

The Expression of the Regulatory T Cell – Specific Forkhead Box Transcription Factor FoxP3 Is Associated with Poor Prognosis in Ovarian Cancer

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Abstract Purpose: The forkhead box transcription factor FoxP3 is specifically expressed in T cells with regulatory properties (Treg). Recently, high numbers of Treg were described to be associated with poor survival in different malignancies. The aim of the presented study was determine the prognostic effect of FoxP3 mRNA expression (reflecting the tissue content of Treg) in ovarian carcinoma and its relation with cytokines, such as IFN- γ .

Experimental Design: Total RNA was isolated from 99 ovarian carcinoma and from 14 healthy ovarian biopsies. Real-time PCR for FoxP3 was done and correlated with IFN- γ -, CD3-, IRF-1-, SOCS-1-, HER-2-, and iNOS expression as well as patients' outcome. The mRNA data was corroborated by FoxP3 immunohistochemistry.

Results: Quantitation of FoxP3 expression identified a patient subgroup (>81th percentile), which is characterized by a significantly worse prognosis in terms of overall survival (27.8 versus 77.3 months, $P = 0.0034$) and progression-free survival (18 versus 57.5 months; $P = 0.0041$). FoxP3 expression correlated with IFN- γ , IRF-1, and CD3 expression. High FoxP3 expression represents an independent prognostic factor for overall survival ($P = 0.004$) and progression-free survival ($P = 0.004$).

Conclusions: High expression levels of FoxP3 might represent a surrogate marker for an immunosuppressive milieu contributing to tumor immune escape. Strategies selectively depleting Treg might improve the antitumor activity of endogenously arising tumor-reactive T cells and immunotherapies using vaccines or antibodies.

Cancer patients can harbor significant numbers of CD8 and CD4 T cells specific for tumor antigens. In most cases, tumor-reactive T cells fail to eradicate the tumor *in vivo* because they seem to be actively maintained in an unresponsive state. Several immune-evasion strategies of malignant tumors have been described thus far (reviewed in ref. 1). Recently, regulatory T cells (Treg), which are characterized by coexpression of CD4 and CD25, have also been attributed to contribute to cancer-related immunosuppression (2–5). Treg represent 5% to 10% of total CD4⁺ T cells and are crucial for the repression of autoimmune disorders and transplant rejection (6). The activation of Treg is antigen specific;

however, inhibition of CD4⁺ and CD8⁺ T cells seems to be antigen nonspecific. Very recent evidence showed that Treg abrogate CD8⁺ T cell-mediated tumor rejection in a transforming growth factor- β -dependent manner (7). The suggestion of a role for Treg in cancer-induced immunosuppression in humans arises from the observation that patients suffering from a variety of cancer types have an enlarged pool of Treg in the peripheral blood, tumor-draining lymph nodes, and in the tumor itself (8–12). Exact characterization of Treg has been hampered by the lack of specific cell surface markers. The observation that autoimmune diseases occur in both humans and mice lacking functional FoxP3 (13) indicates that this transcription factor plays a crucial role in the regulation of Treg function. It was found that FoxP3^{-/-} mice lack the CD4⁺CD25⁺ Treg population, which leads to hyperactivation of CD4⁺ T cells. Several recent reports have further shown that expression of FoxP3 is sufficient to confer suppressive activity on naive T cells (14, 15). Hence, FoxP3 fulfills the criteria of a Treg-specific marker, which is, at least in differentiated Treg, not known to be substantially regulated and represents a suitable surrogate marker for the indirect quantitation of Treg tissue content. Thus far, only two recent reports immunohistochemically quantified tumor Treg content in larger patient cohorts suffering from Hodgkin disease (16) and ovarian cancer (17). Our current report provides evidence that quantitation of FoxP3 expression in ovarian cancer patients by means of real-time PCR is a more convenient way to

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determine FoxP3 expression compared with time-consuming enumeration of Treg using immunofluorescence and confocal microscopy. Our report extends the findings of Curiel et al. (17) by showing that high FoxP3 expression is not only associated with a dismal prognosis in ovarian cancer but also represents an independent prognostic variable in terms of overall survival (OS) and progression-free survival (PFS).

