The 16,121 genes that passed the filtering criteria were then considered for further analysis. The *t*-test ($p<0.001$) was used to identify the genes differentially expressed between NED and ED. Clustering for 50 identified genes used centered correlation and average linkage.

Both mRNA and DNA copy numbers were validated using real-time PCR in the 107 independent cases of EOC. PFS was calculated by the Kaplan-Meier method. Univariate and multivariate Cox's proportional hazard test was applied to identify variables associated with PFS. A p value of <0.05 was considered to be significant (SAS software ver. 9.1.3; SAS Institute Inc., Cary, NC).

Results

Clinical backgrounds of 140 EOC

The clinical backgrounds of the 33 aEOC samples used for array analysis are as follows: median age (54 years: range 33–80), stage (II:4, III:19, IV:10), histologic types (endometrioid: 4, serous: 22, undifferentiated: 7), histologic grade (1+2:9, 3:24), and operation status (optimal:14, suboptimal:19). The median follow-up period is 907 days (range: 292–2136 days), and 11 patients are alive without relapse.

The clinical backgrounds of 107 aEOC samples assayed for real-time PCR are as follows: median age (54 years: range 29–85), stage (II:12, III:74, IV:21), histologic types (endometrioid: 27, serous: 61, undifferentiated: 19), histologic grade (1+2:46, 3:61), operation status (optimal: 61, suboptimal: 46). The median follow-up period is 699 days (range: 92–2885 days) and 49 patients are alive without relapse.

Identification of 50 candidate disease progression-related genes by microarray analysis

To identify candidate disease progression-related genes from 54,675 transcripts, microarray analysis was performed on a training set of 33 samples. A total of 16,121 genes passed the filtering criteria and were further analyzed. Fifty genes were significantly correlated with disease progression, with a p value of <0.001 (Fig. 1A, Table 1). The chromosome distribution of the gene set analyzed and a selected subset are shown in Fig. 1B. Fifty selected genes on chromosome 8 and 20 show a higher than expected chromosomal distribution (Fig. 1B). Of these 50 genes, 6 are located at chromosome 8q24 and 9 (12 probes) at 20q11–13. The ratio of selected gene set/analyzed gene set in chromosome 20 is significantly higher than that in other chromosome regions (12/383 vs. 32/13656, $p=1.3\times10^{-16}$) and the selected gene set/analyzed gene set in chromosome 8 is significantly higher than that in other chromosome regions (6/606 vs. 32/13656, $p=0.01$). We speculate that the abnormal chromosomal distribution is due to genomic alteration and that these genes may play an important role in aEOC.

The results suggested that 8q24 and 20q11–13 loci are amplified in the ED group and that this might be related to treatment of aEOC.

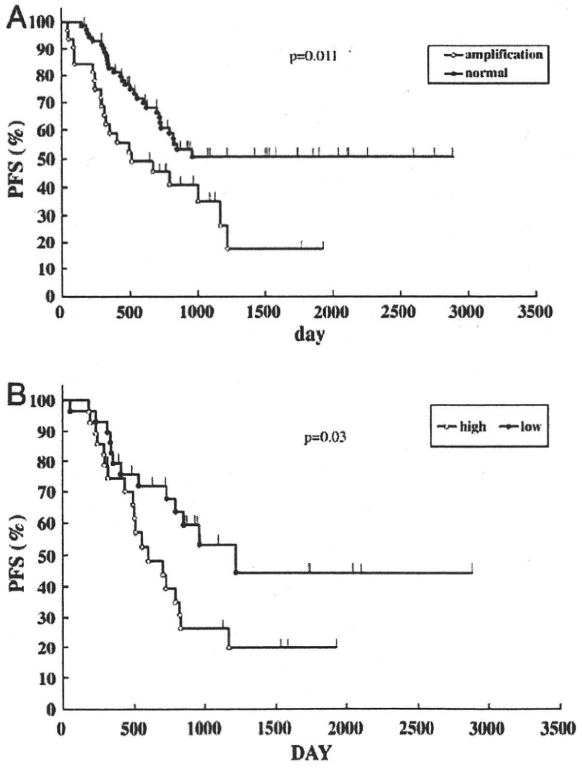

Fig. 2. The relationship between amplification and expression status of GNAS and PFS. (A) The relationship between copy number change of GNAS and PFS. (B) The relationship between mRNA expression of GNAS and PFS.

Table 2
Relationship between gene amplification and clinical factors.

Clinical Factor	GNAS	
	Frequency	*p* Value
Age		
Young	30%(16/53)	0.999
Old	30%(16/53)	
Grade		
1+2	33%(15/45)	0.689
3	28%(17/61)	
Operation		
Optimal	26%(16/61)	0.392
Suboptimal	36%(16/45)	

Relationship between GNAS gene amplification and PFS

DNA marker is thought to be more reliable and qualitative than RNA, because RNA expression changes according to the condition and environment of cancer cells. Therefore, we focused on detecting genomic alterations of chromosome 20q11–13 loci using real-time quantitative PCR on the 107 independent validation samples. The amplification of GNAS is significantly related to poor PFS ($p=0.011$) (Fig. 2A). GNAS shows gain of gene copy number rates of 30% (32/106), respectively. Correlations between amplification of GNAS and clinical factors are shown in Table 2. There are no significant associations between gene amplification at this loci and clinical factors such as age, histological grade, histologic type, and operation status. In addition, we performed multivariate Cox's proportional hazard test to identify variables including age, histologic grade, debulking status, histologic type, and GNAS copy number change associated with PFS. Finally, GNAS amplification was independent prognostic factor in aEOC (Table 3).

Table 3
Univariate and multivariate analysis of the effect of various prognostic factors on PFS.

Variable[a]	Univariate			Multivariate		
	Hazards Ratio[b]	95% CI	*p*	Hazards Ratio[b]	95% CI	*p*
GNAS	2.035	1.164–3.558	0.013	1.906	1.081–3.36	0.026
Age	0.975	0.563–1.688	0.927	0.765	0.434–1.348	0.354
Histologic grade	1.376	0.774–2.447	0.277	1.248	0.697–2.233	0.22
Debulking status	0.26	0.143–0.471	<0.001	0.256	0.139–0.493	<0.001

[a] GNAS: amplification vs. non-amplification, AGE: >median vs.<median, histologic grade: G3 vs. G1+2, debulking status: optimal vs. suboptimal.

[b] Hazard ratio refers to risk of survival, with values<1.0 indicating reduced risk. CI, confidence interval.

Relationship between GNAS gene expression and PFS

Of the 107 independent samples, RNA from 62 was available for real-time RT-PCR analysis. There are no significant associations between GNAS expression and clinical factors such as age, histological grade, and operation status. GNAS expression correlates significantly with PFS of aEOC ($p=0.03$) (Fig. 2B).

Discussion

The platinum/taxane regimen has improved the prognosis of EOC; however, this remains poor. For this reason, it is very important to find new prognostic markers for EOC receiving standard therapy, so that future clinical trials can be focused on patients with a poor prognosis. In this study, we used oligonucleotide microarrays to identify new prognostic biomarkers for aEOC excluding clear cell or mucinous types receiving standard therapy. In the past decade, several studies have analyzed chromosomal imbalances using comparative genomic hybridization (CGH) in EOC [10,17–20]. Arnold et al. investigated 47 malignant ovarian tumors and 2 ovarian tumors of low malignant potential using CGH and demonstrated that common genetic changes include DNA gains of chromosome arms 8q24 (51%) and 20q13.2-qter (40%) [10]. Iwabuchi et al. presented CGH data from 31 ovarian carcinomas and reported that increased copy numbers were most commonly observed in their cases at 3q26 (42%), 8q24 (35%), and 12q11.1–12 (25%) [17], while Sonoda et al. demonstrated that the most frequent sites of copy number increase were 8q24.1 (56%) and 20q13.2-qter (48%) in tumor DNA from 25 malignant ovarian carcinomas and 2 tumors of low malignant potential [18]. Tanner et al. focused on 20q12–q13 amplification in 24 sporadic, 3 familial and 4 hereditary ovarian carcinomas, and 8 ovarian cancer cell lines [19]. They demonstrated high-level amplification of at least one of the five non-syntenic regions at 20q12–q13.2 in 13 sporadic (54%) and in all four hereditary tumors [19]. Hu et al. focused on ovarian serous carcinomas and demonstrated DNA copy number gain at 8q22q24 and 20q12q13 in 60% and 45% of samples, respectively [20] In our study, amplification rates of GNAS at 20q13 is 30%, respectively.

Furthermore, Tanner et al. showed a tendency toward correlation between amplification and poor survival (not significant) and Hu et al. reported that 20q12q13 amplification may indicate a high risk for recurrence of serous ovarian cancer [19,20]. These reports included various histologic types and stages and even cell lines as well as primary and recurrent cases. Furthermore, they provide no information on therapy or operation status, include small number of patients, and do not perform validation assays. As it is well established that patients with ovarian carcinoma of different histologic types vary in their response to chemotherapy [8,9], it is important to take this into account in testing new biomarkers for their utility in clinical practice. In this study therefore, we focused on patients receiving standard therapy, excluding those with mucinous and clear cell tumors, and performed microarray analysis in 33 patients and validation assays in 107 patients.

Recent attempts to develop accurate predictors of clinical outcome in ovarian cancer have focused on techniques that are capable of assessing global gene status such as expression profiling and array CGH [4–7]. Birrer et al. performed oligonucleotide array CGH on 42 microdissected high-grade serous ovarian tumors and reported that amplification at 5q31–5q35.3 exhibited the strongest correlation with overall survival, identifying FGF-1 on 5q31 as a prognostic marker in 81 independent samples [7]. These data are not in agreement with our own. However, Birrer et al.'s report provides no information about the chemotherapy used, so that we speculate that prognostic biomarkers may be dependent on the chemotherapeutic regimen. Spentzos et al. also reported expression profiles for EOC and established a Chemotherapy Response Profile (CRP) and Ovarian Cancer Prognostic Profile (OCPP) [4,6].

We selected GNAS gene based on the p value and fold change in array data and examined the amplification status of GNAS as prognostic marker of aEOC. GNAS gene amplification was an independent prognostic factor. The GNAS locus encodes the G (alpha) protein, which stimulates the formation of cyclic AMP (cAMP). The cAMP pathway mediates pleiotropic effects including regulation of apoptosis and proliferation [21–23] and different genotypes of the single nucleotide polymorphism (ANP) T393C in the GNAS gene predict the clinical outcome of urothelial carcinoma, sporadic colorectal cancer, renal cell carcinoma, and chronic lymphocytic leukemia [24–27]. However, the role of GNAS in EOC remains unclear.

In conclusion, we identified amplification of GNAS on 20q13 as markers of prognosis in patients with aEOC treated with standard therapy. Our finding identifies qualitative and reproducible biomarker to predict the PFS of aEOC.

Conflict of interest statement

All of the authors are aware of and agree to the content of the article and have no conflict interest.

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Gene Expression Profile for Predicting Survival in Advanced-Stage Serous Ovarian Cancer Across Two Independent Datasets

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Abstract

Background: Advanced-stage ovarian cancer patients are generally treated with platinum/taxane-based chemotherapy after primary debulking surgery. However, there is a wide range of outcomes for individual patients. Therefore, the clinicopathological factors alone are insufficient for predicting prognosis. Our aim is to identify a progression-free survival (PFS)-related molecular profile for predicting survival of patients with advanced-stage serous ovarian cancer.

