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Forkhead box protein 3 (FoxP3) mRNA as a diagnostic marker in ovarian tumors: a pilot study

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Summary

Purpose: To assess the diagnostic performance of forkhead box protein 3 (FoxP3) mRNA in ovarian tumors. *Materials and Methods:* Using quantitative real time reverse transcription PCR (q-RT-PCR), FoxP3 mRNA level was measured in fresh frozen ovarian tumors and its diagnostic performance was compared to those of preoperative serum CA125 and risk of malignancy index (RMI). *Results:* FoxP3 mRNA was differentially expressed in the malignant (n = 25) and benign (n = 25) groups, yet without statistically significant differences; positivity rate: 15/25 *vs*. 10/25; $p = 0.157$, median: 0.429 *vs*. 0.046; $p = 0.684$, and mean ± SD: 73.75 ± 234.68 *vs*. 247.09 ± 792.17; $p = 0.301$. Although it showed much less diagnostic performance (AUC: 0.534), FoxP3 mRNA enhanced the diagnostic sensitivity and specificity of both CA125 and RMI (96% and 100%, for both). *Conclusion:* FoxP3 mRNA may not be good diagnostic marker in ovarian tumors; however it may prove valuable in defining underlying tumor molecular signature.

Key words: Ovarian tumor; q-RT-PCR; FoxP3 mRNA; CA125; $T_{reg.}$

Introduction

Ovarian cancer presents a great challenge for the gynecologic oncologist and oncology researcher, as apparently perceived from epidemiologic and clinical data. It accounts for 3.7% of all cancers in women [1] and is considered as the most lethal among all gynecological malignancies with an overall five-year survival of 45% [2]. Despite the recent advances in medical care and managements, the survival rate remained relatively unchanged over the past 30 years [3, 4].

As late-case diagnosis almost frequently complicates the illness, the clinical impact of early detection and treatment of ovarian tumor patients may not be underestimated based on that literary background that signifies the pressing need for novel diagnostic and therapeutic tools. Over the past few decades, several diagnostic parameters have been used, either separately or in combinations, including ultrasound findings, menopausal status, and serum tumor markers with less than satisfactory outcomes [5].

The transcription factor forkhead box protein 3 (FoxP3) gene is located at Xp11.23 and is a member of forkhead-box/winged-helix transcription factor family [6]. It is specifically expressed by the thymically-derived naturally occurring regulatory T cells, Tregs [7]. However, there are few reports on its expression by other cell types: pancreatic carcinoma cells [8] and in other human cancer cell lines; namely lung, colon, and breast cancers, melanoma, erythroid leukemia, and acute T-cell leukemia [9]. While FoxP3 is known mainly to modulate, i.e. maintain a balance, the immune response being important for self-tolerance and protection against chronic infection and tumor formation [10], recent views suggest that it could, in other contexts, have a tumor suppressor role [6, 11].

FoxP3 was found to be expressed in different cancer types where it was correlated to unfavorable patients' outcome (e.g., in melanoma [12] and ovarian cancer [13]). Nonetheless, others reported the potential usefulness of the gene as therapeutic target (rheumatoid arthritis [14], graft *vs*. host disease [15], and cancer [16]). In the present study the authors address the question whether FoxP3 mRNA expression could have possible diagnostic value in ovarian tumors which, in such a case, might prove clinically relevant marker in disease prediction and/or characterization.

Materials and Methods

Participants

This study was performed in female patients with ovarian masses who were admitted to Ain Shams University Maternity Hospital through the Gynaecology Outpatient Clinic from October 2012 to February 2014. An informed consent was taken from each patient. The protocol of this study was approved by the Ethics Committee of Ain Shams Faculty of Medicine. As inclusion criteria each patient was/had: 1) established diagnosis of an ovarian mass and 2) planned for surgical intervention. Patients who were pregnant or had tumors other than ovarian were excluded from this study.

All patients were subjected to detailed history taking, general and local examination, blood sample analysis of routine laboratory

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tests, serum CA125, and pelvic ultrasonography. Risk of malignancy index (RMI) was calculated for each patient as described elsewhere [17].