Materials and Methods

Patients. Tissue samples from patients with invasive ovarian cancer were collected during primary debulking surgery at the Department of Obstetrics and Gynecology in Innsbruck between 1992 and 1999 after informed consent. Tumor slices of 5 mm were subsequently stored at -70°C . Two opposite margins were checked by standard histology in all cases and were proved to consist of $>90\%$ cancer tissue. Moreover, normal ovaries removed for reasons other than ovary-related disease were used as normal controls ($n = 14$). Patient characteristics are given in Table 1. Ninety-two percent of patients received chemotherapy consisting of either cisplatin and cyclophosphamide (41 patients), carboplatin and paclitaxel (43 patients), or carboplatin alone (7 patients). All patients had their follow-up in the outpatient clinic of the Department of Obstetrics and Gynecology, University Hospital Innsbruck. The median observation period of surviving ovarian cancer patients was 5.8 years.

RNA extraction and reverse transcriptase reaction. Total cellular RNA was extracted from the tumor specimens and purified by using the acid guanidium thiocyanate-phenol-chloroform method. RNA concentration was measured by spectrophotometric analysis and its integrity was evaluated by assessing the 18S- and 28S-rRNA bands in 2% ethidium bromide-stained agarose gel.

Table 1. Patient characteristics

Characteristics	FoxP3 ^{low} (<i>n</i> = 81)	FoxP3 ^{high} (<i>n</i> = 18)	<i>P</i>
Age (y \pm SD)	64.8 \pm 10.9	67.9 \pm 10.2	0.228
Stage (%)			
FIGO I	17 (21)	1 (5.6)	0.071
FIGO II	3 (3.7)	1 (5.6)	
FIGO III	52 (64.2)	10 (55.6)	
FIGO IV	9 (11.1)	6 (33.3)	
Grade (%)			
I	3 (3.7)	0 (0)	0.121
II	49 (60.5)	7 (38.5)	
III	29 (35.8)	11 (61.1)	
Histology (%)			
Serous	29 (35.8)	8 (44.4)	0.59
Mucinous	24 (29.6)	6 (33.3)	
Other	28 (34.6)	4 (22.2)	
Residual tumor mass (%)			
No	31 (38.3)	6 (33.3)	0.229
<2 cm	21 (25.9)	2 (11.1)	
>2 cm	29 (35.8)	10 (55.6)	

NOTE: Clinical characteristics of 99 patients with invasive ovarian cancer are given. Tissue was taken during primary debulking surgery at the Department of Obstetrics and Gynecology in Innsbruck between 1992 and 1999. Important clinical characteristics are shown for both the FoxP3^{high} and FoxP3^{low} group. Abbreviation: FIGO, Federation Internationale des Gynaecologistes et Obstetristes.

Reverse transcription of total RNA was done in a final volume of 20 μL containing $1\times$ reverse transcription buffer [50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, and 5 mmol/L MgCl_2], 40 units of RNasin RNase inhibitor (Promega, Madison, WI), 10 mmol/L DTT, 200 units of M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD), 5 $\mu\text{mol/L}$ random hexamers (Applied Biosystems, Foster City, CA), and 1,000 ng of total RNA. The samples were first incubated at 65°C for 5 minutes and then quickly chilled on ice. After addition of the M-MLV enzyme, the samples were incubated at 25°C for 10 minutes and 37°C for 50 minutes, followed by heating at 70°C for 15 minutes to inactivate the reverse transcription enzyme.