Methodology/Principal Findings: Advanced-stage serous ovarian cancer tissues from 110 Japanese patients who underwent primary surgery and platinum/taxane-based chemotherapy were profiled using oligonucleotide microarrays. We selected 88 PFS-related genes by a univariate Cox model ($p<0.01$) and generated the prognostic index based on 88 PFS-related genes after adjustment of regression coefficients of the respective genes by ridge regression Cox model using 10-fold cross-validation. The prognostic index was independently associated with PFS time compared to other clinical factors in multivariate analysis [hazard ratio (HR), 3.72; 95% confidence interval (CI), 2.66–5.43; $p<0.0001$]. In an external dataset, multivariate analysis revealed that this prognostic index was significantly correlated with PFS time (HR, 1.54; 95% CI, 1.20–1.98; $p=0.0008$). Furthermore, the correlation between the prognostic index and overall survival time was confirmed in the two independent external datasets (log rank test, $p=0.0010$ and 0.0008).

Conclusions/Significance: The prognostic ability of our index based on the 88-gene expression profile in ridge regression Cox hazard model was shown to be independent of other clinical factors in predicting cancer prognosis across two distinct datasets. Further study will be necessary to improve predictive accuracy of the prognostic index toward clinical application for evaluation of the risk of recurrence in patients with advanced-stage serous ovarian cancer.

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Introduction

Patients with advanced-stage ovarian cancer generally undergo primary debulking surgery followed by platinum/taxane-based chemotherapy. Although postoperative introduction of taxane drug has improved the 5-year survival rate for advanced-stage ovarian cancer, patients with this cancer have a 5-year survival rate of only 30% [1–3]. Clinicopathological characteristics, such as debulking status after primary surgery, are clinically considered important indicators of prognosis [4,5]. However, recurrence after optimal debulking surgery occurs in some patients, while disease-free status after incomplete surgery is maintained in others. In fact, it has been reported that 34% of patients treated with optimal surgery and platinum-taxane combination chemotherapy for advanced-stage ovarian cancer recur within 12 months [4]. Therefore, these clinicopathological factors alone are insufficient for predicting prognosis and elucidating the pathological mechanisms of disease progression or recurrence. Molecular biology

approaches can be used to identify new prognosis-related profiles leading to elucidation of pathological issues of advanced-stage serous ovarian cancer.

Microarray technology has been developing very rapidly, and it has become relatively easy to analyze the expression levels of thousands of genes within cancer cells. Although many studies have reported the associations of gene expression profiles with prognoses in cancer patients [6–10], a limited number of such profiles are used in clinical settings. Microarray technology is clinically applied for predicting prognosis in breast cancer patients. MammaPrint ™ (Agendia BV, Amsterdam, the Netherlands) has been already put to practical use for the purpose. Meanwhile, there are no microarray kits for clinical diagnosis and management in patients with ovarian cancer yet.

Three studies have recently reported gene expression profiles that predict overall survival (OS) in ovarian cancer patients using microarray techniques [11–13]. These studies use a relative large sample size (n>80) for establishing a survival-related profile in a discovery phase of the experiment and an external independent dataset as the validation set to solve the problem that the number of the genomic variables examined is much larger than that of subjects. Thus, research on the overall survival-related profiles in ovarian cancer patients has progressed, whereas there are no extensive studies based on multicenter validation of gene expression profiles for prediction of disease progression or recurrence in patients with ovarian cancer [14–15]. Prediction of the risk of recurrence in patients with advanced-stage ovarian cancer receiving standard treatments (primary surgery+platinum/taxane-based chemotherapy) is more important with respect to optimization of clinical management [16].

We have recently reported that there are high similarities in gene expression between early-stage and a subset of advanced-stage serous ovarian cancer patients that have favorable prognoses, and two molecular subgroups among patients with advanced-stage serous ovarian cancer according to gene expression profiles reflecting tumor progression and prognosis [17]. In this study, we focused on progression-free survival (PFS) time in a larger number of patients only with advanced-stage serous ovarian cancer treated with platinum/taxane-based chemotherapy, and tried to identify PFS-related gene expression profile using a new survival analysis method: ridge regression Cox model [18]. We then assessed the correlation between our PFS-related genes expression profile and survival time in an external independent dataset of advanced-stage serous ovarian cancer.

Results

Clinical Characteristics

The clinical characteristics of 110 Japanese patients with advanced-stage serous ovarian cancer are summarized in Table 1. In the discovery set, 93 patients (84.5%) were diagnosed as the International Federation of Gynecology and Obstetrics (FIGO) stage III, and 17 patients (15.5%) as FIGO stage IV [19]. All patients received platinum/taxane-based chemotherapy after primary surgery. The median progression-free and overall survival times were 17 and 31 months, respectively.

On the other hand, we used a part of publicly available microarray data (GSE9891) as an external independent dataset (See Materials and Methods) [20]. The clinical characteristics of 87 patients with advanced-stage serous ovarian cancer in the external dataset are listed in Table S1 [20]. Kaplan-Meier survival analysis showed that there were no significant differences in PFS and OS time between patients of the discovery dataset and those of the external dataset (Figure S1). When we compared clinicopathological characteristics between the discovery set and the external dataset, there were significant differences in frequencies of stage (Table S1). Because grading system adopted in the external dataset was distinct from that in the discovery set [21–23], we could not make a simple comparison of malignant grade between the two datasets. Then we examined the association between clinicopathological features and PFS time in patients with advanced-stage serous ovarian cancer of each dataset. Multivariate analysis revealed that only optimal surgery was an independent prognostic factor for PFS in the discovery dataset (Table S2) and that there was marginally significant correlation between debulking status of primary surgery and PFS time in the external dataset (Table S2). Therefore, we planned first to develop a prognostic index based on PFS-related genes in the discovery dataset, secondarily to evaluate the prognostic ability of our index in the external dataset using multivariate analysis, and then thirdly to assess predictive performance of the prognostic index again after the stratification of patients according to the debulking status of primary surgery.

Table 1. Clinical characteristics of advanced-stage serous ovarian cancer patients.

	Present Dataset (n=110)	Percentage
Median age, years (range)	58 (23–85)	
Stage		
Stage III	93	84.5
Stage IV	17	15.5
CA125 (IU) (n=99)	1960±3519	
Optimal Cytoreduction		
Optimal (<1cm)	57	51.8
Not optimal	53	48.2
Grade		
Grade 1	26	23.6
Grade 2	41	37.3
Grade 3	43	39.1
Median survival time, months (range)	31 (1–81)	

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Identification of PFS-Related Profile

Using Agilent Whole Human Genome Oligo microarray, we generated gene expression data for 110 advanced-stage serous ovarian cancer patients. Then this dataset was used as a discovery set for the identification of PFS-related profile in patients with advanced-stage serous ovarian cancer. To further evaluate the PFS-related profile, we prepared a part of the GSE9891 dataset as an external independent dataset using Affymetrix Human Genome U133 Plus 2.0 Array (See Materials and Methods) [20]. To deal with cross-platform microarray data appropriately, we analyzed only common genes (28304 probes in Agilent platform; 38497 probes in Affymetrix platform) between the two platforms in this study. Of 28304 Agilent probes, 18178 probes with expression levels marked as "Present" in all of the 110 microarray data from the discovery set was further extracted to remove missing and uncertain signals on gene expression, and then the data were per-gene normalized in each dataset by transforming the expression of each gene to a mean of 0 and standard deviation of 1 (Figure S2).

A univariate Cox proportional hazard model showed that expression levels of 97 probes (representing 88 nonredundant genes) were correlated with PFS time ($p<0.01$). In case of multiple-tagged 8 genes (represented by 17 probes), we selected 8 probes (one probe per gene) with the largest sum of the squares of individual expression values for the respective genes as representatives [24]. A total of 88 genes (represented by 88 unique probes) were thereby identified as PFS-related profile. Furthermore, we applied the ridge regression model to estimate optimal regression coefficients (β) for 88 genes of the PFS-related profile (Table 2), and calculated the prognostic index for each sample using equation (1) as reported previously [18]. The 88-gene prognostic indices obtained were in the range of -5.09 to 4.14 (median, 0.11), and the frequency distribution of the indices among 110 patients was unimodal.

To assess the prognostic index as a categorical variable, we attempted to divide this dataset into two groups based on median prognostic index of 0.11 [9]. Patients were assigned to the "high-risk" group if their prognostic index was greater than or equal to the median value, whereas "low-risk" group was composed of cases with the prognostic indices that were less than the median. As shown in Figure 1A, patients with high-risk prognostic indices had shorter median PFS times than those belonging to low-risk group (median PFS, 12 months vs. 51 months; log rank test, $p<0.0001$).

We then performed univariate and multivariate Cox proportional hazard analyses to prove that the 88-gene prognostic index was an independent prognostic factor (Table 3). A univariate Cox's proportional hazard analysis showed that the prognostic index, stage, optimal surgery, and histological grade were correlated with PFS ($p<0.0001$, $p=0.022$, $p<0.0001$ and $p=0.016$, respectively). Moreover, a multivariate analysis showed that the prognostic index was most significantly associated with PFS time [hazard ratio (HR), 3.80; 95% confidence interval (CI), 2.68–5.61; $p<0.0001$].

Validation by Quantitative Real-Time RT-PCR

To validate the microarray expression data, we performed quantitative real-time RT-PCR for a subset of the discovery dataset (53 samples). The four genes, *E2F2*, *FOXJ1*, *DNAH7*, and *FILIP1*, were randomly selected for this purpose. There were significant correlations between microarray expression data and real-time RT-PCR expression data (Figure 2). In spite of the smaller sample size, we confirmed a significant association between PFS time and each of the real-time RT-PCR data for the four genes in the univariate Cox hazard model (data not shown).

Appling PFS-Related Profile to the External Dataset

We translated the 88 prognostic genes with Agilent Probe IDs to Affymetrix 196 probes using a translation function in GeneSpring GX 10 and evaluated the present PFS-related profile in the external dataset (Figure S2). We calculated the prognostic index for each sample in the external dataset by the weighted sum of the expression values of 88 PFS-related genes according to the equation (1), in which the ridge regression coefficients (β) identified in the discovery set were used as weights for the respective genes (See Materials and Methods). We obtained prognostic indices ranging from -5.37 to 4.56 in the external dataset. The frequency distribution of the prognostic indices was not statistically different from that in the discovery set by Kolmogorov Smirnov test ($p>0.05$).

When we divided the external dataset into two subgroups by the median prognostic index (0.11) in the discovery set, a significant correlation was observed between risk classification and PFS (log rank test, $p=0.0004$) (Figure 1B). In univariate analysis of the external data, the estimated prognosis index and optimal surgery were correlated with PFS time ($p=0.0001$ and 0.049, respectively) (Table 3). Multivariate analysis showed that prognostic index was an independent prognostic factor for PFS time (HR, 1.64; 95% CI, 1.27–2.13, $p=0.0001$).