Patients were classified according to the histopathological diagnoses of the ovarian tumors into two main groups: malignant patients group and benign patients group. Tumor pathological staging and grading were done according to TNM classification. Clinical staging of the disease was done according to TNM classification [18].

Ovarian tumor tissue samples were obtained directly at the operating theater in a Petri dish on ice. These were selected to be representative of the tumor. Blood was washed by ice cold saline. The fat, necrotic tissue, skin, and muscle tissue were rapidly dissected from tissue of interest. The tissue samples were chilled on ice and were wrapped in aluminum foil and immediately frozen at -80°C until their use for RNA extraction.

RNA extraction of all samples was performed according to the instructions provided by the manufacturer in the product insert. RNA was kept at -80°C until its use for quantitative-Real Time PCR (qRT-PCR) of FoxP3 and β actin as a house keeping gene in each sample.

Measurement of RNA concentration in µg/µl included 75 µl of DEPC-water that was added to three µl of RNA solution (dilution 1:25). The sample was pipetted up and down several times to ensure adequate mixing; 40 µg RNA/ml was equivalent to one absorbance (O.D), so the concentration of RNA in a sample (µg/ µl) equals O.D. (at 260 nm)×40x dilution factor (25)/1000. The sample was read at 260 nm and 280 for RNA detection and protein detection, respectively using the spectrophotometer. The samples were considered with good RNA quality if RNA: protein ratio (260:280 ratio) was more than 1.5. The authors added one ug of RNA sample to the RT-PCR reaction. RT was performed using Quantitect Reverse Transcription. The steps were done according to the instructions provided by the manufacturer in the product insert.

The volume of the first-strand reaction was brought to 20 µL with RNAase free water, and template cDNA (one ug/reaction) was amplified on an iCycler using ten µL 2x QuantiTect SYBR Green PCR Master Mix and two µl of the gene-specific oligonucleotide primers. All PCRs were done by initial activation step at 95°C for 15 minutes followed by 45 cycles of 15, 30, and 45 seconds at 95°C, 50°C, and 72°C, respectively. Bio-Rad software was used to calculate threshold cycle (Ct) values for the target gene and for the reference β actin gene. The expression values for the tumor samples are presented as fold expression in relation to the control sample; the actual values were calculated using the $2^{-\Delta\Delta Ct}$ equation, where ΔΔCT = [Ct FOXp3 – Ct β actin] (malignant sample) − [Ct FOXp3 – Ct β actin] (control sample). Then calculation of the relative quantification (RQ) or fold change is done by the following equation: Relative quantity (RQ) = $2^{-\Delta\Delta CT}$. The following primer sequences were used: Homo sapiens forkhead box P3 (>NM_014009.3) sense primer: CCCACTTACAGGCACTCCTC and antisense primer: CTTCTCCTTCTCCAGCACCA [19], and Homo sapiens β actin (>XM_005249820.1) sense primer: CTACGTCGCCCTGGACTTCGAGC and antisense primer: GATGGAGCCGCCGATCCACACGG [20].

Positivity rate, Spearman's correlation for continuous variables, and the chi-square analysis (X^2) of the association variables in the patients' category groups were estimated. Median or mean values were compared using nonparametric (Mann-Whitney and Kruskal Wallis) or parametric (Student *t*-test and ANOVA) tests, respectively, according to the number of the comparisons' groups. The threshold values for optimal sensitivity and specificity of FoxP3 mRNA, CA125 or RMI were determined by receiver operating characteristics (ROC) curve and were used for discrimination between benign and malignant groups. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated according to standard statistical methods. All statistical analysis of data was performed using the software package SPSS, version 15.0. *P* value ≤ 0.05 was considered significant.

Table 1. — *Clinicopathological characteristics in the two study groups.*

		Patient groups		*p*
		Malignant n = 25	Benign n = 25	
Parity	Nulliparous	10	7	0.55[a]
	Multiparous	15	18	
Breast feeding	Positive	10	15	0.23[a]
	Negative	15	10	
MS	Premenopausal	12	18	0.214[a]
	Postmenopausal	13	7	
FH	Positive	5	10	0.22[a]
	Negative	20	15	
Smoking	Smoker	2	3	0.86[a]
	Non smoker	13	10	
	Passive smoker	10	12	
OCT	Past administration	4	10	0.109[a]
	Never	21	15	

MS: menopausal state, FH: family history, OCT: oral contraception.
[a] Chi square test (X^2).