Primers and probes. Gene expression assay mixes for CD3, IFN- γ , and HER-2 were purchased from Applied Biosystems [assay ID: Hs00167894_m1 (CD3E), Hs00174143_m1 (IFN- γ), and Hs00170433_m1 (HER-2)]. Primer for SOCS-1, IRF-1, iNOS (18), and FoxP3 were designed using Primer Express software (Applied Biosystems). As control genes, primers and probes for the TATA box-binding protein (a component of the DNA-binding protein complex transcription factor IID as endogenous RNA control) as well as glyceraldehyde-3-phosphate dehydrogenase were used. The nucleotide sequences of these oligonucleotides are shown in Table 2.

Real-time PCR. TaqMan PCRs for CD3, IFN- γ , HER-2, SOCS1, IRF1, iNOS, and FoxP3 were done using ABI Prism Detection System (Applied Biosystems) as described previously (18). In the case of FoxP3, magnetically bead isolated (Miltenyi, Bergisch-Gladbach, Germany; purity $>90\%$) CD4⁺CD25⁺ Tregs served as positive control on every plate and as internal reference which was arbitrarily set as 1. For amplification, 2.5 μL of cDNA were incubated with 12.5 μL of $2\times$ TaqMan Master Mix (8% glycerol, $1\times$ TaqMan buffer, 200 $\mu\text{mol/L}$ dATP, 200 $\mu\text{mol/L}$ dGTP, 200 $\mu\text{mol/L}$ dCTP, 400 $\mu\text{mol/L}$ dUTP, 0.05 units/ μL AmpErase uracil *N*-glycosylase, 5 mmol/L MgCl_2 , and 0.01 units/ μL Amplitaq Gold; Perkin-Elmer, Wellesly, MA), 2.5 μL TaqMan probe, sense and reverse primers at a final concentration of 10 pmol/ μL . The reaction mixture was brought up to a final volume of 25 μL with RNase-free distilled water. For quantitation of the human housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase*, the Perkin-Elmer predeveloped assay kit was used. The amplification was done and analyzed using an AbiPrism 7700 Sequence detector. To rule out contamination from buffers and tubes, a negative control with water instead of the cDNA template was used on every plate. Real-time PCR efficiencies were acquired by amplification of a standardized dilution series of cDNA isolated from separated CD4⁺CD25⁺ Tregs. The amount of FoxP3 was calculated by the method of Pfaffl (19).

Immunohistochemical detection of FoxP3. After deparaffinization, sections were placed in Epitope Retrieval Solution [Dakocytomation, Glostrup, Denmark 10 mmol citrate buffer (pH 6.0), part of HercepTest] in a preheated water bath (98°C , 40 minutes.) Endogenous peroxidase was blocked by Chemmate Peroxidase Blocking Solution (Dakocytomation) for 5 minutes.

Primary antibody FoxP3 (16) diluted 1:20 in Chemmate Antibody Diluent (Dakocytomation) was applied for 40 minutes, followed by Biotinylated Link (Dakocytomation LSAB2 System-HRP), streptavidin HRP (Dakocytomation LSAB2 System-HRP), and 3,3'-diaminobenzidine (Dakocytomation Chemmate Dako Envision), 10 minutes each. After counterstaining with hematoxylin (diluted 1:2 in demineralized water for 5 minutes; Dakocytomation) sections were dehydrated and mounted. All incubation steps were done at room temperature. Two variants of negative controls were done with antibody diluent omitting primary antibody and Negative Control Reagent (part of HercepTest) instead of the specific primary antibody.