Assessment of Our Prognostic Index

To assess the sensitivity and specificity of our prognostic index, we used ROC curves for the index. An area under ROC curve of 0.5 (indicated by diagonal dotted lines in Figure S3) represents equality between true positive and false positive test results. The extent to which the ROC curve departs from the diagonal line to left and top axes is a measure of the effectiveness of the 88-gene prognostic index in the prediction of clinical outcome. The area under the ROC curves to distinguish early-relapse patients with less than 18 months of PFS times from late-relapse patients was 0.959 and 0.674 in the discovery set and the external dataset, respectively (Figure S3). When we used median value of prognostic index in the discovery set as the cut-off, the sensitivity and specificity were 88.9% and 85.7% in discovery dataset and 64.4% and 69.2% in the external dataset.

We performed survival analysis after the stratification of patients according to the status of debulking surgery which was an independent prognostic factor in multivariate analysis of the discovery dataset (Table 3). We divided patients into two groups ("optimal group" and "suboptimal group") in each of the discovery and external datasets, and assigned each patient to "high-risk" or "low-risk" based on the median value of the current prognostic index in each stratum according to the debulking status. Kaplan-Meier survival analysis showed that high-risk patients had significant shorter PFS time than low-risk patients in each of the four strata from the two datasets (Figure 3) as follows: optimal group ($p<0.0001$) and suboptimal group ($p<0.0001$) in our dataset; optimal group ($p=0.0034$) and suboptimal group ($p=0.015$) in the external dataset. This stratified analysis also indicated that the prognostic index was associated with PFS time independently of the debulking status.

Correlation between This Prognostic Index and Overall Survival

Overall survival is another important endpoint in patients with advance-stage ovarian cancers, and hence we further examined if the present 88-gene prognostic index could be extended to use for predicting the overall survival of patients. To evaluate correlation between this prognostic index and overall survival time, we performed Kaplan-Meier survival curve analysis. Patients with high-risk prognostic indices had shorter overall survival times than the low-risk patients in the two datasets (log rank test, $p<0.0001$ and $p=0.0010$, respectively) (Figure 1C, D). Furthermore, the prognostic index was significantly associated with overall survival time in both the discovery set and the external dataset in multivariate analysis (Table 4).

In addition, we examined the predictive ability of our prognostic index in publicly available Dressman's dataset [25], in which patients were longer followed-up (median overall survival, 31 months; range, 1–185 months). Dressman's dataset [25] was composed of 119 advanced-stage serous ovarian cancer patients treated with platinum-based chemotherapy (including non-taxane chemotherapy). Because their data were generated by a different platform with the foregoing two datasets, 75% of 88 PFS-related genes were translated for survival prediction in this dataset. When we divided Dressman's dataset [25] into two subgroups by the median prognostic index in discovery dataset, a significant association was observed between risk classification and overall survival (log rank test, $p=0.0008$) (Figure S4). Its prognostic index

Table 2. Eighty-eight genes composing the progression-free survival-related profile.

GenBank Acc.	GeneSymbol	Cytoband	β_{ridge}[a]	Description
NM_001123	*ADK*	10q22.2	0.006	adenosine kinase
NM_006408	*AGR2*	7p21.1	0.128	anterior gradient homolog 2 (Xenopus laevis)
NM_080429	*AQP10*	1q21.3	−0.162	aquaporin 10
NM_001040118	*ARAP1*	11q13.4	0.141	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1
NM_006420	*ARFGEF2*	20q13.13	0.032	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)
NM_181575	*AUP1*	2p13.1	0.129	ancient ubiquitous protein 1
NM_004776	*B4GALT5*	20q13.13	0.215	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5
NM_138639	*BCL2L12*	19q13.33	−0.189	BCL2-like 12 (proline rich)
NM_020643	*C11orf16*	11p15.4	0.221	chromosome 11 open reading frame 16
NM_145061	*C13orf3*	13q12.11	−0.107	chromosome 13 open reading frame 3
NM_024032	*C17orf53*	17q21.31	−0.184	chromosome 17 open reading frame 53
NM_001144956	*C1orf230*	1q21.3	0.012	chromosome 1 open reading frame 230
NM_022106	*C20orf177*	20q13.33	0.167	chromosome 20 open reading frame 177
NM_000715	*C4BPA*	1q32.2	−0.505	complement component 4 binding protein, alpha
NM_012337	*CCDC19*	1q23.2	−0.162	coiled-coil domain containing 19
NM_015603	*CCDC9*	19q13.32	0.263	coiled-coil domain containing 9
NM_005408	*CCL13*	17q12	−0.228	chemokine (C-C motif) ligand 13
NM_001252	*CD70*	19p13.3	−0.204	CD70 molecule
NM_078481	*CD97*	19p13.12	−0.137	CD97 molecule
NM_006383	*CIB2*	15q25.1	0.359	calcium and integrin binding family member 2
NM_182848	*CLDN10*	13q32.1	−0.292	claudin 10
NM_001316	*CSE1L*	20q13.13	−0.220	CSE1 chromosome segregation 1-like (yeast)
NM_024295	*DERL1*	8q24.13	0.007	Der1-like domain family, member 1
NM_001042517	*DIAPH3*	13q21.2	0.022	diaphanous homolog 3 (Drosophila)
NM_021120	*DLG3*	Xq13.1	−0.039	discs, large homolog 3 (Drosophila)
NM_020877	*DNAH2*	17p13.1	−0.378	dynein, axonemal, heavy chain 2
NM_018897	*DNAH7*	2q32.3	0.226	dynein, axonemal, heavy chain 7
NM_001394	*DUSP4*	8p21.1	0.007	dual specificity phosphatase 4
NM_004091	*E2F2*	1p36.12	0.220	E2F transcription factor 2
NM_006795	*EHD1*	11q13.1	0.248	EH-domain containing 1
NM_020819	*FAM135A*	6q13	0.142	family with sequence similarity 135, member A
NM_032181	*FAM176A*	2p12	−0.096	family with sequence similarity 176, member A
NM_015687	*FILIP1*	6q14.1	−0.188	filamin A interacting protein 1
NM_021784	*FOXA2*	20p11.21	0.184	forkhead box A2
NM_001454	*FOXJ1*	17q25.1	−0.344	forkhead box J1
NM_000819	*GART*	21q22.11	0.140	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase
NM_178172	*GPIHBP1*	8q24.3	0.147	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
NM_000189	*HK2*	2p13.1	−0.087	hexokinase 2
NM_002118	*HLA-DMB*	6p21.32	−0.288	major histocompatibility complex, class II, DM beta
NM_022465	*IKZF4*	12q13.2	−0.092	IKAROS family zinc finger 4 (Eos)
NM_016584	*IL23A*	12q13.2	0.493	interleukin 23, alpha subunit p19
NM_006801	*KDELR1*	19q13.32	−0.001	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1
NM_014895	*KIAA1009*	6q14.3	−0.150	KIAA1009
NM_017527	*LY6K*	8q24.3	0.226	lymphocyte antigen 6 complex, locus K
NM_005906	*MAK*	6p24.2	0.271	male germ cell-associated kinase
NM_024871	*MAP6D1*	3q27.1	−0.038	MAP6 domain containing 1
NM_031417	*MARK4*	19q13.32	0.040	MAP/microtubule affinity-regulating kinase 4
NM_024298	*MBOAT7*	19q13.42	−0.058	membrane bound O-acyltransferase domain containing 7
NM_002421	*MMP1*	11q22.2	−0.336	matrix metallopeptidase 1 (interstitial collagenase)
NM_181526	*MYL9*	20q11.23	0.058	myosin, light chain 9, regulatory

Table 2. Cont.

GenBank Acc.	GeneSymbol	Cytoband	β_{ridge}[a]	Description
NM_032344	*NUDT22*	11q13.1	0.198	nudix (nucleoside diphosphate linked moiety X)-type motif 22
NM_007224	*NXPH4*	12q13.3	−0.310	neurexophilin 4
NM_015311	*OBSL1*	2q35	−0.045	obscurin-like 1
NM_014982	*PCNX*	14q24.2	−0.098	pecanex homolog (Drosophila)
NM_014317	*PDSS1*	10p12.1	0.001	prenyl (decaprenyl) diphosphate synthase, subunit 1
NM_024420	*PLA2G4A*	1q31.1	0.107	phospholipase A2, group IVA (cytosolic, calcium-dependent)
NM_016341	*PLCE1*	10q23.33	0.029	phospholipase C, epsilon 1
NM_001031745	*RIBC1*	Xp11.22	0.209	RIB43A domain with coiled-coils 1
NM_015653	*RIBC2*	22q13.31	0.053	RIB43A domain with coiled-coils 2
NM_006987	*RPH3AL*	17p13.3	−0.043	rabphilin 3A-like (without C2 domains)
NM_001025070	*RPS14*	5q33.1	0.013	ribosomal protein S14
NM_152732	*RSPH9*	6p21.1	−0.102	radial spoke head 9 homolog (Chlamydomonas)
NM_014433	*RTDR1*	22q11.22	0.034	rhabdoid tumor deletion region gene 1
NM_005500	*SAE1*	19q13.32	0.038	SUMO1 activating enzyme subunit 1
NM_020150	*SAR1A*	10q22.1	0.277	SAR1 homolog A (S. cerevisiae)
NM_031469	*SH3BGRL2*	6q14.1	−0.281	SH3 domain binding glutamic acid-rich protein like 2
NM_003951	*SLC25A14*	Xq25	−0.344	solute carrier family 25 (mitochondrial carrier, brain), member 14
NM_014585	*SLC40A1*	2q32.2	0.065	solute carrier family 40 (iron-regulated transporter), member 1
NM_052910	*SLITRK1*	13q31.1	−0.314	SLIT and NTRK-like family, member 1
NM_172312	*SPAG8*	9p13.3	−0.123	sperm associated antigen 8
NM_145263	*SPATA18*	4q12	0.041	spermatogenesis associated 18 homolog (rat)
NM_006100	*ST3GAL6*	3q12.1	−0.192	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
NM_018414	*ST6GALNAC1*	17q25.1	−0.175	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3) -N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
NM_032872	*SYTL1*	1p36.11	−0.084	synaptotagmin-like 1
NM_014466	*TEKT2*	1p34.3	−0.226	tektin 2 (testicular)
NM_005424	*TIE1*	1p34.2	0.250	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
NM_198276	*TMEM17*	2p15	0.025	transmembrane protein 17
NM_199203	*TMEM189 -UBE2V1*	20q13.13	0.174	TMEM189-UBE2V1 readthrough transcript
NM_033550	*TP53RK*	20q13.12	0.054	TP53 regulating kinase
NM_139075	*TPCN2*	11q13.2	0.034	two pore segment channel 2
NM_018430	*TSNAXIP1*	16q22.1	0.170	translin-associated factor X interacting protein 1
NM_014023	*WDR37*	10p15.3	0.296	WD repeat domain 37
NM_018053	*XKR8*	1p35.3	0.106	XK, Kell blood group complex subunit-related family, member 8
NM_015896	*ZMYND10*	3p21.31	0.052	zinc finger, MYND-type containing 10
NM_005773	*ZNF256*	19q13.43	0.048	zinc finger protein 256
NM_024691	*ZNF419*	19q13.43	−0.042	zinc finger protein 419
NM_021089	*ZNF8*	19q13.43	0.093	zinc finger protein 8
NM_017975	*ZWILCH*	15q22.31	−0.074	Zwilch, kinetochore associated, homolog (Drosophila)

[a]A regression coefficient of each gene in ridge regression extension of multivariate Cox hazard model.