Results

The present study included 50 patients (age mean ± SD: 45.8 ±14.6 years). Table 1 shows the clinicopathological parameters of the patients groups. The benign patients were 22 benign neoplastic and three benign non-neoplastic (age mean ± SD: 43.2 ± 14.4 and 37.6 ± 7.7 years, respectively; $p = 0.524$). The malignant patients were 15 serous epithelial, five mucinous epithelial, and five sex cord stromal tumors (age mean ± SD: 51.1 ± 12.1, 45.4 ±16.1 and 46.8 ± 23.1 years, respectively; $p = 0.724$).

The frequencies of disease stages and grades in the malignant patients were: Stage IA-C and IIA-C: 15, and Stage III: ten patients; grade Gx-G1: eight patients, and grade G2-3: 17 patients. The distribution of disease grade and stage in the malignant pathological subgroups were: serous epithelial subgroup: Stage IA-C and IIA-C: six patients and Stage III: nine patients; grade Gx-G1: 3 and grade G2-3: 12 patients; mucinous epithelial subgroup: Stage IA-C and IIA-C: four patients, and Stage III: one patient; grade Gx-G1: two patients, and grade G2-3: three patients; sex cord stromal subgroup: Stage IA-C and IIA-C: five patients, and Stage III: no patients; grade Gx-G1: three patients, and grade G2-3: two patients (*p*-values: 0.036 and 0.230, respectively).

Comparisons of patients clinicopathological parameters (age, menstrual cycle, parity, breast feeding, pills intake,

smoking, and family history) in patients groups did not show statistically significant differences (X^2: p-values: 0.258, 0.041, 0.370, 0.157, 0.355, 0.679, and 0.059, respectively) except for menstrual cycle where malignant patients had higher (13/25 *vs*. 6/25) proportion of postmenopausal women. At the benign or malignant subgroups level, there were – with only one exception – also no statistically significant differences in the distribution of those parameters (benign subgroups: X^2: p-values: 0.102, 0.299, 0.826, 0.802, 0.918, 0.798, and 0.024, respectively and malignant subgroups: X^2: p-values: 0.574, 0.808, 0.435, 0.435, 0.072, 0.330, and 0.551, respectively). Benign nonneoplastic patients (n= 3) all had positive family history of ovarian masses while the neoplastic patients were mainly with negative family history (15/22).

There were no statistically significant differences in the different overall patients' clinicopathological groups (age, menstrual cycle, parity, breast feeding, pills intake, smoking, and family history) with regards to the distribution of marker's level; below and above cutoff; CA125 (X^2: p-values: 0.693, 0.545, 0.149, 0.010, 0.538, 0.398, and 0.366, respectively) except for breast feeding where non-breast feeding patients had higher proportion of above cutoff level of CA125; RMI (X^2: p-values: 0.153, 0.093, 0.616, 0.258, 0.902, 0.774, and 0.278, respectively) or FoxP3 (X^2: p-values: 0.571, 0.771, 0.765, 0.396, 0.355, 0.363, and 1.000, respectively). These findings were also true for the patient pathological subgroups: benign (neoplastic and non-neoplastic) and malignant (serous, mucinous, and sex cord stromal; data not shown).

Using Spearman's correlations, patients' age correlated with RMI (correlation coefficient = 0.316; p = 0.025) but not with CA125 (correlation coefficient = 0.005; p = 0.978) or FoxP3 (correlation coefficient = - 0.030; p = 0.837).

CA125 positively correlated with RMI (correlation coefficient = 0.867; p = 0.000), FoxP3 did not correlate with CA125 (correlation coefficient = - 0.007; p = 0.966) or RMI (correlation coefficient = 0.009; p =0.951). Concordance assessments (X^2) showed again the same findings: CA125 and RMI: p = 0.000, CA125, and FoxP3: p = 0.914, and RMI and FoxP3: p = 0.258.