Statistical analysis. Differences in expression levels between normal and malignant tissues were evaluated with the Mann-Whitney *U* test. Data were expressed as median and interquartile range. Because FoxP3 expression showed neither a clear negative value nor a biphasic distribution, the optimal cutoff point had to be determined. For this purpose, each percentile from 10 to 90 was calculated for FoxP3 expression and used as cutoff point. At each of these cutoff points, the

data were divided into two groups and differences in OS were analyzed using the log-rank test. The greatest difference between the two groups was at the 82nd percentile. Therefore, this percentile was chosen as cutoff point for discrimination between FoxP3 low and high expression. Groups characterized by high and low FoxP3 expression were tested for differences with the Mann-Whitney *U* test for quantitative data and the χ^2 test for categorical data. Spearman's correlation coefficient was calculated to show the correlation in the expression of FoxP3, IFN- γ , CD3, HER-2, and STAT-induced genes (*SOCS-1*, *IRF-1*, and *iNOS*). Survival curves were estimated with the use of the Kaplan-Meier method, and differences between the groups were evaluated in a multivariate analysis by means of the log-rank test. The Cox proportional hazard model was used for assessment of the predictive value of FoxP3 expression with adjustment for confounding variables.

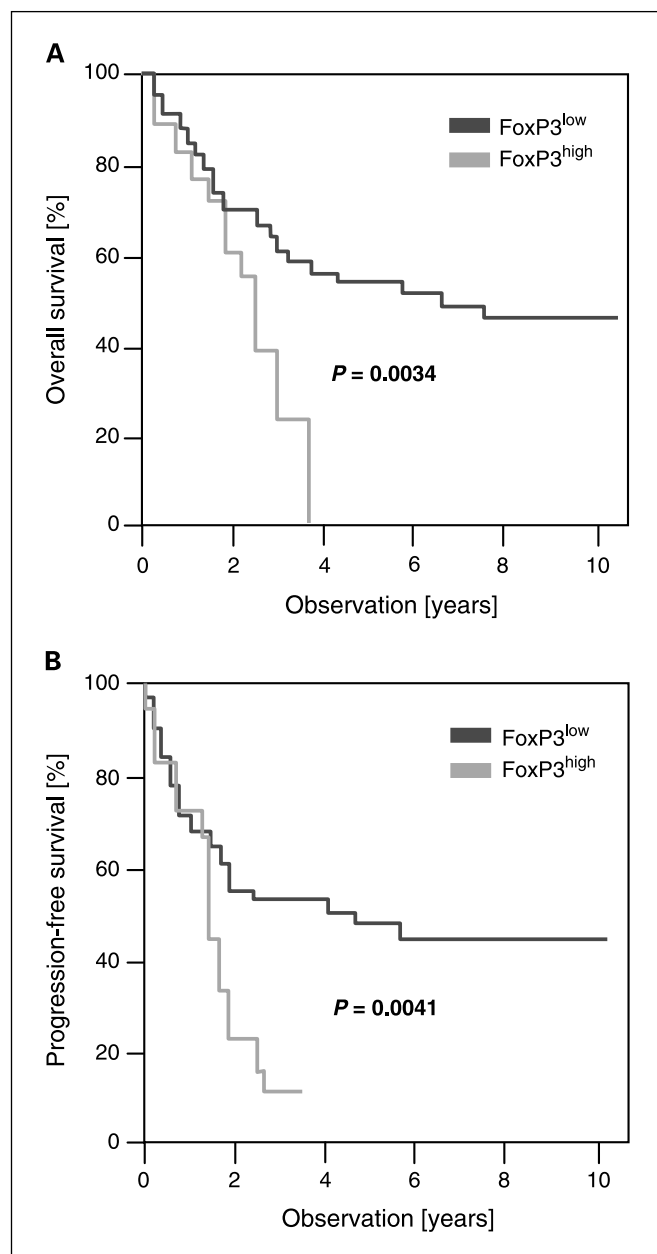


Fig. 1. High FoxP3 expression is associated with inferior OS and PFS. OS (A) and PFS (B) curves for 99 patients suffering from invasive ovarian cancer based on expression of FoxP3: the cases were divided into two groups (i.e., FoxP3^{high} and FoxP3^{low}). *P* value was determined by log-rank test.