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was significantly correlated with overall survival time in multivariate analysis (HR, 1.51; 95% CI, 1.19–1.93, $p = 0.0008$).

Characterization of PFS-Related Profile

We conducted GO analysis to understand the biological characteristics of 88 PFS-related genes. To characterize the gene list based on GO classification on 'biological process', 'molecular function', and 'cellular component', we examined which categories were highly associated with the 88 genes. After multiple testing corrections using the FDR method [26], 8 categories were significantly overrepresented (FDR q-value<0.10) (Figure 4). In the 88 PFS-related genes, genes involved in GTPase binding (GO17016, GO31267 and GO51020), cellular localization (GO51649 and GO51641), intracellular transport (GO46907 and GO6886), and/or ciliary or flagellar motility (GO1539) were notably enriched. We investigated similarities in overrepresented GO categories between our 88 PFS-related genes and the previously reported gene expression profiles which were correlated to prognosis in ovarian cancer [11,13]. However, we could not identify common GO categories between our profile and the previously reported profiles (data not shown).

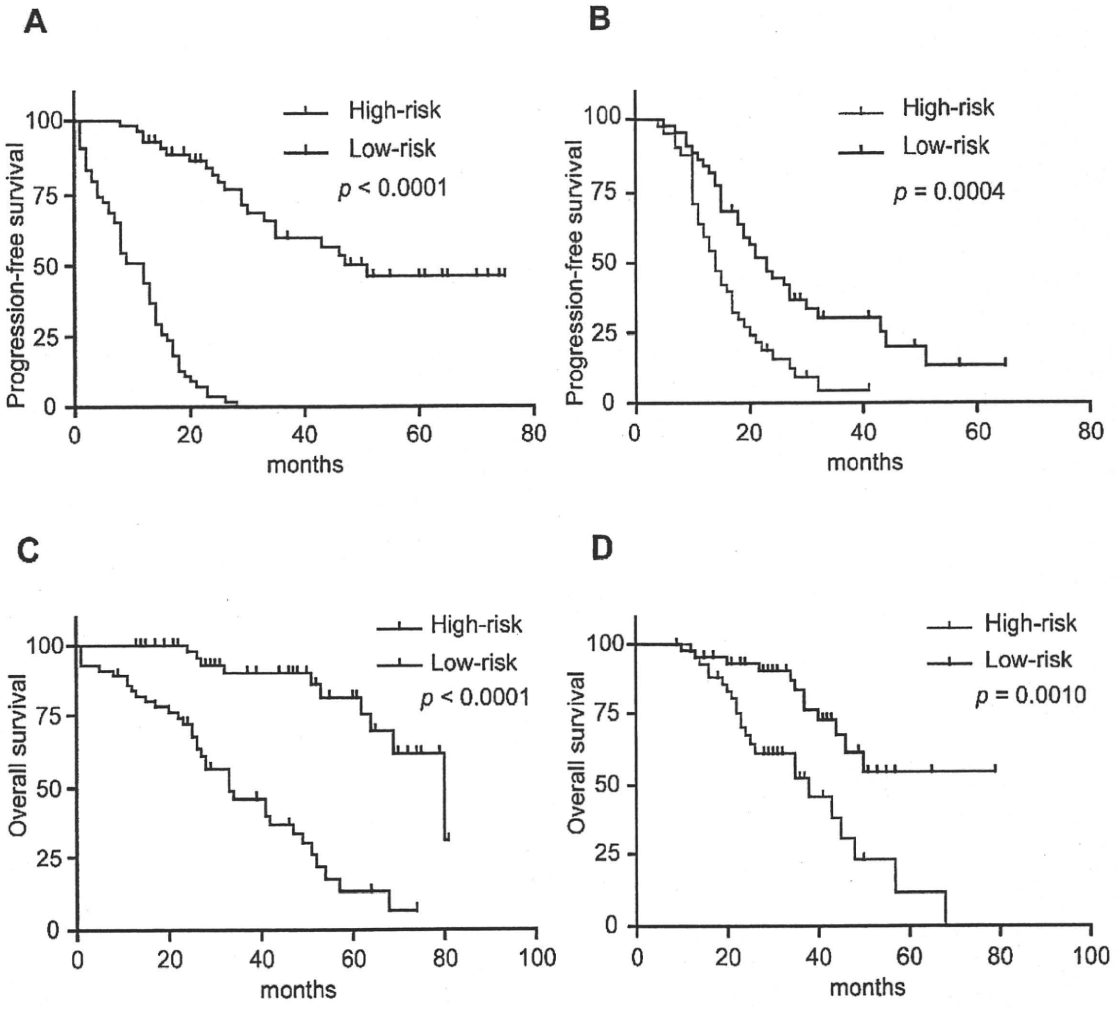

Figure 1. Prediction of prognosis in high-risk and low-risk patients based on the prognostic index. High-risk patients had significantly short progression-free survival times compared to low-risk patients (A) in the discovery set (log rank test, $p<0.0001$) and (B) in the external set (log rank test, $p=0.0004$). Similarly, high-risk patients had significantly shorter overall survival times compared to low-risk patients (C) in the discovery set (log rank test, $p<0.0001$) and (D) in the external set (log rank test, $p=0.0010$).
doi:10.1371/journal.pone.0009615.g001

We further used IPA software to analyze 88 PFS-related genes from the viewpoint of molecular interaction or pathway. Top three significant networks (score>25) are shown in Figures S5-7. The network 1 included 15 of the 88 prognostic genes, and was significantly associated with IPA-defined several networks: cell death, neurological disease, and cellular assembly and organization (Figure S5). Fourteen prognostic genes were included in the network 2, which was defined as networks related to cancer, cell morphology, and renal and urological disease (Figure S6). The network 3 displayed significant interactions and interrelations between genes involved in cell-to-cell signaling and interaction, hematological system development and function, and immune cell trafficking (Figure S7). In the 88 genes, we found several genes interacting with *SRC* or *MYC* (Figure S6), each of which was reported as a representative gene in oncogenic pathways of ovarian cancer [25,27].

Discussion

In this study, we identified the prognostic index for predicting PFS time in patients with advanced-stage serous ovarian cancer treated with platinum/taxane-based adjuvant chemotherapy across two types of microarray expression data from the present discovery set and publicly available external set by using the ridge regression Cox model. The significant correlation between our prognostic index and OS time was also indicated in the two independent datasets.

In expression microarray analysis, there is a so-called "curse of dimensionality" problem that the number of genes is much larger than the number of samples. To improve the reliability of a gene expression-based prognostic model, it is necessary to avoid overfitting to the dataset, and to confirm the reproducibility of the predictive ability in external independent datasets [28]. Until now, several bioinformatics approaches have been proposed to establish a model for survival prediction using microarray data [18,29]. Bøvelstad *et al.* [18] recently examined the prediction performance of the following seven methods: univariate selection, forward stepwise selection, principal components regression, supervised principal components regression, partial least squares regression, ridge regression and the lasso using three microarray datasets [Dutch breast cancer data (n = 295), diffuse large B-cell lymphoma data (n = 240), and Norway/Stanford breast cancer data (n = 115)] [7,30–32]. They concluded that the univariate Cox model alone was insufficient for predicting survival and that the ridge regression

Table 3. Univariate and multivariate Cox's proportional hazard model analysis of prognostic factors for progression-free survival.

	Univariate analysis		Multivariate analysis	
Prognostic factor	**Hazard ratio (95%CI*)**	***p*-value**	**Hazard ratio (95%CI)**	***p*-value**
A) Present study (n = 110)				
Age	0.99 (0.97–1.01)	0.41	1.00 (0.99–1.02)	0.68
Stage IV (vs Stage III)	1.40 (1.05–1.81)	0.022	0.93 (0.69–1.24)	0.65
Optimal Surgery (vs not optimal)	0.57 (0.45–0.72)	<0.0001	0.73 (0.56–0.94)	0.016
Grade				
Grade2 (vs Grade1)	1.21 (0.89–1.67)	0.23	1.08 (0.78–1.50)	0.66
Grade3 (vs Grade1)	1.44 (1.07–1.98)	0.016	1.34 (0.98–1.88)	0.065
Prognostic Index				
High (vs Low)	3.95 (2.85–5.74)	<0.0001	3.80 (2.68–5.61)	<0.0001
B) Tothill's dataset [20] (n = 87)				
Age	1.01 (0.98–1.03)	0.61	1.00 (0.98–1.03)	0.82
Stage IV (vs Stage III)	1.26 (0.51–2.28)	0.55	0.83 (0.33–1.55)	0.60
Optimal Surgery (vs not optimal)	0.78 (0.62–0.99)	0.049	0.76 (0.60–0.98)	0.035
Prognostic Index				
High (vs Low)	1.62 (1.26–2.09)	0.0001	1.64 (1.27–2.13)	0.0001

*CI denotes confidence interval.

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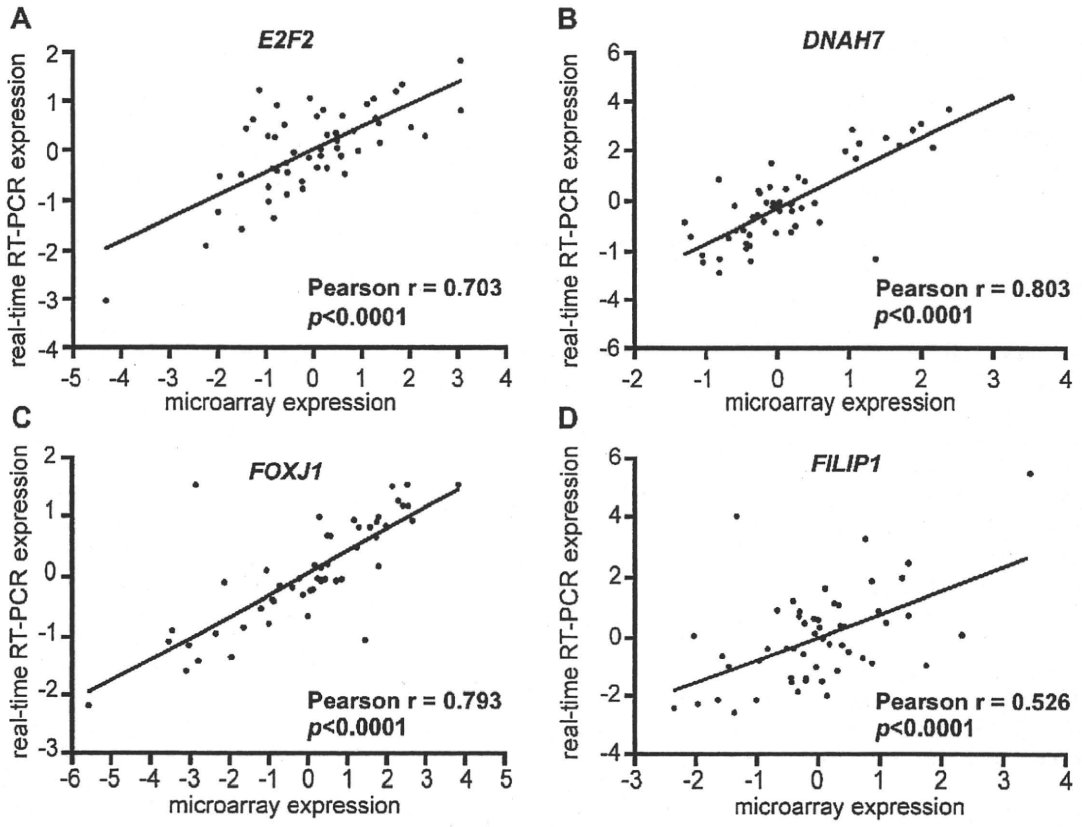

Figure 2. Validation of microarray expression data using quantitative real-time reverse transcript polymerase chain reaction (RT-PCR) analysis. There were significant correlations between microarray expression and real-time RT-PCR expression in (A) *E2F2*, (B) *DNAH7*, (C) *FOXJ1*, and (D) *FILIP1*.

