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Original Article

Prognostic value of PDCD-1 and CTLA-4 in ovarian cancer patients

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Abstract: Therapeutic effectiveness of treatments for ovarian cancer is not optimal. PDCD-1 and CTLA-4 offers the potential as a prognostic marker in addition to being a target for therapy. To assess the prognostic roles of PDCD-1 and CTLA-4 Gene in ovarian cancer, we utilized the Kaplan Meier plotter, a biomarker assessment tool with large quantities of data. The relationship between PDCD-1 and overall survival (OS) as well as CTLA-4 and OS were presented using Hazard Ratio, 95% CI and logrank *P* value. Then gene expression level was compared using H-Test and U test. The results were as follows: PDCD-1 and CTLA-4 gene expressions among 1582 ovarian cancer patients were shown with median gene expression value as the cut-off. Expression of PDCD-1 and CTLA-4 did not differ with regard to stages and P53 gene mutation. But the expression of CTLA-4 was higher in endometrioid than in serous cancer patients. Different grades of both PDCD-1 and CTLA-4 had different mean values. Higher expression of the PDCD-1 was not significantly correlated with better OS with HR 0.88 (95% CI: 0.77-1.01, P=0.061) but higher CTLA-4 was associated with better survival with HR 0.84 (95% CI: 0.73-0.96, P=0.0099) on the transcriptome level. In conclusion, lower expression of CTLA-4, but not PDCD-1 predicts worse survival.

Keywords: PDCD-1, CTLA-4, ovarian cancer, prognosis

Introduction

Ovarian cancer (OC) is a kind ofmalignant tumor of the female reproductive system. It is the most common cause of death in women with gynecological malignancy which is responsible for the highest mortality among US women [1, 2]. Despite extensive researches in this area, the therapeutic effectiveness of treatments for ovarian cancer is not optimal [3]. But recently, a large number of biomarker candidates have been suggested for predicting clinical outcomes in ovarian cancer patients and have shown the potential in the treatment of OC [1, 4-9].

Among them, the roles of PDCD-1 (Programmed cell death 1, also known as PD-1 and CD279) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, also known as CD152) are brought forward in tumor progression and suppression. The translational products of PDCD-1 and CTLA-4 gene are PDCD-1 and CTLA-4, respectively. PDCD-1 binds two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) and can compete with B7.1, resulting in inhibition of T cell activation [10, 11]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is another immune inhibitory pathway protein that results in T-cell down regulation and can act as a co-inhibitory receptor with PDCD-1 [12].

Studies have suggested that some tumors have high levels of expression of PD-L1, possibly suppressing anti-tumor T cell responses [13]. Hamanishi J et al. [14] studied the expression of PD-L1 and PD-L2 on ovarian tumors and found a significantly worse overall survival (OS) in patients whose tumor expressed one or both of these ligands. It has been indicated that

Table 1. A summary of the clinical characteristics of PDCD-1 and CTLA-4

	PDCD-1					CTLA-4				
	N	Mean	95% Conf. (±)	Std. Error	Std. Dev.	N	Mean	95% Conf. (±)	Std. Error	Std. Dev.
Stage										
1	77	45.23	14.08	7.07	62.05	77	56.48	12.17	6.11	53.61
2	65	29.02	9.80	4.90	39.54	65	49.06	12.18	6.10	49.17
3	1004	60.54	6.11	3.11	98.65	1004	58.22	6.41	3.27	103.47
4	169	47.35	10.39	5.27	68.45	169	48.95	9.10	4.61	59.89
Entire sample	1315	56.39	4.96	2.53	91.62	1315	56.47	5.11	2.61	94.48
Subtype (1_serous2_endometrioid)										
1	1143	44.72	4.49	2.29	77.44	1143	53.95	4.43	2.26	76.25
2	36	63.31	29.55	14.56	87.35	36	64.75	18.23	8.98	53.89
Entire sample	1179	45.28	4.44	2.27	77.79	1179	54.28	4.32	2.20	75.67
Grade										
0	10	34.00	20.85	9.22	29.15	10	77.70	59.58	26.34	83.29
1	56	67.29	27.41	13.68	102.35	56	63.66	20.60	10.28	76.92
2	315	77.32	10.92	5.55	98.54	315	71.37	8.19	4.16	73.87
3	973	52.75	5.62	2.87	89.39	973	63.15	7.26	3.70	115.41
4	2	136.00	1651.81	130.00	183.85	2	23.00	228.71	18.00	25.46
Entire sample	1356	59.04	4.93	2.51	92.52	1356	65.13	5.62	2.86	105.46
P53 mutation (1_mutated, 0_wild type)										
0	87	17.51	4.14	2.08	19.44	87	21.66	5.34	2.69	25.05
1	441	17.19	1.84	0.94	19.70	441	23.95	2.47	1.26	26.41
Entire sample	528	17.24	1.68	0.85	19.64	528	23.57	2.24	1.14	26.18