The overall data of CA125 (n = 34) were as follows: positivity rate: 23/34 (67.6%), range (min.-max.): 2-17596 U/ml, median: 14.30 U/ml, and mean ±SD: 551.1+3012.3 U/ml. Table 2 shows CA125 levels (distribution of cutoff level, minimum, maximum, median, and mean ± SD) in the two patient's pathologic groups.

The marker's level showed statistically significant difference in benign (median: neoplastic patients: 7.20 U/ml; non-neoplastic patients: 2.65 U/ml, p = 0.040) but not in malignant (median: serous epithelial patients: 21.80 U/ml; mucinous epithelial patients: 28.30 U/ml; sex cord stromal patients: 9.60 U/ml, p = 0.121) subgroups.

There was statistically significant difference of CA125 median in malignant patients' Stage (median: IA-C and IIA-C: 15.20 U/ml; III: 38.40 U/ml, p = 0.049) but not in disease grade (Gx-G1: 14.40 U/ml; G2-3: 28.30 U/ml, p = 0.210) groups.

Table 2. — *Serum CA125, RMI and FoxP3 mRNA levels in the two patient's groups.*

	Malignant n = 25	Benign n = 9-25	p
CA125 cutoff (8.85 U/ml)			
Below	3	8	0.000[a]
Above	22	1	
Min - max	4-17596	2-75	
Median	21.00	5.60	0.002[b]
Mean ± SD	744.76±3511.34	13.17±23.26	0.540[c]
RMI cutoff (17.7)			
Below	2	24	0.000[a]
Above	23	1	
Min - max	12-158364	0-75	
Median	58.50	0.00	0.000[b]
Mean ± SD	6495.04±31640.17	4.10±15.16	0.310[c]
FoxP3 mRNA cutoff (0.186)			
Below	10	15	0.157[a]
Above	15	10	
Min - max	0.00-1097.76	0.00-3717.20	
Median	0.429	0.046	0.684[b]
Mean ± SD	73.75±234.68	247.09±792.17	0.301[c]

RMI: risk of malignancy index.
[a] Chi Square test (X^2), [b] Mann-Whitney test, [c] Student t-test.

The overall data of RMI (n = 50) were as follows: positivity rate: 24/50 (48%), range (min-max): 0-158364, median: 15.2, and mean ± SD: 3249.5 ± 22384.8. Table 2 shows RMI scores (distribution of cutoff level, minimum, maximum, median, and mean ± SD) in the two patient's pathologic groups. The marker's level showed statistically significant difference in malignant (median: serous epithelial patients: 136.50; mucinous epithelial patients: 84.90; sex cord stromal patients: 21.00, p = 0.007) but not in benign (median: neoplastic patients: 0.00; non-neoplastic patients: 0.00, p = 0.432) subgroups. There were statistically significant differences of RMI median in malignant patients' Stage (IA-C and IIA-C: 36.90; III: 241.20, p = 0.010) and grade (Gx-G1: 29.90; G2-3: 145.89, p = 0.006) groups.

The overall data of FoxP3 (n = 50) were as follows: positivity rate: 25/50 (50%), range (min-max): 0.00- 3717.20, median: 0.1865, and mean ± SD: 160.4 ± 586.6. Ten samples (six malignant and four benign neoplastic) out of 50 showed undetectable levels of FoxP3 mRNA. Table 2 shows FoxP3 mRNA levels (distribution of cutoff level, minimum, maximum, median, and mean ± SD) in the two patient's pathologic groups. The samples having odd FoxP3 mRNA levels in comparison to the trend in their pathologic group were seven benign (six neoplastic and one non-neoplastic) that had very high levels: min-max: 2.57-3717.20, median: 218.07 and mean: 882.04, and six malignant (five