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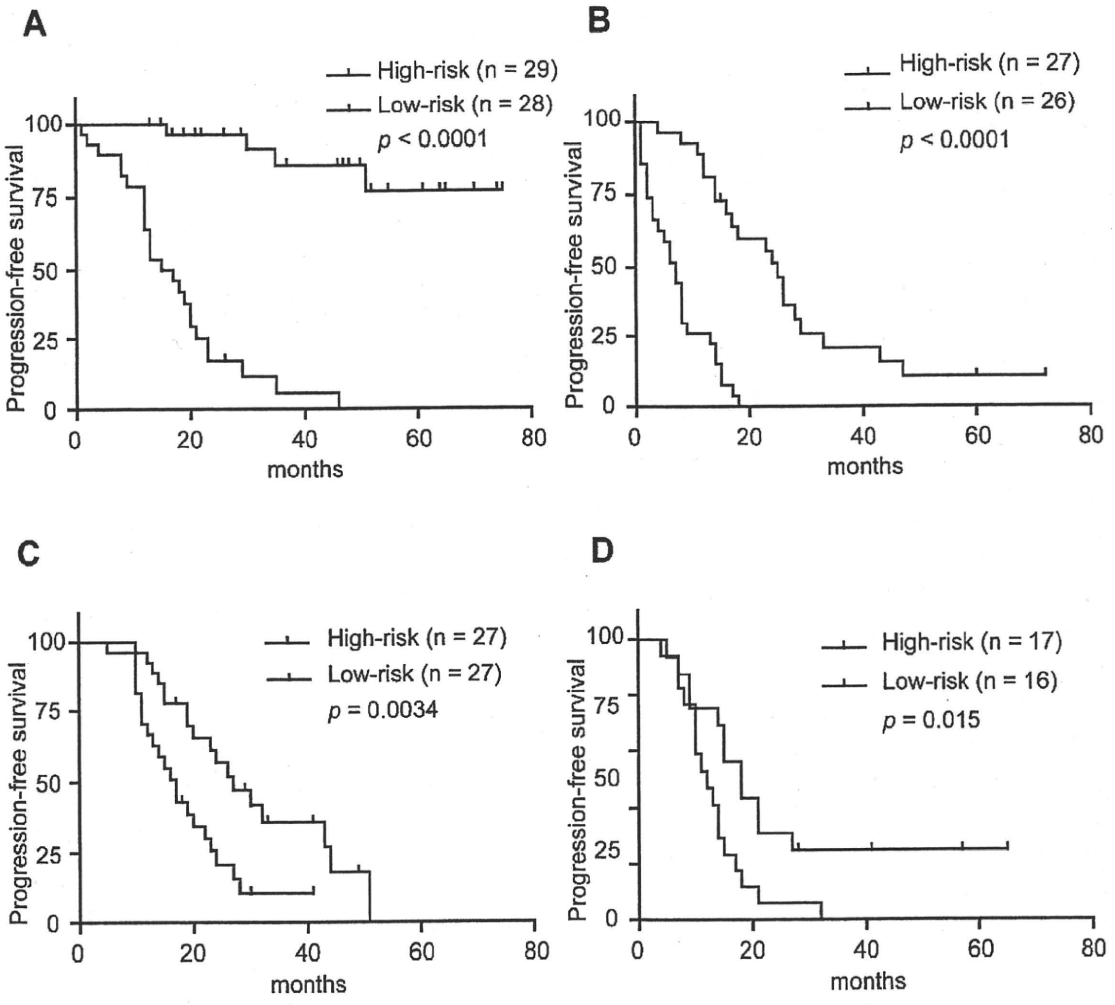

Figure 3. Prediction of prognosis in high-risk and low-risk patients based on the prognostic index after the stratification of patients according to the status of debulking surgery. High-risk patients had significantly short progression-free survival times compared to low-risk patients (A) in optimal (log rank test, $p<0.0001$) and (B) suboptimal group of discovery dataset (log rank test, $p<0.0001$). Similarly, high-risk patients had significantly shorter overall survival times compared to low-risk patients (C) in optimal (log rank test, $p=0.0034$) and (D) suboptimal group of the external dataset (log rank test, $p=0.015$).
doi:10.1371/journal.pone.0009615.g003

Cox model demonstrated the best performance in three datasets. Therefore, we used univariate Cox model only for selecting genes related to PFS time, and adjusted the regression coefficients by the ridge regression Cox model in order to increase the predictive performance of the prognostic index in our dataset.

The current study is intended to identify gene expression profile with a superior ability to predict prognosis than other clinicopathological factors. The stratification of patients with ovarian cancer according to clinicopathological prognostic factors is one of important analysis methods for the identification of highly accurate prognostic index [11]. After we stratified patients according to grade, FIGO stage, and status of debulking surgery, we investigated gene expression profile for predicting PFS time in stage III grade 2/3 serous ovarian cancer patients received optimal surgery or suboptimal surgery. However, we could find poorer predictive performance of the prognostic indices from the stratified analyses than that from the non-stratified analysis (Table S3). Besides the reduction of sample size in the discovery and external datasets after the stratification, a variety in clinical features and grading systems between the two datasets (Table S1) might influence the results from these stratified analyses. This is the main reason why we planned to identify prognostic index based on PFS-related genes in 110 advanced-stage serous ovarian cancers and then evaluate the significance of the prognostic index using multivariate analysis including grade, stage, and status of debulking surgery.

Although we enrolled ovarian cancer patients screened carefully by the following three categories: advanced-stage, histological serous-type, and platinum/taxane-based chemotherapy after primary surgery, we established no inclusion or exclusion criterion of histological grade for the enrollment as well as Crijns and colleagues did [12]. This is because a standard system for grading ovarian carcinomas is still under construction in the world, although several grading systems have been proposed for epithelial ovarian cancer [21–23,33,34]. According to the three criteria above, we recruited 110 Japanese ovarian cancer patients as a discovery set for the PFS analysis. The prognostic index for each patient was simply calculated by the ridge-regression-weighted sum of 88-gene expression values, and the prognostic power of our index was assessed using Tothill's dataset [20]. Further, subsequent stratified analysis according to debulking status, which was an independent prognostic factor in multivariate analysis of the discovery dataset, indicated that our prognostic index was associated with PFS time

Table 4. Univariate and multivariate Cox's proportional hazard model analysis of prognostic factors for overall survival.

	Univariate analysis		Multivariate analysis	
Prognostic factor	**Hazard ratio (95%CI*)**	***p*-value**	**Hazard ratio (95%CI)**	***p*-value**
A) Present study (n = 110)				
Age	1.01 (0.98–1.03)	0.56	-	-
Stage IV (vs Stage III)	1.14 (0.78–1.59)	0.49	0.75 (0.50–1.08)	0.12
Optimal Surgery (vs not optimal)	0.69 (0.50–0.92)	0.012	0.98 (0.70–1.35)	0.90
Grade				
Grade2 (vs Grade1)	1.30 (0.85–2.09)	0.23	1.23 (0.80–2.01)	0.35
Grade3 (vs Grade1)	1.68 (1.12–2.68)	0.012	1.83 (1.18–3.02)	0.0065
Prognostic Index				
High (vs Low)	2.72 (1.91–4.08)	<0.0001	2.99 (2.02–4.65)	<0.0001
B) Tothill's dataset [20] (n = 87)				
Age	1.01 (0.97–1.05)	0.73	1.00 (0.97–1.04)	0.88
Stage IV (vs Stage III)	2.13 (0.85–3.95)	0.093	1.60 (0.62–3.21)	0.28
Optimal Surgery (vs not optimal)	0.89 (0.62–1.23)	0.42	0.94 (0.66–1.37)	0.74
Prognostic Index				
High (vs Low)	1.76 (1.24–2.55)	0.0013	1.71 (1.20–2.49)	0.0029

*CI denotes confidence interval.

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independently of the debulking status. However, the sensitivity and specificity of the prognostic index for discriminating between early- and late-relapse patients were lower in Tothill's dataset than those in the discovery set. This might be caused by different backgrounds in respects of ethnicity or microarray platform. Although the differences in gene expression of cancer tissues among ethnicities have not been reported previously, several studies indicate that the proportions of clear cell and endometrioid histological types in

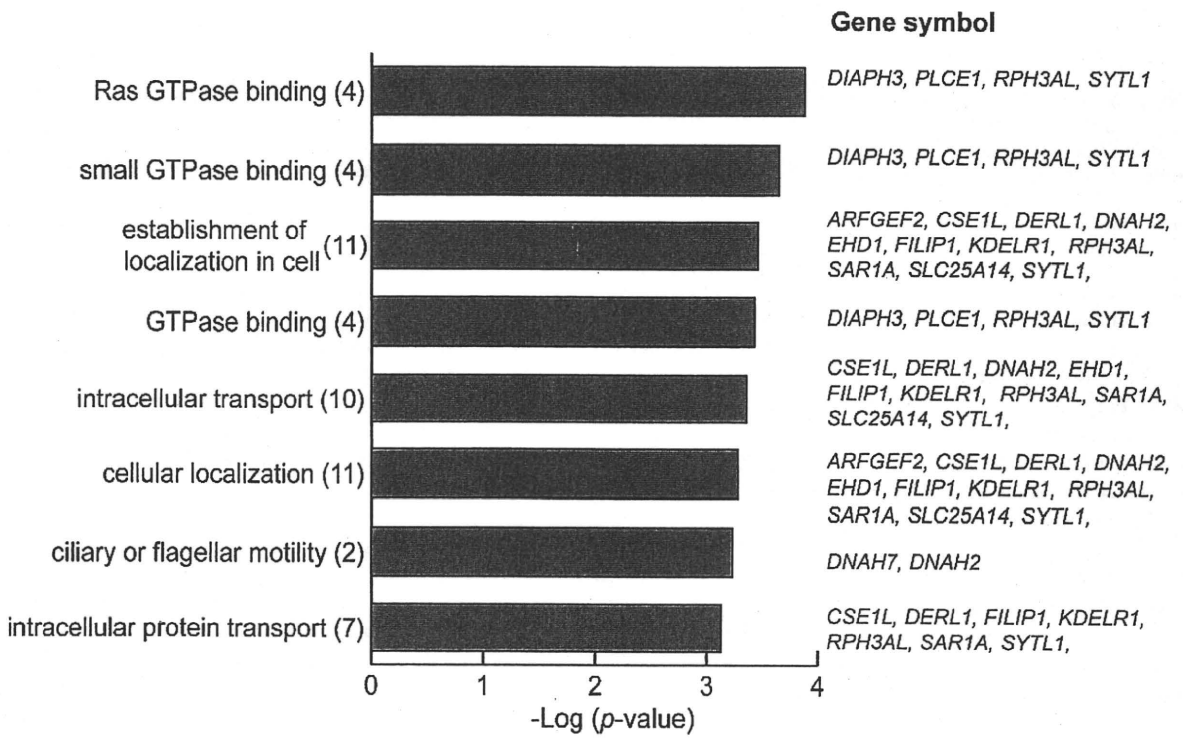

Figure 4. Biological characteristics of 88 progression-free survival-related genes. Significantly over-represented 8 gene ontology (GO) categories in GO-based profiling of 88 genes after multiple testing correction of the Benjamini–Hochberg false discovery rate method (FDR *q*-value<0.10). Over-represented GO categories were identified using all genes on Agilent platform as a background set of genes for the determining *p*-values. The actual number of the PFS-related genes involved in each category is given in parentheses.