PDCD-1/PD-L1 pathway (CD274) blockade augments tumor inhibition by increasing effector T cell activity, while attenuating Treg cell suppression [15]. The blockage and silencing of PDCD-1, CTLA-4 or both PDCD-1 and CTLA-4 molecules could significantly reduce arginase I activity and expression induced with tumor-associated factor [16]. As a result, PD-L1 offers the potential as a prognostic marker in addition to being a target for therapy.

What's more, blockade of PDCD-1, CTLA-4 or both slowed tumor growth and improved the survival rate of tumor-bearing mice. Monoclonal antibodies that bind such targets have been used to resist tumor. For example, FDA has approved αCTLA-4 antibody for treatment of melanoma [16].

However, the studies described above enrolled a relatively small number of patients and some of them appeared to be promising only in animal models without being confirmed in human clinical trials. Larger and more specific studies are required to define the prognostic roles. In this study, we estimated the prognostic roles of PDCD-1 and CTLA-4 Gene in ovarian cancer using a large-scale database. We utilized the Kaplan Meier plotter, a biomarker assessment tool, of which the large quantities of data are from the Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA).

Methods

Construction of ovarian cancer microarray database

The way of the database's (www.kmplot.com) construction was described in a previous report [37]. To summarize, we explored GEO (http://www.pubmed.com/geo) and TCGA (http://cancergenome.nih.gov) to identify ovarian datasets suitable for the analysis and extracted the data. Subtypes of ovarian cancer are available of different stages, histology, grades and P53 gene mutation. Detailed demographic and treatment information of each patient is not shown. Datasets included in the online database are as follows: GSE14764, GSE15622,

Table 2. Prognostic roles of PDCD-1 and CTLA-4 in ovarian cancer patients and subgroup analysis

	PDCD-1					CTLA-4				
	Low Expression (n)	High Expression (n)	Hazard Ratio	95% CI	logrank P	Low Expression (n)	High Expression (n)	Hazard Ratio	95% CI	logrank P
Entire cohort	792	790	0.06	0.77-1.01	0.06	803	779	0.84	0.73-0.96	0.01
Stage										
1	44	30	0.69	0.21-2.29	0.54	37	37	1.30	0.41-4.11	0.65
2	43	16	0.74	0.2-2.73	0.65	33	26	0.62	0.19-2.02	0.42
3	576	406	0.83	0.7-0.99	0.04	568	414	0.78	0.66-0.93	0.01
4	74	92	1.22	0.83-1.81	0.31	67	99	0.82	0.56-1.2	0.31
Subtype (1_serous2_endometrioid)										
1	728	410	0.91	0.77-1.07	0.27	571	567	0.91	0.78-1.07	0.91
2	22	14	1.16	0.19-6.94	0.87	9	27	0.22	0.04-1.32	0.07
Grade										
1	168	162	1.70	0.88-3.31	0.11	28	28	1.03	0.39-2.73	0.94
2	159	156	0.91	0.67-1.23	0.53	158	157	0/81	0.59-1.1	0.18
3	496	472	0.95	0.8-1.13	0.60	491	477	0.89	0.75-1.05	0.16
P53 mutation (1_mutated, 0_wild type)										
0	45	41	1.37	0.76-2.49	0.29	45	41	0.94	0.52-1.68	0.83
1	224	215	1.08	0.84-1.39	0.56	222	217	0.90	0.7-1.16	0.43

GSE18520, GSE19829, GSE23554, GSE26193, GSE26712, GSE30161, GSE3149, GSE9891, GSE27651 and TCGA. All the microarray are based on transcriptome.

