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Research Article

Transforming Growth Factor β1 (TGF β1) mRNA Expression and Tumor Necrosis Factor mRNA Expression (TNF) in Endometrial Cancer Versus Normal Endometrial Tissue: Macrophages Polarization Markers?

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Abstract

Objectives: Tumor Associated Macrophages (TAMS) infiltration is a prominent component of peri-tumor microenvironment. Macrophages affect cancer initiation, progression and invasion: depending on different triggers, two distinct subsets of macrophages work differently and in antagonism: macrophage type 1 (M1) and macrophage type 2 (M2). M1 suppress tumor growth and metastases and express Tumor Necrosis Factor-α (TNF). In contrast, M2 exhibit pro-tumor activity repressing the immune system and produce TGF-β. Among the tumor microenvironment chemo attractants, Macrophage Migration Inhibitory Factor (MIF) has been found modulate immune responses of Tumor Associated Macrophages (TAMs) in many cancers. In this study we decided to analyze TGFb1 mRNA expression, as a marker of M2, TNF mRNA expression as a marker of M1 and MIF mRNA expression due to its actions on TAMs in order to understand which subset of polarized macrophages could be prevalent in peri-tumor microenvironment of human endometrial cancer (EC) in comparison with normal endometrial (NE) counterpart.

Materials and Methods: Fresh specimens from 15 patients with EC and corresponding NE were stored at -80°. One mcg of mRNA was reverse-transcribed in cDNA. A Real-Time PCR determined relative cDNA levels of targeted gene mRNA.

Results: In EC vs NE, we observed a down-regulation of TGFβ 1 mRNA in 81% ($P < .01$) and a down-regulation of TNF mRNA in 73% ($P = .056$) of samples. In EC vs NE, MIF mRNA was over-expressed in 100% ($P < .001$) and inversely related to TNF mRNA ($P < .001$) and TGFb1 expression. In NE, TGFb1 was over-expressed vs TNF mRNA ($P = .003$) while in EC sample this difference was no more statistically significant.

Conclusion: In NE, considering TGFb1 and TNF as TAM's markers, the prevalence of M2 over M1 (P.003) might favor tumor progression and invasion. In EC this difference is not yet significant: probably expression of relative increase of M1 in the tumor microenvironment. In EC, MIF mRNA expression seems down-regulate all TAMS, but especially M2. In order to became possibly therapeutic target, further analysis need to identify exactly in which TAMS cell subpopulation of endometrial tumor microenvironment this cytokines are over/down - expressed.

Keywords *Endometrial Cancer; Peritumor Microenvironment; mRNA; Tumor Necrosis Factor; Macrophages Type 1; Macrophages Type 2; Transforming Growth Factor β1; Migration Macrophages Inhibitory Factor; Macrophage Polarization*

Abbreviations

TNF: Tumor Necrosis Factor; TNFβ 1: Transforming Growth Factor β 1; MIF: Migration macrophage Inhibitory Factor; TAMS: Tumor Associated Macrophages; EC: Endometrial Cancer; NE: Normal Endometrium; M1 Macrophages Type 1; M2 Macrophages Type 2; FIGO: International Federation of Gynecologic Oncology

Introduction

In solid tumors, the Tumor Associated Macrophages (TAMS) infiltration, is a prominent component of the mononuclear leukocyte population which are recruited and modulated by many growth factors and chemokines/cytokines produced by tumor cells or stroma in response to the tumor itself [1].

Accumulating evidence proves that macrophages affect cancer initiation, progression and invasion: depending on different triggers, two distinct subsets of macrophages work differently and in antagonism in peritumoral microenvironment and are therefore so called "polarized". They include: macrophage type 1 (M1) and macrophage type 2 (M2). Macrophages type 1, whose prototypical activating stimuli are IFN gamma and LPS, and macrophages type 2 further subdivided in M2a (after exposure to IL-4 or IL-13), M2b (produce immune complexes in combination with IL-1beta or LPS) and M2c (produce IL-10, TGFbeta or glucocorticoids) [2]. The macrophages M1 suppress tumor growth and metastases by direct killing of cancer cells and express Tumor Necrosis Factor-α (TNF) [3,4]. In contrast, macrophages M2 exhibit pro-tumoral activity repressing the immune system and producing a wide variety of anti-inflammatory molecules, including TGF-β [5].

MIF Among the tumor microenvironment chemo-attractans, Macrophage Migration Inhibitory factor (MIF) has been found to enhance tumor cells metastases by modulating immune responses of macrophages and by promotion of angiogenesis in many non-hormone dependent cancers [6-12]. Vice versa, in breast cancer versus normal tissue, up-regulated MIF was reported statistically significantly directly related with progesterone and estrogen receptors expression of good prognosis and indirectly related with tumor size [13].

In this study we decided to analyse TGFb1 mRNA gene expression, as a marker of M2, TNF mRNA gene expression as a marker of M1 and MIF mRNA expression due to its actions on TAMs in order to understand which subset of polarized macrophages could possibly be supposed prevalent in peritumoral microenvironment of human endometrial cancer in comparison with normal endometrium counterpart.