doi:10.1371/journal.pone.0009615.g004

epithelial ovarian cancer in Asian population are higher than those in non-Asian populations [35,36]. Recent genome-wide association study has identified a single nucleotide polymorphism at 9p22 associated with ovarian cancer risk in subjects with European ancestry but not in non-European descendants [37]. This type of differences between studies could be also attributed to genetic as well as environmental factors. In addition, we cannot rule out the possibility that the present PFS-associated classifiers with ridge-regression-based weights still have insufficient generalization properties on the external dataset due to the problem of overfitting. Therefore, we will reconsider these important issues such as between-study differences in ethnicities and microarray platforms and the overfitting problem using a larger number of microarray data from advanced-stage serous ovarian cancer patients in order to obtain better classifiers for the prediction of prognosis. And to improve the accuracy of prognostic index, development of prognostic index after the stratification of patients will be a research agenda for further study.

Interestingly, the present 88-gene prognostic index for prediction of PFS time was also significantly associated with overall survival time in both our dataset and Tothill's dataset [20]. Moreover, we examined the predictive ability of our prognostic index in Dressman's dataset [25] since patients in their dataset received longer-term follow-up than those in the above two datasets. Although Dressman's dataset (n = 119) [25] included 34 patients treated with platinum/cyclophospamide chemotherapy and 3 with single-agent platinum, the significance of this prognostic index for overall survival was still statistically supported in the longer followed-up dataset. As treatments for recurrent ovarian cancer patients remain an open area of investigation aiming to lead to survival benefit [38], our prognostic index for patient with advanced-stage serous ovarian cancer displays a potential to predict not only PFS time but also overall survival time. In the future, we may apply the prognostic indices to estimation of risk of recurrence for serous ovarian cancer patients and select a novel treatment such as dose-dense chemotherapy [39] or molecular-targeted agent for the purpose of improving prognosis of high-risk patients.

There are small number of genes overlapped between our 88 PFS-related profile and previously reported expression-profiles that were related to prognosis or sensitivity of platinum/taxane-based chemotherapy [11–15,40,41]. Konstantinopoulos *et al.* [6] have discussed that these discrepancies might be related to the use of different microarray platforms with different normalization methods and different degree of contamination by noncancerous cells in a tumor sample, as well as differences in the patient populations under study. Nevertheless, several survival-associated genes such as *E2F2* and *HLA-DMB* [42,43] are included in 88 PFS-related genes. Reimer *et al.* [42] have reported that *E2F2* is associated with grade 3 ovarian tumors and residual disease (more than 2cm in diameter) after initial surgery, and that low *E2F2* expression is significantly associated with favorable disease-free and overall survival in epithelial ovarian cancer. Callahan *et al.* [43] have recently reported that the high expression of HLA-DMB in ovarian cancer cells is correlated with increased numbers of tumor-infiltrating CD8-positive T lymphocytes, and with good prognosis in advanced-stage high-grade serous ovarian cancer.

We performed GO analysis and IPA to assess biological characteristics of PFS-related genes. GO analysis revealed the significant associations of GTPase binding, intracellular transport, and ciliary or flagellar motility with PFS (Figure 4). *PLCE1* belongs to the GTPase binding category and activates MAP kinase or ERK as shown in IPA network 3 (Figure S7). In particular, previous report indicates that *PLCE1* activates the small G protein Ras/MAP kinase signaling [44], which is one of important pathways associated with cell growth and differentiation. Intriguingly, *CSE1L* included in the intracellular transport category is involved in the regulation of multiple cellular mechanisms, proliferation, and apoptosis [45]. Tanaka *et al.* [46] have reported that *CSE1L* is associated with regulated expression of p53 target genes, and that downregulation of *CSE1L* protects cancer cell from DNA damage-induced apoptosis. *DNAH2* and *DNAH7* are components of the inner dynein arm of cilialy axonemes, and axonemal dyneins are molecular motors that drive the beating of cilia and flagella. Plotnikova *et al.* [47] have reported that loss of cilia in cancer cells may contribute to the insensitivity of cancer cells to environmental repressive signals, partly owing to derangement of cell cycle checkpoints governed by cilia and centrosomes. On the other hand, IPA analysis showed several genes interacting with *SRC* or *MYC* (Figure S6), each of which was reported as a representative gene in oncogenic pathways of ovarian cancer [25,27]. Dressman *et al.* [25] have demonstrated that Src pathway activity is associated with chemotherapy response because of a significant correlation between the activation of Src pathway and poor prognosis in patients with platinum-resistant ovarian cancer. *MYC* is a multifunctional proto-oncogene and activated in about 30% of ovarian cancer by several mechanisms [48]. Iba *et al.* [49] report that MYC expression is associated with responsiveness to platinum-based chemotherapy and with prognosis in patients with epithelial ovarian cancer. Our PFS-related profile might have potentially functional relevance to altered activities of several oncogenic pathways. Although we identified several genes whose molecular function could be linked to prognosis in ovarian cancer patients, further functional study will be necessary to clarify the biological and pathological implications of the PFS-related profile.

These results suggest that the gene expression profile could be a useful tool to predict disease progression or recurrence of advanced-stage serous ovarian cancer. To apply the gene expression profile in clinical practice, we will need to improve the predictive ability of the profile and confirm the reliability of survival profile in a prospective multi-center study. Nevertheless, the survival-related profile could provide an optimization of the clinical management and development of new therapeutic strategies for the serous ovarian cancer patients.

Materials and Methods

Tissue Samples

One hundred ten Japanese patients who were diagnosed with advanced-stage serous ovarian cancer between July 1997 and June 2008 were included in this study. Fresh-frozen samples were obtained from primary tumor tissues during primary debulking surgery prior to chemotherapy. All patients with advanced-stage serous ovarian cancer were treated with platinum/taxane-based chemotherapy after surgery. In principle, patients were seen every 1 to 3 months for the first 2 years. Thereafter, follow-up visits had an interval of 3 to 6 months in the third to fifth year, and 6 to 12 months in the sixth to tenth year. At every follow-up visits, general physical and gynecologic examination were performed. CA125 serum levels were routinely determined. Staging of the disease was assessed according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) [19]. Optimal debulking surgery was defined as ≤1cm of gross residual disease. The histological characteristics of surgically resected specimens were assessed on formalin-fixed and paraffin-embedded hematoxylin and eosin sections by two or three gynecological pathologists belonging to the Japanese Society of Pathology at each institute,

and frozen tissues containing more than 80% of tumor cells upon histological evaluation were used for RNA extraction. In this study, the degree of histological differentiation is determined according to the increase in the proportion of solid growth within the adenocarcinoma as follows: grade 1, less than 5% solid growth; grade 2, 6-50% solid growth; grade 3, over 50% solid growth based on grading system proposed by Japan Society of Gynecologic Oncology.

PFS time was calculated as the interval from primary surgery to disease progression or recurrence. Based on standard Response Evaluation Criteria In Solid Tumors (RECIST) guidelines [50], disease progression was defined as at least 20% increase in the sum of the longest diameters of all target lesions or as the appearance of one or more new lesions and/or unequivocal progression existing non-target lesions. Overall survival time was calculated as the interval from primary surgery to the death due to ovarian cancer. This study was approved by the institutional ethics review board at Niigata University (No. 239, 282, 285, and 318), Niigata Cancer Center Hospital (No. 25), Jichi Medical University (G07-01), Kagoshima City Hospital (H19-21), Hiroshima University (Hi-11), Nagasaki University (080509), Kumamoto University (No. 309), and Tokai University (07I-29). All patients provided written informed consent for the collection of samples and subsequent analysis.

Microarray Experiments

Total RNA was extracted from tissue samples as previously described [17]. Five hundred nanograms of total RNA were converted into labeled cRNA with nucleotides coupled to a cyanine 3-CTP (Cy3) (PerkinElmer, Boston, MA, USA) using the Quick Amp Labeling Kit, one-color (Agilent Technologies). Cy3-labeled cRNA (1.65 μg) was hybridized for 17 hours at 65°C to an Agilent Whole Human Genome Oligo Microarray, which carries 60-mer probes to more than 40,000 human transcripts. The hybridized microarray was washed and then scanned in Cy3 channel with the Agilent DNA Microarray Scanner (model G2565AA). Signal intensity per spot was generated from the scanned image using Feature Extraction Software version 9.1 (Agilent Technologies) in the default settings. Spots that did not pass quality control procedures were flagged as "Absent". The MIAME-compliant microarray data were deposited into the Gene Expression Omnibus data repository (accession number GSE17260).

Microarray Data Analysis

We analyzed our dataset as a "discovery set" and the publicly available dataset as an "external dataset". Considering differences in microarray platforms, we selected common genes between the Agilent Whole Human Genome Oligo Microarray and Affymetrix Human Genome U133 Plus 2.0 Array, which was the platform in an external dataset (GSE9891) [20].

Data normalization was performed in GeneSpring GX 10 (Agilent Technologies) as follows: (i) Threshold raw signals were set to 1.0. (ii) 75th percentile normalization was chosen as normalized algorithm. (iii) Baseline was transformed to median of all samples. Furthermore, the expression level was normalized by Z-transformation (the mean expression was set to 0 and standard deviation to 1 for each gene in each dataset). In our dataset, 18,178 probes with expression levels marked as "Present" in all microarrays were used to remove missing and uncertain signals on gene expression.