Data collection

To present the association between the gene under investigation and survival, we retrieved the data using gene expression cutoff value as median over entire dataset. We also excluded the biased arrays by checking if two or more of the following parameters were out of the 95% range of all arrays: percentage of present calls, the raw Q, presence of bioB-/C-/D-spikes, GAPDH and ACTB 3' to 5' ratio, thus the array quality could be controlled. Because our data were from the online database, no informed consents would be needed.

Statistical analysis

We analyzed the relationship between PDCD-1 and overall survival (OS) as well as CTLA-4 and OS using Cox's proportional hazards regression model. Hazard Ratio, 95% CI and logrank P were calculated. Then the analysis was restricted to different subtypes by stage, histology, grade and P53 mutation and association of each category with OS was also presented with entire reported data online (see **Table 2**). Gene expression level was compared using the H-Test (Kruskal-Wallis) and U test (Mann-Whitney) (see **Table 3**). All the above analyses were conducted using the SPSS software (version 19.0 Chicago, IL, USA).

Results

The Kaplan Meier plotter databases include 1,648 ovarian cancer patients with a mean follow-up of 40 months. The Overall survival analysis was run on 1,582 patients meeting our criteria. Altogether, 77, 65, 1005 and 169 patients were stage I, II, III, and IV; 1143 patients were serous cancer, whereas 36 were endometrioid cancer; the number of people in Grade 0, 1, 2, 3, 4 were 10, 56, 315, 973, 2; 441 patients had P53gene mutation, in contrast to 87 wild types. Mean expression value, (±) 95% Confidence Interval, Standard Error and standard Deviation were analyzed. A summary of the clinical characteristics of the database is presented in **Table 1** and **Figure 1**. Grade 0 and 4 patients were excluded because of relatively small numbers of patients enrolled in the database. Mean CTLA-4 expression value was higher in endometrioid, compared with serous cancer patients (mean value 53.95 vs. 64.75).

Low expressions of PDCD-1 and CTLA-4 were associated with poor outcomes

Using KMplot software, PDCD-1 and CTLA-4 gene expressions among 1582 ovarian cancer patients were shown with median gene expression value as the cut-off. The number of patients

Table 3. Gene expression level comparison using H-Test (Kruskal-Wallis) and U test (Mann-Whitney)

PDCD-1							CTLA-4				
H-Test (Kruskal-Wallis)											
		Patient Number	Mean Rank	H	Degrees of Freedom	*P* Value	Patient Number	Mean Rank	H	Degrees of Freedom	*P* Value
Stage	1	77	683.27	3.27	3.00	0.35	77	735.38	4.43	3.00	0.22
	2	65	605.68				65	658.24			
	3	1004	665.14				1004	657.50			
	4	169	624.16				169	625.60			
U-Test (Mann-Whitney)											
		N	Mean Rank	U	Z	P	N	Mean Rank	U	Z	P
Subtype (1_serous2_ endometrioid)	1	1143	586.99	17131.50	1.71	0.09	1143	585.46	15386.50	2.58	0.01
	2	36	685.63	24016.50			36	734.10	25761.50		
H-Test (Kruskal-Wallis)											
		N	Mean Rank	H	Degrees of Freedom	P	N	Mean Rank	H	Degrees of Freedom	P
	0	10	722.40	23.68	4.00	<0.01	10	787.10	26.67	4.00	<0.01
	1	56	725.68				56	714.97			
	2	315	767.10				315	771.66			
	3	973	646.48				973	645.61			
Grade	4	2	762.50				2	444.50			
U-Test (Mann-Whitney)											
		N	Mean Rank	U	Z	P	N	Mean Rank	U	Z	P
P53 mutation (1_mutated, 0_wild type)	0	87	258.15	18631.00	-0.43	0.67	87	255.90	18435.50	-0.58	0.56
	1	441	265.75	19736.00			441	266.20	19931.50		