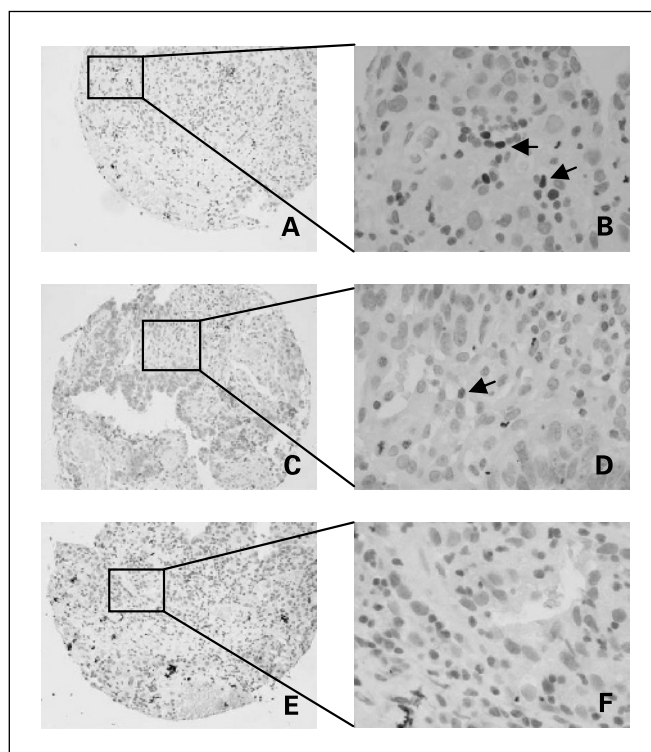


Fig. 2. Detection of FoxP3 expression by immunohistochemistry. Representative immunohistochemical stainings of FoxP3^{high} (A and B) and FoxP3^{low} (C and D) tumors are shown. The negative control is shown in (E and F). Arrows, nuclear FoxP3 staining in tumor-infiltrating Treg (A and B) or FoxP3-negative tumor-infiltrating lymphocytes (C and D). Magnification, $\times 4$ (A, C, and D); $\times 40$ (B, D, and F).

The final model included performance status, grading, residual disease, and FoxP3 expression. The relative risk of recurrence or death is expressed as ratio with 95% confidence interval. Statistical significance was defined as $P < 0.05$. SPSS for Windows 11.0 software (SPSS, Inc., Chicago, IL) was used for all analyses.

Results

FoxP3 expression is associated with inferior survival in ovarian cancer patients. FoxP3 mRNA expression was quantified using quantitative reverse transcription-PCR as surrogate for tissue infiltration of Treg. FoxP3 mRNA was readily detectable in ovarian tissue from healthy volunteers ($n = 14$, data not shown). However, a 1.8 ± 0.7 -fold increase of FoxP3 expression was detectable in tissue samples ($n = 99$) obtained from ovarian cancer patients. FoxP3 expression neither showed a clear negative value nor a biphasic distribution, thus a distinct cutoff point had to be determined. We detected a highly significant inferior OS (Fig. 1A) and PFS (Fig. 1B), respectively, in patients from the 19th highest FoxP3 (FoxP3^{high}) expression percentile compared with patients from the 81th lowest FoxP3 (FoxP3^{low}) percentile ($P = 0.0034$ for OS and $P = 0.0041$ for PFS). Median survival time was not achieved in the FoxP3^{low} fraction (mean 77.3 ± 6.1 months) compared with a median survival time of 30.2 ± 3.9 months in FoxP3^{high}-expressing patients (mean 27.8 ± 3.4 months). Median PFS was 57.5 ± 27.3 months in the FoxP3^{low} and 18.0 ± 0.7 months in the FoxP3^{high} group. Immunohistochemical staining of FoxP3 on a

