfection) leads to growth inhibition, as measured by colony formation. *P* = .01. **C**) siRNA-mediated suppression of ABCA1 impairs wound closure ability in 27/87 cells. Measurement was taken at 16 hours after chamber removal and is expressed as area of wound covered (arbitrary units). *P* = .004. **D**) siRNA-mediated suppression of ABCA1 impairs cell migratory ability in SKOV3 cells. Forty-eight hours after siRNA transfection, cells were allowed to migrate across the Transwell membrane. *P* = .01. **E**) Inhibition of the cholesterol synthesis pathway impairs SKOV3 cell migration. Eighteen hours after treatment with lovastatin (LVS) or simvastatin (SVS), cells were allowed to migrate across the Transwell membrane. *P* < .001. For (**D**) and (**E**), the number of cells migrated in five fields was counted for each of three replicate wells and expressed as a percentage of control cells migrating. *P* values were derived from Student *t* test in parts (**A**), (**C**), and (**D**) and one-sample *t* test in parts (**B**) and (**E**). All data shown represent the mean of at least three independent experiments. Error bars represent standard deviations.

cohorts, and for ABCA1 this was further confirmed by immunohistochemical staining. Suppression of ABCA1 with siRNA altered the growth and migratory capacity of EOC cells, and we showed for the first time that statins inhibit ovarian cancer cell migration.

The A branch of the ABC superfamily comprises 12 functional transporters related by sequence homology and, at least for the most-well-studied members, by having discrete roles in cellular lipid trafficking (22). ABCA1 is the most intensively studied, and its normal role in the maintenance of cellular cholesterol homeostasis through transfer of phospholipids and cholesterol to ApoA1, leading to the formation of high-density lipoprotein, is well established (23). In cancer cells, the role of ABCA1 is unclear. An essential component of cellular membranes, cholesterol is rate-limiting for the rapid growth and division of tumor cells (24) and migration (20,21) and can influence cell–cell signaling and drug sensitivity (25,26). Curiously, inhibitory effects of mitochondrial ABCA1 expression on colon cancer cells were observed in the context of *Ras*-driven disease (27), although *Ras* mutation in ovarian cancer is predominantly associated with low-grade cases. Further experimentation should define the precise function for ABCA1 in high-grade EOC. Of relevance to EOC, omental adipose-derived lipids promote growth and metastases by providing energy to ovarian cancer cells (7). Investigating the importance of ABCA transporters and lipid balance in this cycle could highlight new therapeutic targets.

Several cancers are associated with faulty cholesterol metabolism (28), and a recent study of the Danish population linked statin use in cancer patients to reduced cancer-related mortality (29). In EOC, a retrospective study suggested statins are associated with improved outcome and elevated serum low-density lipoprotein cholesterol levels with poor prognosis (30). These findings concur with our observations that statin treatment or suppression of ABCA1 expression reduced EOC cell motility, and it would be prudent to examine association of metastatic disease with statin use in clinical studies.

The combined expression pattern of high *ABCA1*, low *ABCA5*, and high *ABCA8* or *ABCA9* identified tumors from patients with particularly poor outcome. A limitation of this finding is that, because of small patient numbers in the affected groups, we were unable to test for validation of this effect in the TCGA dataset. Further investigation of this relationship may require analysis of an independent cohort by RT-qPCR or similar quantitative measure.

Although the specific roles or substrates of ABCA5, ABCA6, ABCA8, and ABCA9 are largely unknown, their genes cluster on chromosome 17q24 (31,32), suggesting that they could be coregulated. However, *ABCA5* expression was independently associated with outcome after adjustment for the expression of *ABCA6*, *ABCA8*, or *ABCA9*, and we found no relationship between the SNP genotype and any ABCA gene expression in the dataset from TCGA (data not shown). Nevertheless, further fine-mapping at the *ABCA5* locus and investigation of the functional significance of rs536009 or other nearby SNPs (Supplementary Figure 1, available online), some of which lie in potential enhancer sequences, may shed light on regulation of this region in EOC.

High-level expression of *ABCA* genes was associated with ovarian cancers displaying an active stromal component and poor outcome (12), and *ABCA1*, *ABCA5*, and *ABCA6* levels were also linked to outcome within this class. The development of many solid tumors,

including those of the ovary, is facilitated by growth factors and other inflammatory mediators within the immediate tumor microenvironment (33). High-level expression of *ABCA* genes could contribute to the reactive stroma. For example, among the phospholipids transferred to high-density lipoprotein by ABCA1 is the signaling lipid, sphingosine-1-phosphate (S1P). High-density lipoprotein–derived S1P appears to account for much of the angiogenic activity associated with ovarian follicular fluid (34) and also promotes ovarian cell migration (35–37). Inhibition of S1P efflux could explain the observed inhibitory effects on cell growth and migration reported here after ABCA1 knockdown, and S1P efflux from tumor cells could also contribute to the desmoplastic tumor phenotype (38–40). Testing these hypotheses should be a major focus of future work in this area.

Given the potential for pharmacological modulation using novel or existing agents, establishing the influence of ABCA family transporter activity on the tumor microenvironment, as well as on EOC cells themselves, should be a priority for future study. Identification of the key transported substances could also provide a tool for detection or monitoring of disease.

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