with lower PDCD-1 expression was 792, and the number with higher was 790 (see **Table 2**). We observed that shorter OS in those patients with low gene expressions (see **Figure 2**). In detail, higher expression of the PDCD-1 was not significantly correlated with better OS with HR 0.88 (95% CI: 0.77-1.01) but higher CTLA-4 was associated with better survival with HR 0.84 (95% CI: 0.73-0.96) on the transcriptome level.

In addition, by utilizing an extended version of a database of public microarray datasets (http://kmplot.com/analysis), which is meta-analysis based biomarker assessment software, we generated Kaplan-Meier curves (see **Figure 2**). We also drew the bee swarm plot using the bee swarm package (www.cbs.dtu.dk/weklund/beeswarm/). The bee swarm plot can visualize gene expression as non-overlapping points in a one-dimensional scatter plot which is useful in identifying outlier samples and genes with bimodal distribution.

Comparison of gene expression level

The non-parametric (see **Table 3**) tests, H-Test (Kruskal-Wallis) and U test (Mann-Whitney) were used to compare gene expression level. Expression of PDCD-1 and CTLA-4 did not differ with regard to stages and P53 gene mutation. But the expression of CTLA-4 was higher in endometrioid than in serous cancer patients. H-Test (Kruskal-Wallis) showed that different grades of both PDCD-1 and CTLA-4 had different mean values.

Discussion

In this study with a large number of patients, we found that lower CTLA-4 was associated with poorer survival (HR 0.84, 95% CI: 0.73-0.96, P=0.0099), but the relationship between expression of PDCD-1 and survival (HR: 0.88, 95% CI: 0.77-1.01, P=0.061) was not statistically significant. Although significant improvement has been achieved in the new chemotherapeutical agents in the therapy of ovarian cancer and the 5-year survival rate has been increasing, the mortality of this malignant disease remains unchanged [6], current treatment strategies are still far from optimum, and we can only improve OS by identifying more robust targets [17].