Materials and Methods

Immediately after surgery, fresh samples of endometrial cancer (EC) and their normal endometrial counterpart (NE) were obtained from patients submitted to primary surgery for endometrial cancer at RCCS Humanitas Clinical Institute in Milan (Italy). Parts of the samples were used for the histologic diagnosis and other parts were immediately treated with RNA later (Ambion) for 24 - 36h at 4°C, and subsequently dried and stored at 80°. The study was approved by the Ethical Committee of Humanitas Research Institute and informed, written consent was obtained for all patients. All the clinical and surgical data were recorded on a data base. The total RNA was isolated both from endometrial cancer and normal endometrial specimen using TRI Reagent (Ambion). RNA was quantified by Nanodrop spectrophotometer ND-1000and its quality was examined by 1.5% agarose gel electrophoresis. According to the manufacturer's instructions, 1 mg of total RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems), treated with DNase I, quantified and reverse - transcribed into cDNA using random primers. A real-time quantitative polymerase chain reaction, using Syber Green I (Applied Biosystem) as detection dye, was used to deter-mine the relative cDNA levels of genes in each samples. The amplification protocol was used as following: 2 minutes at 50°C to activate uracil-DNA glycosylase, 10 minutes at 94.5°C (activation), 40 cycles of denaturation al 97°C for 30s and annealing and extension at 59.7°C for 1 minute. The relative amount of each target gene mRNA to the housekeeping

gene (18S) was calculated as 2 (DCt), where DCt@Ct gene ACt housekeeping gene. The fold-change of each tar-get gene mRNA to the corresponding normal tissue was calculated as 2 (DDCt), where DDCt@DCt target gene in tumor tissue - DCt target gene in normal tissue. The threshold cycle Ct was automatically given by the SDS2.2 software package (Applied Biosystems).

We analyzed TNF, MIF and TGFβ 1 gene expression.

Statistical analysis

Statistical significance was determined by T- test and considered significant at a P value of < 0.05.

Results and Discussion

We collected tissue samples from endometrial cancer (EC) and from normal corresponding endometrium (NE) in 15 patients with endometrial cancer FIGO stage I-IIIC. All patients were submitted to primary laparoscopic total hysterectomy and bilateral salpingectomy with pelvic lymphadenectomy. Four patients dropped out from the study: two because the endometrial sample was damaged during the storage making it impossible to process, and two because no residual tumor was found in the samples, despite an initial histological diagnosis by endometrial biopsy. Table 1 and 2 describe the clinical characteristics. Three patients (27%) underwent adjuvant chemotherapy and pelvic radiotherapy and one patient (9%) underwent adjuvant pelvic radiotherapy (Table 2). At a median 3 years follow-up, the median disease free survival was 25 months (range 18 - 36). Only one patient with clear cell adenocarcinoma FIGO stage IIIA and no residual disease after surgery relapsed at 18 months (Table 2).

No. of patients	11
Median Age	63 (range 53 - 81)
Median BMI (Kg/m^2)	28 (range 25 - 31)
FIGO stage I	8 (73%)
IA	7 (64%)
IB	1 (9%)
FIGO stage III	3 (27%)
III A	2 (18%)
III C	1 (9%)
Histotype	
Endometrioid	7 (64%)
Clear Cell	2 (18%)
Villoglandular	1 (9%)
Endometrioid with squamous differentiation	1 (9%)

Table 1: *Evaluable patients' clinical characteristics.*

BMI: Body Mass Index.

Pt.	Age	FIGO stage	LVS	N	G	Histotype	ADJ	DFS mths
1	66	IA	-	-	G3	AE	FU*	30
2	65	IA	-	-	G3	ACC	PAC + RT	32
3	75	IA	-	-	G1	AV	FU	36
4	63	IA	-	-	G2	AE	FU	35
5	58	IA	-	-	G2	AE	FU	23
6	68	IA	-	-	G2	AE	FU	24
7	61	IA	-	-	G2	AE	FU	36
8	81	IB	-	-	G2	AE	FU	22
9	53	IIIA	-	-	G2	AS	PAC + RT	25
10	81	IIIA	+	-	G2	ACC	CT +RT	18°
11	63	IIIC	+	+	G2	AE	RT	23

Table 2: *Clinic characteristics of 11 evaluable patients.*

*LVS: Lymphovascular Space; N: Lymph Nodes; G: Histological Grade; ADJ: Adjuvant Therapy; DFS: Disease Free Survival in Months; PAC: Cisplatin, Paclitaxel; CT: Carbo Taxol; RT: Radio Therapy; AE: Endometrioid Adenocarcinoma; ACC: Clear Cell Adenocarcinoma; AV: Villoglandular Adenocarcinoma; AS: Squamous Adenocarcinoma; *Patient refused RT; ° Abdomino-pelvic relapse after 18 months.*