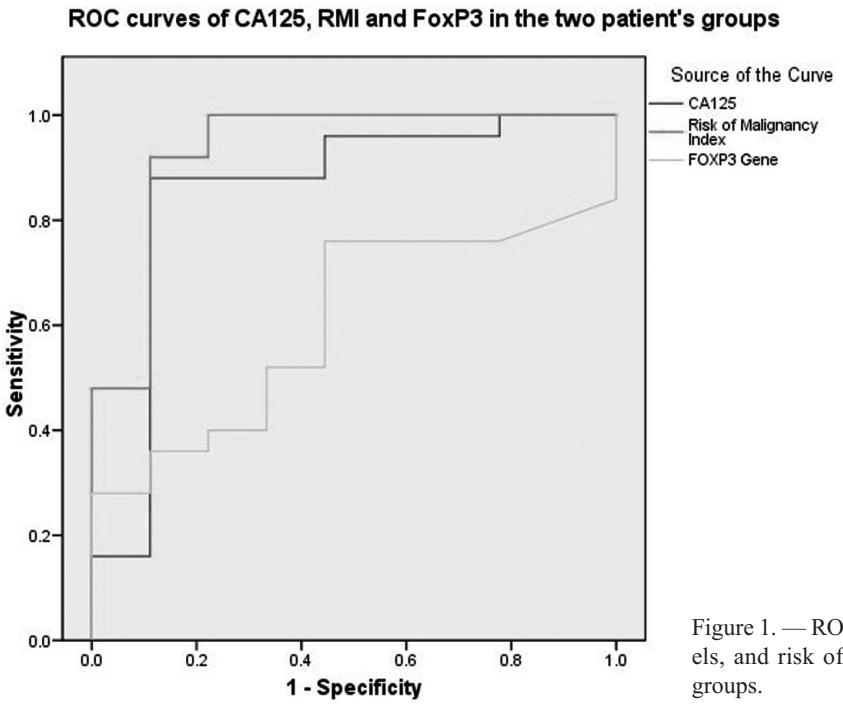

Figure 1. — ROC curves for FoxP3 mRNA, serum CA125 levels, and risk of malignancy index (RMI) in the two patient groups.

serous and one sex cord stromal) samples that had undetectable levels. The frequencies of disease stage and grade in those samples were: serous epithelial (n = 5): Stage IA-C and IIA-C: two patients, and Stage III: three patients; grade Gx-G1: one patient, and grade G2-3: four patients; and the sex cord stromal sample had Stage IA-C and grade G2.

The marker's level showed no statistically significant difference in benign (median: neoplastic patients: 0.0390; non-neoplastic patients: 1.26, $p = 0.277$) or malignant (median: serous epithelial patients: 0.19; mucinous epithelial patients: 0.48; sex cord stromal patients: 1.5, $p = 0.454$) subgroups.

There were no statistically significant differences of FoxP3 mRNA median in malignant patients' Stage (IA-C and IIA-C: 1.0763; III: 0.3405, $p = 0.618$) or grade (Gx-G1: 0.7812; G2-3: 0.1930, $p = 0.485$) groups.

ROC curve analysis of each of the three markers showed statistically significant diagnostic ability for CA125 (n = 32; AUC: 0.853; $p = 0.002$; sensitivity: 88%; specificity: 88.9%) and RMI (n = 50; AUC: 0.976; $p = 0.000$; sensitivity: 92%; specificity: 96%) but not for FoxP3 (n = 50; AUC: 0.534; $p = 0.684$; sensitivity: 60%; specificity: 60%). The best cutoff values were as follows: CA125: 8.85 U/ml, RMI: 17.7, and FoxP3: 0.186. Figure 1 shows the ROC curves of the three markers in the benign and malignant samples.

Although the diagnostic ability for FoxP3 mRNA alone was not satisfactory, its combination with CA125 or RMI showed enhancement of the diagnostic ability: sensitivity: 96% and specificity: 100% for both. Combination of CA125 and RMI did not show enhancement because the increase in their combined sensitivity (96%) was complicated by decrease in specificity (88%).

Discussion

FoxP3 has recently gained considerable interest as immune modulator molecule implicated in different types of cancer through the establishment of tumor immune evasion [21]. Indeed, assessing FoxP3 expression in peripheral blood or tumor tissue has been shown valuable indicator for disease progress [22, 23] and the efficiency of surgical treatment [24].