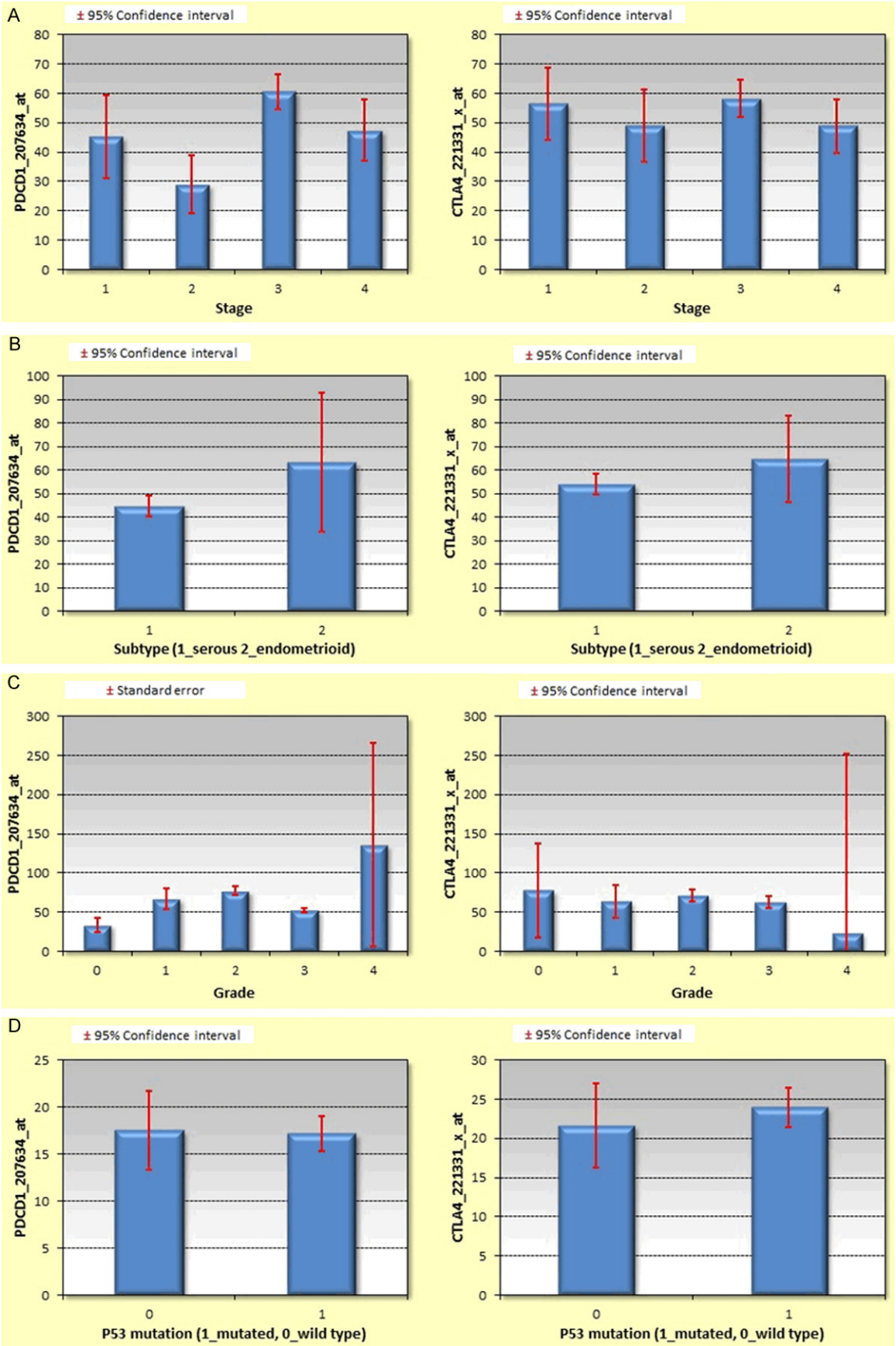
A
± 95% Confidence interval
PDCD1_207634_at
Stage
± 95% Confidence interval
CTLA4_221331_x_at
Stage
B
± 95% Confidence interval
PDCD1_207634_at
Subtype (1_serous 2_endometrioid)
± 95% Confidence interval
CTLA4_221331_x_at
Subtype (1_serous 2_endometrioid)
C
± Standard error
PDCD1_207634_at
Grade
± 95% Confidence interval
CTLA4_221331_x_at
Grade
D
± 95% Confidence interval
PDCD1_207634_at
P53 mutation (1_mutated, 0_wild type)
± 95% Confidence interval
CTLA4_221331_x_at
P53 mutation (1_mutated, 0_wild type)

Figure 1. PDCD-1 and CTLA-4 expression based on (A) stage, (B) subtype, (C) grade and (D) P53 mutation. Histogram plot error bar represents 95% CI.