Table 2. Primers and probes used for real-time PCR

Gene	Direction	Sequence
FoxP3	Forward	5'-TGGCTAGGAAAATGGCA-3'
	Reverse	5'-GCAGGAGCCCTTGTCGG-3'
	Probe	5'-FAM-TGACCAAGGCTTCATCTGTGGCATCA-TAMRA-3'
iNOS	Forward	5'-CCAACAATGGCAACATCAGG-3'
	Reverse	5'-TCGTGCTTGCCATCACTCC-3'
	Probe	5'-FAM-CGGCCATCACCGTGTCCCC-TAMRA-3'
SOCS-1	Forward	5'-TTTTCGCCCTTAGCGTGAAG-3'
	Reverse	5'-CATCCAGGTGAAAGCGGC-3'
	Probe	5'-FAM-CCTCGGGACCCACGAGCATCC-TAMRA-3'
IRF-1	Forward	5'-AAGGATGCCTGTTTGTCCG-3'
	Reverse	5'-CAGCGAAAGTTGGCCTTCC-3'
	Probe	5'-FAM-CTGGGCCATTACACAGCCGATAC-TAMRA-3'

tissue microarray corroborated our mRNA data in terms of the identification of tumors with either high (Fig. 2A and B) or low levels of FoxP3-positive Treg per high-power field (Fig. 2C and D). Figure 2E and F shows the negative control.

High FoxP3 expression is an independent prognostic variable for progression-free survival and overall survival in ovarian cancer patients. FoxP3 expression was not correlated with other well-defined prognostic variables, such as tumor grade, FIGO stage, histologic subtype, or the age of the patients (Table 2). Using Cox multivariate regression analyses, we were able to show that FoxP3 expression is an independent prognostic factor for PFS as well as for OS (Table 3). Patients with low FoxP3 expression have a ~60% reduced risk of recurrence or death ($P = 0.004$ for PFS and OS). In addition, residual disease also seemed to be an independent risk factor for PFS and OS. Tumor grading predicted recurrence only, whereas a good performance status was associated with improved survival.

FoxP3 expression correlates with IFN- γ and CD3. FoxP3-expressing Treg have been shown to exert potent immunosup-

pressive effects on activated T cells, such as down-regulation of IFN- γ (6). Thus, we hypothesized that FoxP3^{high}-expressing tumors would have low levels of IFN- γ expression. Surprisingly, there was a highly significant positive correlation between FoxP3 and IFN- γ ($\rho = 0.424$, $P < 0.001$) and CD3 ($\rho = 0.417$, $P < 0.001$), respectively (Table 4). According to the correlation between FoxP3 and IFN- γ expression, its expression was also strongly correlated to IRF-1 expression ($\rho = 0.237$, $P < 0.018$; Table 4), which is known as a central regulator of the IFN- γ pathway. No significant correlation could be detected between FoxP3 expression and SOCS-1, iNOS, and Her2 mRNA in ovarian cancer tissue or CA-125.

FoxP3 expression identifies a subgroup of patients with inferior survival in IFN- γ ^{high}-expressing tumors. We recently provided evidence that high IFN- γ expression is associated with a superior outcome (18). We now show that in this group of patients (IFN- γ ^{high}), a subgroup of patients with concomitant high FoxP3 expression (IFN- γ ^{high}/FoxP3^{high}) is characterized by a significantly inferior OS (Fig. 3, $P = 0.0001$). Due to low patient

Table 3. Cox regression analysis of several variables for OS and PFS

Variable	No. patients	PFS		OS	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
FOXp3 expression					
High	18	1.00	0.004	1.00	0.004
Low	81	0.41 (0.23-0.76)		0.39 (0.20-0.74)	
Residual disease					
No tumor left	37	1.00	0.003	1.00	0.002
Tumor left	62	0.38 (0.20-0.72)		0.34 (0.18-0.66)	
Grade					
3	40	1.00	0.366	1.00	0.719
1 + 2	59	0.78 (0.45-1.34)		1.11 (0.63-1.94)	
Performance status					
0	68	1.00		1.00	
1	17	0.63 (0.29-1.37)	0.245	0.46 (0.22-0.94)	0.033
>1	14	0.49 (0.19-1.28)	0.145	0.19 (0.23-1.34)	0.552