The PFS-related genes from the 18,178 probes were identified by univariate Cox proportional hazard analysis, followed by a ridge regression, a penalized Cox regression analysis for survival prediction (Figure S2). We first identified 97 probes with expression levels correlating with the PFS time determined using the univariate Cox proportional hazard model ($p<0.01$). In case of multiple probes representing a given gene (so-called multiple tagged gene) in microarrays, only the probe with the largest magnitude (i.e., sum of the squares of per-individual expression values) was extracted as a representative probe for the gene [24]. To avoid the problem of overfitting, ridge regression extension of the multivariate Cox model was employed [18]. The ridge regression shrinks regression coefficients (β) of genes in multivariate Cox model by imposing a penalty on squared values of the coefficients, and is able to handle the problem of having larger number of expression values than individuals in an appropriate way [30]. We estimated regression coefficients of the prognostic genes by the ridge regression Cox model using M-files (available at http://www.med.uio.no/imb/stat/bmms/software/microsurv/) for MATLAB (Mathworks, Natick, MA, USA). Using 10-fold cross-validation, we obtained regression coefficients with optimal penalty parameter for the penalized Cox model, and calculated a prognostic index for each patient as defined by

$$\text{Prognostic index} = \sum_{i=1}^{88} \beta_i \times X_i \qquad (1)$$

where β_i is the estimated regression coefficient of each gene in discovery dataset under ridge regression multivariate Cox model and X_i is the Z-transformed expression value of each gene [18]. The estimated regression coefficient of each PFS-related gene given by ridge regression in the discovery set was also applied to calculate a prognostic index for each patient in external dataset using the equation above. We classified all patients into the two groups (high- and low-risk groups) by the median of the prognostic index in discovery set [9]. PFS between high- and low-risk groups was compared using Kaplan-Meier curves and the log rank test using GraphPad PRISM version 4.0 (GraphPad Software, San Diego, CA, USA). Furthermore, We then evaluated the prognostic index in the multivariate Cox proportional hazard model using JMP version 6 (SAS Institute, Cary, NC, USA). We also examined the discrimination performance of the prognostic index between early and late relapse in patients by plotting a receiver operating characteristic (ROC) curve for each dataset (JMP). Because 18 months is the median PFS time for advanced-stage ovarian cancer patients treated with cisplatin-paclitaxel [1], we used 18 months as the cut-off between early and late relapse. We performed ROC curve analysis for our prognostic index in only patients with follow-up for more than 18 months (Discovery set 103 samples; External dataset 84 samples).

To investigate the biological functions of PFS-related gene expression profiles, we used GO Ontology Browser, embedded in GeneSpring GX [17,51]. The GO Ontology Browser was used to analyze which categories of gene ontology were statistically overrepresented among the gene list obtained. Statistical significance was determined by Fisher's exact test, followed by multiple testing corrections by the Benjamini and Hochberg false discovery rate (FDR) method [26]. Furthermore, we tried to explore molecular interaction networks among the PFS-related genes using Ingenuity Pathway Analysis (IPA) [17].

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Real-time PCR was performed on *E2F2* (Hs00231667_m1, Applied Biosystems), *FOXJ1* (Hs00230964_m1, Applied Biosystems), *DNAH7* (Hs01022427_m1, Applied Biosystems), and *FILIP1* (Hs00325074_m1, Applied Biosystems) for a subset of serous

ovarian cancer (n = 53) as previously described [17]. The relative quantification method [52] was used to measure the amounts of the respective genes in serous ovarian cancer samples, normalized to *ACTB* (Hs99999903_m1, Applied Biosystems) and *TBP* (Hs99999910_m1, Applied Biosystems).

Evaluation of PFS-Related Genes in the External Dataset

To confirm whether our expression profile could predict prognosis of serous ovarian cancer patients in an independent data set, we selected to use publicly available microarray data (GSE9891) only because the data also disclosed individual clinical characteristics including PFS time. We examined clinical information of these dataset using supplementary data [20]. From this original dataset (n = 285), we selected 87 samples that were (i) diagnosed as advanced-stage serous adenocarcinoma, (ii) treated by platinum/taxane-based chemotherapy, (iii) obtained from primary lesion, and (iv) followed-up for more than 12 months (Table S1). Their samples are histologically graded by Silverberg classification [22] whose grading system is different from that in this study.

Supporting Information

Figure S1 Kaplan-Meier survival curves between 110 patients in this dataset and 87 in Tothill's dataset.
Found at: doi:10.1371/journal.pone.0009615.s001 (0.24 MB TIF)

Figure S2 Analytical process to develop a prognostic index for predicting survival.
Found at: doi:10.1371/journal.pone.0009615.s002 (0.48 MB TIF)

Figure S3 Assessment of the sensitivity and specificity of 88-gene prognostic index using receiver-operating characteristic (ROC) curves. When early relapse is positive in the analysis, the area under ROC curve to distinguish early-relapse patients with less than 18 months of progression-free survival times from late-relapse patients was 0.959 and 0.674 in (A) discovery set (early, n = 54; late, n = 49) and in (B) external set (early, n = 45; late, n = 39), respectively.
Found at: doi:10.1371/journal.pone.0009615.s003 (0.42 MB TIF)

Figure S4 Appling PFS-related gene expression profile to Dressman's dataset [25]. (A) Multivariate analysis showed a significant association of overall survival with the prognostic index estimated using the 88-gene linear combination model with the ridge regression coefficients from the present discovery set in Dresssman's dataset (HR, 1.51; 95% CI, 1.19–1.93, p = 0.0008) (B) Kaplan-Meier survival curves and the log rank test showed that high-risk patients had shorter overall survival compared to low-risk patients (median survival, 31 and 87 months for high- and low-risk patients, respectively; p = 0.0008).
Found at: doi:10.1371/journal.pone.0009615.s004 (0.23 MB TIF)

Figure S5 Molecular interaction networks of 88 progression-free survival-related genes using Ingenuity Pathway Analysis (IPA) software. The prognostic genes incorporated into the respective networks were marked as gray-colored.
Found at: doi:10.1371/journal.pone.0009615.s005 (2.42 MB TIF)

Figure S6 Molecular interaction networks of 88 progression-free survival-related genes using Ingenuity Pathway Analysis (IPA) software. The prognostic genes incorporated into the respective networks were marked as gray-colored.
Found at: doi:10.1371/journal.pone.0009615.s006 (1.68 MB TIF)

Figure S7 Molecular interaction networks of 88 progression-free survival-related genes using Ingenuity Pathway Analysis (IPA) software. The prognostic genes incorporated into the respective networks were marked as gray-colored.
Found at: doi:10.1371/journal.pone.0009615.s007 (1.82 MB TIF)

Table S1 Clinical characteristics of advanced-stage serous ovarian cancer patients in Tothill's dataset [20] (n = 87).
Found at: doi:10.1371/journal.pone.0009615.s008 (0.04 MB DOC)

Table S2 Univariate and multivariate Cox's proportional hazard model analysis of prognostic factors for progression-free survival.
Found at: doi:10.1371/journal.pone.0009615.s009 (0.04 MB DOC)

Table S3 Univariate Cox's proportional hazard model analysis of prognostic index for progression-free survival in the two datasets.
Found at: doi:10.1371/journal.pone.0009615.s010 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: KY AT TY II KT. Performed the experiments: KY AT. Analyzed the data: KY AT. Contributed reagents/materials/analysis tools: KY TY SK HF MS YO MH KS HF YK KK HM HT HK II KT. Wrote the paper: KY AT TY II KT.

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Less Impact of Adjuvant Chemotherapy for Stage I Clear Cell Carcinoma of the Ovary

A Retrospective Japan Clear Cell Carcinoma Study

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Introduction: Ovarian clear cell carcinoma (CCC) is regarded as grade 3 tumor, and the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines recommend adjuvant chemotherapy for the tumor even at stage IA. However, CCC often showed chemo-resistant phenotype, and the effect of adjuvant chemotherapy still remained uncertain.

Methods: Clear cell carcinoma cases treated at collaborating institutions during the period 1992–2005 were retrospectively identified. After a central pathological review, survival analysis was estimated by the Kaplan-Meier method, and prognostic factors were evaluated using a Cox regression model.

Results: Among 219 patients with stage I CCC, 195 patients received adjuvant chemotherapy (C+) and 24 patients (C−) did not. The C+ group had 77 pT1a and 118 pT1c cases, and the C− group included 18 pT1a and 6 pT1c tumors ($P < 0.001$). The median age was 52 years in the C+ group and 57 years in C− group ($P = 0.04$). During the median follow-up period of 48 months (range, 7–160 years), relapse was observed in one patient (4%) in the C− group and in 35 patients (18%) in the C+ group. There were no statistical differences of progression-free survival and overall survival between the C+ and the C− groups. Multivariate analysis revealed that peritoneal cytology status ($P = 0.02$) and pT status ($P = 0.04$) were independent prognostic factors for progression-free survival; however, adjuvant chemotherapy was not a prognostic factor ($P = 0.80$).

Conclusions: Although the present study was a limited retrospective investigation, it suggested that adjuvant chemotherapy had little impact on the survival of stage I CCC patients. Further strategy, such as a molecular targeting agent, is needed to improve survival of CCC, especially in cases with positive peritoneal washing.

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The authors declare no conflicts of interest.

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Ovarian carcinoma is the leading cause of death in all gynecologic malignancies in most developed countries.[1,2] In several histological subtypes, clear cell carcinoma (CCC) is comparatively resistant to anticancer drugs including conventional platinum-based chemotherapy[3–6] and paclitaxel-platinum regimen.[7,8] Low response to chemotherapy seemed to result in poor prognosis, especially in advanced cases. On the other hand, approximately half of CCC tumors were diagnosed at stage I tumor.[9] Surgical staging including lymphadenectomy was inevitable for the International Federation of Gynecology and Obstetrics staging system,[10] and there have been several reports regarding survival of stage I CCC patients.[11–15] Most of the studies indicated that the survival of stage I CCC was not inferior to that of serous subtype. In a large clinical trial comparing adjuvant platinum-based chemotherapy to observation in patients with early ovarian cancer, adjuvant effects were observed in serous subtype but not in the CCC subtype.[16] It still remains unclear whether postoperative adjuvant chemotherapy is clinically beneficial for patients with stage I CCC. The aim of the present study was to evaluate the effect of chemotherapy on the survival of CCC tumors confined to the ovary in a large retrospective multi-institutional analysis.

MATERIALS AND METHODS

Patients and Tumors

Between 1995 and 2005, a total of 219 patients with stage I clear cell carcinoma (CCC) of the ovary were identified by scanning the medical records of the collaborating institutions and a central pathological review by 2 independent pathologists with no knowledge of patients' clinical data. Tumors were diagnosed as CCC if typical clear or hobnail cells growing in a papillary, solid, or tubulocystic pattern are presented in more than 90% of all pathological specimens. The patients received initial treatment and follow-up at 9 institutions belonging to the Japan Clear Cell Carcinoma Study Group: National Defense Medical College Hospital, Tohoku University Hospital, Sapporo Medical University, Aichi Cancer Center Hospital, Fujita Health University Hospital, Osaka City General Hospital, Jichi Medical University Hospital, Tottori University Hospital, and Iwate Medical University Hospital.