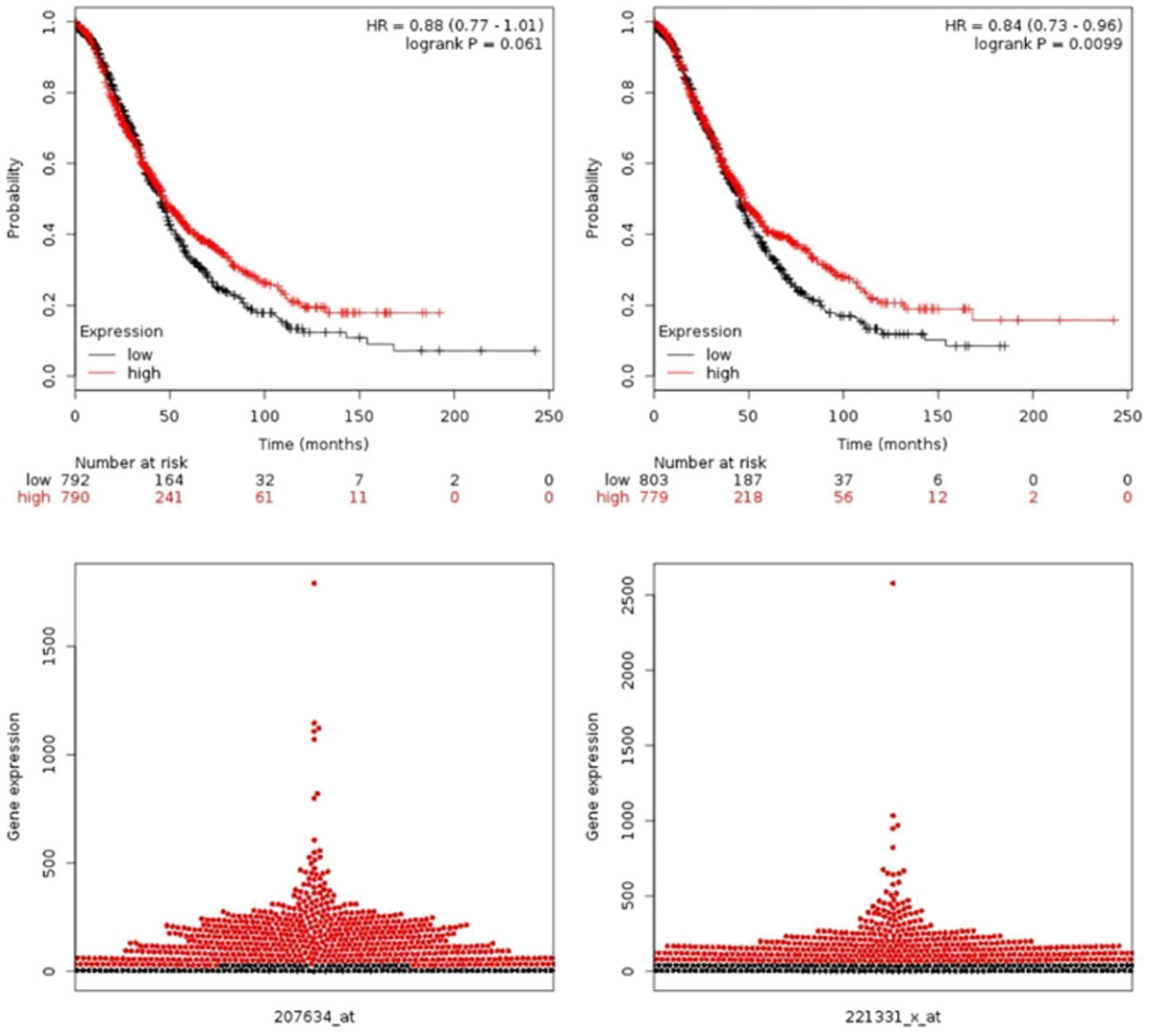

Figure 2. Overall survival curves of PDCD-1 and CTLA-4 gene expressions andbee swarm plot. Red color represents high gene expression; black color represents low expression. PDCD-1 is shown on the left side; CTLA-4 is shown on the right side.