NOTE: High FoxP3 expression as well as residual disease are independent prognostic variables for OS and PFS. FoxP3 expression, tumor grade, residual disease after tumor resection, and performance status were analyzed by Cox proportional hazard model using multivariate analysis.

numbers in the subgroup analysis, we were not able to detect a significant difference by FoxP3 expression level in patients with low IFN- γ expression (data not shown).

Discussion

CD4⁺CD25⁺ Tregs have been shown to be increased in the peripheral blood and in tumor-draining lymph nodes of cancer patients. Detection of Treg is hampered by the problem that these cells share a set of markers with either memory or activated T cells. Recent data in genetically modified animals elegantly showed the specific expression of the forkhead box transcription factor FoxP3 in T cells with immunosuppressive properties. These T cells are almost exclusively represented by CD4⁺CD25⁺ Treg. Thus, detection of FoxP3 might serve as a surrogate marker for the tissue infiltration grade by Treg. In this report, we used quantitative real-time reverse transcription-PCR for the quantitation of FoxP3 in 99 samples from ovarian cancer patients and in 14 control biopsies from nondiseased ovaries. FoxP3 expression was readily detectable in ovaries from healthy women as well as in ovarian cancer tissue. The expression of FoxP3 in normal ovaries and recent findings describing that day 3 thymectomized mice develop autoimmune oophoritis (20–22), which can be prevented by transfer of CD4⁺CD25⁺ Treg (21, 23), suggests a central role for Treg in preventing deleterious effects of autoreactive T cells targeting ovarian self-antigens, thereby maintaining immunotolerance in the female reproductive tract. Of note, malignant transformation of ovarian tissue leads to a significant tissue infiltration of cancer tissue by Treg (11, 17). We provide first evidence that a subgroup of ovarian cancer patients expressing high levels of FoxP3 is characterized by an inferior prognosis. In addition, FoxP3 expression seems to be an independent prognostic factor for OS and PFS. The existence of ovarian cancer tissue with either high (FoxP3^{high}) or low (FoxP3^{low}) Treg content was corroborated by immunohistochemical staining of FoxP3 in an independent set of ovarian cancer tissue samples. Our findings are in keeping with the concept that FoxP3-expressing Tregs are enriched in some malignant epithelial tumors and might contribute to tumor-induced shutdown of an effective antitumor immune response (24, 25). In addition, our data are in line with recently published data showing that increased levels of CD25⁺ T cells coexpressing FoxP3, as determined by semiquantitative enumeration of those cells on tissue sections using confocal microscopy after immunofluorescent staining, are associated with a significantly shortened