The clinicopathological characteristics of the present patients, despite the relatively small patients' number, agreed with the known knowledge in ovarian cancer; e.g. malignant patients had higher postmenopausal status distribution [25], and showed no correlation with contraception or smoking [26]. The present results concerning CA125 and RMI as ovarian diagnostic markers agreed also with those in the previous works, respectively [27, 28]. The much smaller cutoff value for CA125 in the present data could be due to the small sample size and particular pathologic composition in the samples; notably absence of inflammatory and endometriotic masses [29].

In contrast to previous works on FoxP3 that studied its prognostic value in different cancers (e.g. gastric [30] and bladder [31]) including ovarian cancer [32, 33], the authors addressed in the present study, for the first time, the ques-

tion whether FoxP3 would have diagnostic utility in ovarian tumors. For this aim they made a novel combination of serum CA125, RMI, and tumor tissue level of FoxP3 mRNA in benign and ovarian cancer patients. Ovarian cancerous cells showed weak to no expression of FoxP3 [11]. Nonetheless, previous works showed positive correlation in ovarian carcinoma tissues between FoxP3 expression and $CD8^{-}CD4^{+}FoxP3^{+}$ Treg cell infiltrations [32], from one side, and between FoxP3 mRNA level and FoxP3 protein expression [33], from the other side. This is why FoxP3 expression may be considered as surrogate marker for Treg cell infiltration in ovarian tumors [33].

The present results showed no statistically significant correlation between FoxP3 and any of the patients' clinicopathological variables, including CA125 and RMI. This finding would make differences in FoxP3 expression levels attributable to the disease itself rather than some other confounding variable. In the present samples, FoxP3 was differentially expressed in malignant and benign samples as well; however, it could not reach statistical significance in terms of positivity rate, median or mean values. The two pathologic groups interestingly showed nearly equal number of FoxP3 non-expressing samples (six *vs*. four, respectively), and reciprocally shared expression pattern and magnitude: malignant samples had higher positivity rate (levels above cutoff value); 1.5 times that of benign samples, and the highest level in benign samples was ~ threefold higher than that in the malignant ones.

The present FoxP3 expression data agree with those of Curiel *et al.* [32] and Wolf *et al.* [33] that showed strong expression of FoxP3 mRNA in ovarian cancer tissue samples as compared to normal ovarian tissues. Moreover, as noted above, this is the first report on FoxP3 expression in benign ovarian tumors as previous works considered only the prognostic value of FoxP3 in malignant cases. The present data concerning the correlation of FoxP3 expression to disease grade and stage agree with the previous work [33]; however, the comparison may not be justified as the cutoff they used differs from that the present authors chose to differentiate between malignant and benign samples.

Although, in the present samples, FoxP3 mRNA alone did not show good diagnostic performance in ovarian tumors, it enhanced those of CA125 and RMI in terms of increased sensitivity and absolute value (100%) specificity, for both combinations. Indeed, the present results showed no correlation between FoxP3 mRNA level and CA125 or RMI. However, the present authors should be cautious on making any statement while taking into account the obvious limitations in the present study, such as small sample size, lack of normal samples, and non-homogenous distribution of pathological subtypes in benign or malignant groups. For example, the finding that FoxP3 is weakly or non-expressed in normal ovarian tissues [32, 33] may imply possible better diagnostic ability of FoxP3 as regards overall specificity and tumor *vs*. normal sensitivity.

Conclusion

In summary, taking into account the aforementioned limitations in this study, the present results showed weak diagnostic ability of FoxP3 mRNA in ovarian tumors, but it may prove useful to enhance the diagnostic performances of serum CA125 and RMI. Moreover, FoxP3 showed differential expression in benign and cancer tissue samples and did not correlate to patients' clinicopathological variables. Therefore, it could represent a useful marker in defining the characteristic tumor molecular signature that, in turn, would be important in considering treatment modality and patients' outcome. The present results warrant further studies with larger sample size and normal tissue samples, to better evaluate and understand the role of FoxP3 in ovarian tumorigenesis and diagnosis.

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