One hundred forty-five patients underwent complete surgical staging procedures including hysterectomy, bilateral salpingo-oophorectomy, peritoneal washing, omentectomy, pelvic lymphadenectomy, and para-aortic lymphadenectomy. Pelvic lymphadenectomy was done from the common, external, and internal iliac nodes, and the obturator vessel to the femoral ring. Para-aortic lymphadenectomy was done from the bottom of the left renal vessel including the left infrarenal lymph nodes to the bifurcation of the aorta. Seventy-four patients underwent hysterectomy and bilateral salpingo-oophorectomy and only lymph node exploration or sampling. In all the cases, postoperative adjuvant chemotherapy was offered by physicians; however, the patients themselves determined whether they would receive chemotherapy or not. Among all the enrolled patients, 195 (C+) received post-adjuvant chemotherapy, and 24 (C−) received no additional chemotherapy. All the patients enrolled in the C− group refused to receive chemotherapy by their own decisions. The characteristics of the enrolled patients are shown in Table 1. There were disproportions of clinical variables between the C+ group and the C− group. Median age was significantly higher in the C− group ($P = 0.04$). There were more patients with pT1a ($P < 0.001$) and positive malignant washing/ascites ($P = 0.003$). The C+ group had 38 patients with stage IC disease (intraoperative rupture), 3 patients with IC (capsule involvement), and 77 patients with IC (ascites/malignant washing). The C− group included 4 patients with IC (intraoperative rupture) and 2 patients with IC (ascites/malignant washing). There were no significant differences of performance status and completion ratio of surgical staging between the 2 groups.

Chemotherapy

A total of 195 patients received 3 to 6 cycles of postoperative chemotherapy after an initial surgery. The regimen of the chemotherapy was as follows: paclitaxel and platinum in 82 patients, conventional platinum-based regimens in 59 patients, and combination with irinotecan and cisplatin in 54 patients. The paclitaxel-and-platinum regimen consisted of an infusion of 175–180 mg/m^2 paclitaxel and 50–75 mg/m^2 cisplatin or carboplatin (area under the curve = 5–6). Conventional platinum-based regimen included CAP (cyclophosphamide [500 mg/m^2], doxorubicin [50 mg/m^2], and cisplatin [50–70 mg/m^2], every 3–4 weeks) and CP (cyclophosphamide [500 mg/m^2] and cisplatin [50–70 mg/m^2], 3 times a day for 4weeks). The irinotecan and cisplatin regimen consisted of 50 to 60 mg/m^2 irinotecan hydrochloride on days 1, 8, and 15, and 50 to 60 mg/m^2 of cisplatin on day 1, every 4 weeks.

Progressive disease was defined as the appearance of a new lesion evaluated by computed tomography of the chest/abdomen or by pelvic magnetic resonance images. Serum levels of tumor markers including CA125 were not used for survival analysis in the present study. The time to progression was defined as the interval from the date of primary surgery until the date of onset of a progressive disease. Survival

TABLE 1. Characteristics of the patients with stage I ovarian clear cell carcinoma

	Adjuvant Chemotherapy		
	(+)	(−)	*P*
Total	195	24	
Age, yrs			
Median	52	57	0.04
Range	27–76	33–77	
Performance Status			0.54
0	164	19	
1, 2	31	5	
pT Status			<0.001
1a	77	18	
1c	118	6	
Surgical Staging			0.68
Complete	130	15	
Incomplete	65	9	
Ascites/Malignant Washing			0.003
Negative	118	22	
Positive	77	2	
Follow-up Period			
Median	48	43	0.11
Range	7–160	8–98	
Postoperative Chemotherapy			
Paclitaxel + platinum	82	0	
Conventional Platinum*	59	0	
CPT-P†	54	0	

*CAP, cyclophosphamide + doxorubicin + cisplatin; CP, cyclophosphamide + cisplatin.
†CPT-P, cisplatin + irinotecan hydrochloride.

duration was determined as the time from the date of primary surgery until death or the date of last follow-up contact.

Statistical Methods

The Kaplan-Meier method was used for calculation of patients' survival distribution. The significance of the survival distribution in each group was tested by the log rank test. The χ^2 test and the Student *t* test for unpaired data were used for statistical analysis. $P < 0.05$ was considered statistically significant. The Stat View software version 5.0 (SAS Institution Inc, Cary, NC) was used to analyze the data.

RESULTS

A total of 219 patients with stage I CCC were enrolled in the present study. Among them, 195 received adjuvant chemotherapy (C+), and 24 patients (C−) received no additional chemotherapeutic regimens after initial surgery. During the median follow-up period of 48 months (range, 7–160 months), relapse was observed in one patient (4%) in the C− group and 35 patients (18%) in the C+ group ($P = 0.09$). In the C+ group, recurrence was observed in 1 (1.3%) of 77 patients with stage IC disease, 11 (29%) of 38 patients with IC (intraoperative rupture), 2 (67%) of 3 patients with IC (capsule involvement), and 21 (27%) 77 patients with IC (ascites/malignant washing). On the other hand, recurrence was observed in one patient (1 [50%] of 2) with stage IC (ascites/malignant washing) case, and none (0%) of IC (intraoperative rupture; 0 of 4) and IA cases (0/18). There were no statistical differences of progression-free survival (PFS; $P = 0.14$) and overall survival (OS) between the C+ and the C− groups (Fig. 1).

Values of age, performance status, pT status, completion of surgical staging, ascites or peritoneal washing status, and chemotherapy were compared (Table 2). As a result, the peritoneal cytology status ($P = 0.02$) and the pT status ($P = 0.04$) were independent prognostic factors for PFS. However, adjuvant chemotherapy was not a prognostic factor for the PFS of the CCC patients. Other variables including adjuvant chemotherapy ($P = 0.8$) were not prognostic factors for PFS.

The PFS of the patients with malignant ascites/positive peritoneal washing was significantly worse than that of the patients with negative cytology ($P = 0.0002$; Fig. 2A), and pT1c CCC cases showed worse PFS compared with pT1a cases ($P = 0.0013$; Fig. 2B). In addition, the same variables described in Table 2 were analyzed for the OS of the CCC patients; however, there were no significant prognostic factors for OS (data not shown).

DISCUSSION

A large clinical trial named the Adjuvant Chemotherapy in Ovarian Neoplasm (ACTION) trial, the benefit from adjuvant chemotherapy seemed to be restricted to patients without optimal surgical staging.[17,18] The results implied that the patients with more risk of unappreciated residual disease,

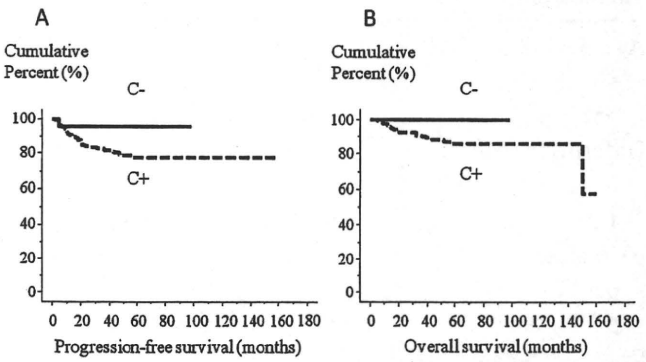

FIGURE 1. A, Progression-free survival of CCC patients who received adjuvant chemotherapy (C+) and those who did not (C−). Relapse was observed in one patient (4%) of the C− group and 35 patients (18%) of the C+ group ($P = 0.09$). Five-year PFS rate was 77% in the C+ group and 96% in the C− group. There was no significant difference of PFS between the C+ and the C− group ($P = 0.14$). B, Overall survival (OS) of CCC patients. Five-year OS rate was 86% in the C+ group and 100% in the C− group.

such as lymph node metastasis or peritoneal implantation, could benefit from adjuvant chemotherapy. A subanalysis of the study showed that disease-free survival of the chemotherapy group was significantly better than that of the observation group in serous adenocarcinoma; however, adjuvant effects were not observed in CCC subtype.[16] Less adjuvant effects might be explained by lower response rate of chemotherapy in the CCC subtype in comparison with the serous subtype.[3–8]

It has been estimated that the incidence of lymph node metastasis of stage I (pT1) ovarian cancer was approximately 20%.[19–21] A report demonstrated that serous tumors had a higher incidence of lymph node metastasis than nonserous tumors.[22] In contrast, the lymph node involvement of the CCC disease was up to 10% in pT1 tumors.[9] In addition to the lower incidence of the lymphatic involvement in CCC, it has been emphasized that the peritoneal cytological status was a strong prognostic factor for early-staged CCC, even after postoperative chemotherapy using multi-agents.[23] There have been several reports describing the impact of peritoneal cytology on survival of early-staged ovarian cancers. A report involving a small number of CCC patients showed no statistical significant difference between stage IC (preoperative rupture) and stage IC (intraoperative rupture).[24] Another report, which involved a higher ratio of CCC patients, revealed that stage IC (intraoperative rupture) patients showed significantly poorer survival than stage IA patients.[25] These studies with more CCC cases implied the importance of peritoneal cytology for the survival of CCC patients.

TABLE 2. Multiple regression analysis for the progression-free survival of patients with stage I ovarian clear cell carcinoma

Variables	Hazard Ratio	95% Confidence Interval	*P*
Age, yrs			0.99
<54	1		
>55	1.28	0.63–2.62	
Performance Status			0.80
0	1		
1,2	1.13	0.43–2.96	
pT Status			0.04
pT 1a	1		
pT 1c	4.67	1.06–20.4	
Complete Surgical Staging			0.95
Complete	1		
Incomplete	1.02	0.51–2.03	
Ascites/Malignant Washing			0.02
Negative	1		
Positive	2.34	1.16–4.72	
Chemotherapy			0.80
No	1		
Yes	1.30	0.16–10.4	

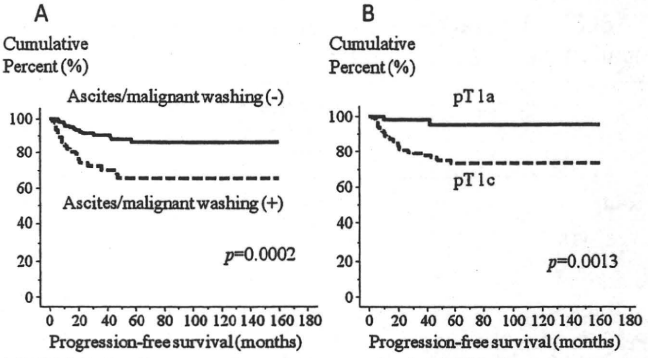

FIGURE 2. Progression-free survival of CCC patient according to peritoneal cytology status (A) and pT status (B). Progression-free survival of the patients with malignant ascites/positive peritoneal washing was significantly worse than that of the patients with negative cytology ($P = 0.0002$), and pT1c CCC cases showed significantly worse PFS compared with pT1a cases ($P = 0.0013$).

In the present study, which involves a large series of pT1 CCC tumors, the significant prognostic factors for PFS were malignant ascites and/or washing and pT status; postoperative chemotherapy could not be a prognostic factor. It is difficult to draw conclusions on the overall effects of all types of the chemotherapy in the management of early-staged CCC because a variety of chemotherapeutic regimens were used when the numbers not receiving chemotherapy was very low. However, the present results suggested that adjuvant chemotherapy had less impact on CCC tumors with pT1a and negative peritoneal cytology. In addition, a more effective therapy is needed to improve survival of CCC tumors with pT1c and positive peritoneal cytology.

CONCLUSIONS

Although the study was a limited retrospective study, it suggested that the postoperative adjuvant chemotherapy had little impact on stage I CCC tumors. Peritoneal cytology and pT status are more important prognostic factors than adjuvant chemotherapy for the PFS of CCC. Further analysis focusing on which subgroup of CCC patients would be candidates not to receive adjuvant chemotherapy is needed.

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