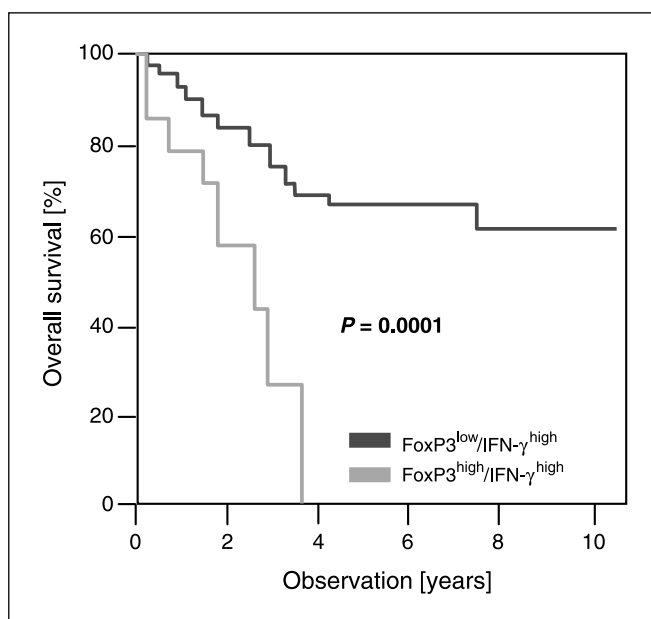


Fig. 3. High FoxP3 expression is a dominant prognostic factor for survival in IFN- γ ^{high}-expressing patients. Patients with both high IFN- γ expression and high FoxP3 expression (IFN- γ ^{high}/FoxP3^{high}) have a significantly worse prognosis when compared with IFN- γ ^{high}/FoxP3^{low} patients. *P* value was determined by log-rank test.

survival (17). However, we propose that detection of FoxP3 using real-time PCR might be a more practicable way for quantitation of Treg tissue content. This strategy has recently been used for the quantitation of Treg in peripheral blood after bone marrow transplantation, a situation where decreased FoxP3 mRNA levels were observed under conditions of graft versus host disease (26). Of note, statistical cutoff determination was chosen due to fact that FoxP3 expression showed neither a clear negative value nor a biphasic distribution. These data now need to be validated in a prospective setting using the defined cutoff values (validation set) to establish a robust predictive clinical marker. In addition, we very recently established an absolute quantitation of FoxP3 transcripts on a large cohort of breast cancer patients using a plasmid standard.⁴ This will facilitate the comparability of the data between different laboratories. Interestingly, the findings from ovarian cancer patients are contradictory to recent data demonstrating that high FoxP3 expression is associated with improved survival rates in patients suffering from Hodgkin lymphoma (16). These data might be due to a direct inhibitory effect of Treg on tumor cell proliferation if the tumor cells are derived from the B cell lineage. This concept is supported by findings from murine models demonstrating a direct effect of Treg on B-cell function and survival (27, 28). However, the presented data clearly show that at least in ovarian cancer, high FoxP3 mRNA expression levels are associated with a bad clinical outcome. Whether FoxP3-expressing Treg are direct inhibitors of T cell effector cells within the tumor compartment remains unknown. Unexpectedly, we found a strong positive correlation between FoxP3 and IFN- γ , and the IFN- γ -regulated gene *IRF-1* and CD3, which argues against our initial hypothesis that the higher the FoxP3 expression the lower the IFN- γ expression.

Table 4. Spearman correlation of FoxP3 with other variables

	CD3	IFN- γ	IRF-1	SOCS-1	iNOS	Her2/neu	CA-125
FoxP3							
ρ	0.417	0.424	0.237	0.183	-0.108	-0.117	0.065
<i>P</i>	0.0001	0.0001	0.018	0.070	0.289	0.249	0.526
<i>n</i>	99	99	99	99	99	99	99

NOTE: FoxP3 expression strongly correlates with CD3, IFN- γ , and IRF-1 expression. Spearman rank correlation coefficient (ρ) relationship between expression levels of FoxP3 and either CD3, IFN- γ , IRF-1, SOCS1, Her2/neu, or CA-125 are shown.

⁴ Manuscript in preparation.

Thus, in ovarian carcinoma tissue, low IFN- γ expression is most likely not due to a higher Treg content. It seems, however, that FoxP3 expression represents a dominant prognostic factor for survival, as patients with both high IFN- γ expression and high FoxP3 expression (IFN- γ^{high} /FoxP3 $^{\text{high}}$) have a significantly worse prognosis when compared with IFN- γ^{high} /FoxP3 $^{\text{low}}$ patients.

In summary, quantitation of FoxP3 expression using real-time PCR in ovarian cancer patients enabled the identification of a patient subgroup (FoxP3 $^{\text{high}}$) that is characterized by a very

poor outcome in terms of OS and PFS. Especially in this patient group, immunotherapeutic strategies modulating or depleting tumor-associated Treg should be considered.

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