The functions of PDCD-1 and CTLA-4's co-expression were shown in four aspects: PDCD-1 and CTLA-4 were associated with obvious dysfunction of antigen-specific T cells, blocking PDCD-1 and CTLA-4 pathways reversed T cell dysfunction, adoptive transfer of $CD8^+$CTLA-4^+PDCD-1^+TILs that had previously been treated in vitro with αPDCD-1 and αCTLA-4 antibodies eliminated tumors, and lastly, blockade of PDCD-1/PD-L1 pathway in regulatory T cells attenuates their suppressive function [15]. Cause tumor cells are known to develop immune escape mechanisms to camouflage themselves from the surveillance of our human bodies, either by down-regulating the activity of T-cells through activation of the inhibitory T-cell receptors CTLA-4 and PDCD-1 or promoting Treg cell activity, they will tip the balance between pro- and anti-immunoactivity towards inhibition of T-cells.

Pioneering studies established that both $CD4^+$ and $CD8^+$ T cells lacking CTLA-4 in vitro and in vivo presented high proliferation and an activated phenotype from the work of laboratories of Allison, Bluestone, and others [18-23].

What's more, a significant body of data suggests that inhibitors of immune checkpoints might have significant utility in treating cancer which has been borne out by the US FDA's approvals of two different antibodies against PDCD-1 and CTLA-4, respectively [24]. Antibody-mediated blockade of PDCD-1 and CTLA-4 augmented T-cell immune responses, and the use of an antibody against CTLA-4 in combination with a cytokine-expressing cellular vaccine was capable of inducing recession of already-established poorly immunogenic tumors like B16 melanoma [25].

Based on the theories mentioned above, PDCD-1 and CTLA-4 expressions should be positively associated with ovarian cancer patients. However, according to our meta-analysis, the HR was 0.88 (95% CI: 0.77-1) for PDCD-1 and 0.85 (95% CI: 0.74-0.97) for CTLA-4, respectively. Lower expressions of the two genes were not significantly correlated with longer relapse-free survival and vice versa. The paradox may be due to the highly sophisticated immunoregulatory pathways involving PDCD-1, CTLA-4, and their ligands [26-29]. The complexity of each pathway and cross-talk between them and the interactions with other pathways make it not so simple in human body.

In a study performed by Li Jiang [30], positive PD-L1 expression showed a trend toward being independently correlated with longer OS (P= 0.080), perhaps due to different choice of cut-point values, and different tumors have different biologic behaviors with the systemic immunologic environment affecting the tumor growth to varying degrees.

In advanced gastric adenocarcinoma patients, it was found that higher up-regulated PD-L1 expression had much better prognosis than low expression patients (65.6% *vs*. 44.7%, P= 0.028). Patients with higher sPD-L1 expression had better overall survival, perhaps because of different strategies in selecting the study population and different kinds of test methods [31]. The association of PD-L1 expression with favorable outcome has also been observed in lung cancer [32, 33], colon cancer [34], Merkel cell carcinoma [35], and melanoma [36].

What's more, analysis of the transcriptome via microarray did not account for the multiple layers of regulation present in the process of transcription, translation, and protein function. All the results were based on transcriptome which may have different functions after processing in human body.

There are some limitations related to our work. First, although this is the largest up-to-date research on the prognostic roles of PDCD-1 and CTLA-4, this study lacks long-enough follow-up data and therefore the impact of both PDCD-1 and CTLA-4 on prognosis could not be accurately evaluated. Second, we did not perform Q-PCR or microarray analysis to substantiate the main conclusions. Additional limitation is the intrinsic property of the database, as it does not show the complicated process present in translation or protein function. Hence, to optimally identify the role of PDCD-1 and CTLA-4, careful delineation of the gene interactions *in vivo and its relationship with the tumor microenvironme*nt is crucial.

In conclusion, we found that lower expression of CTLA-4, but not PDCD-1 predicts worse survival. The development of molecular biomarkers will beneficially allow the selection of those patients who may benefit from novel therapeutic agents against standard therapeutic approaches. However, further large-scale, more comprehensive analyses need to be carried out. Our current findings still merit further investigation.

Disclosure of conflict of interest

None.

Authors' contribution

CJ.Z and XL.M designed the study and wrote the manuscript. Balázs Győrffy analyzed the data and prepared all the tables. WW.L and Y.X prepared all the figures. All authors reviewed the manuscript.

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