In endometrial cancer versus normal counterpart, we observed that mRNA gene expression of TGFβ 1 was significantly down-regulated in 81 % of samples (Figure 1, P < .01) and TNF in 73% of samples (Figure 2, P = .0056). In endometrial cancer versus normal counterpart MIF mRNA was over-expressed in 100% of samples (Figure 3, P < .0001). In the comparison between MIF and TNF mRNA expression in endometrial cancer samples, we found a statistically significant inversely relation (Figure 4, P < .001). The same correlation was found in the comparison between MIF and TGFb1 expression in endometrial cancer (Figure 5, P < .001). More in detail, in normal endometrial samples TGFb1 mRNA was statistically significantly over-expressed in comparison with TNF mRNA (Figure 6, P < .003), while in endometrial cancer this differences was not yet significant (Figure 7, P = NS).

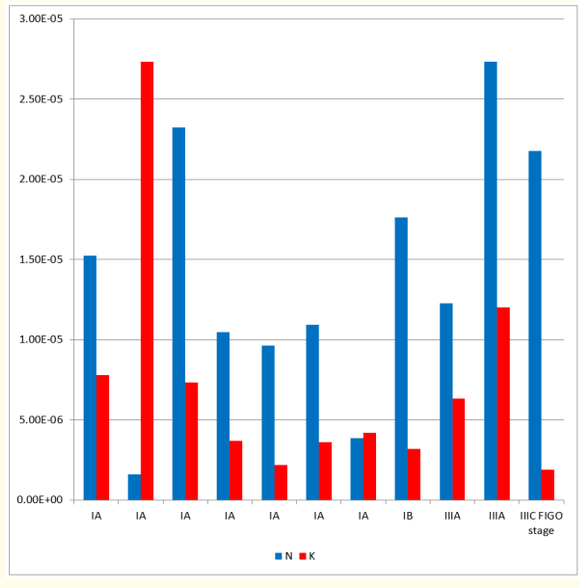

Figure 1: *Transforming growth factor beta (TGFβ1) mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K).*

In 81% Endometrial Cancer (K) versus normal endometrium counterparts (N) TGFβ1 mRNA gene expression was down regulated (P < .01).

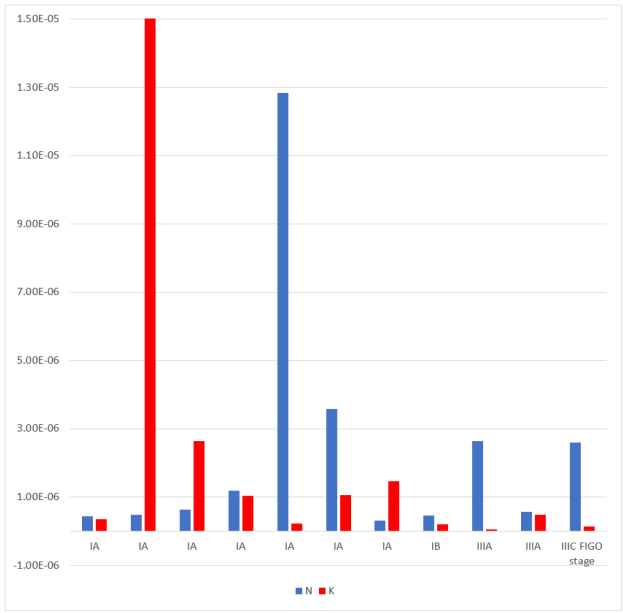

Figure 2: *Tumor necrosis factor (TNF) mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K).*

In 73% endometrial cancer versus normal endometrium counterparts, TNF mRNA gene expression was down- regulated (P = .05).

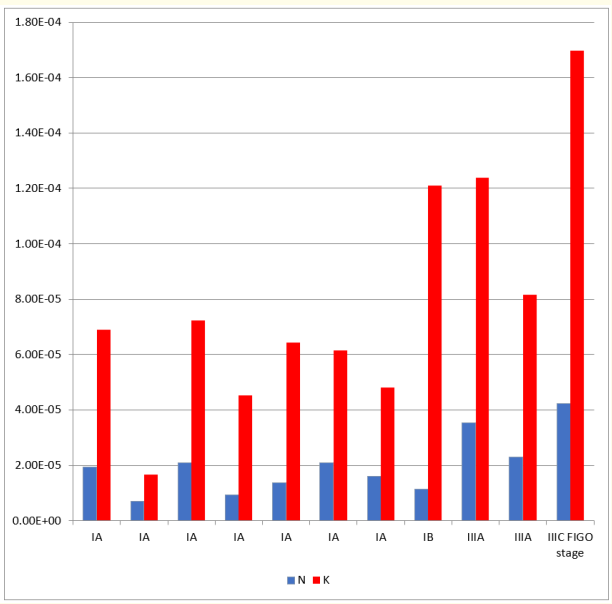

Figure 3: *Migration macrophages inhibitory factor (MIF) mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K).*

MIF was over-expressed in 100% endometrial cancer (P < .001).

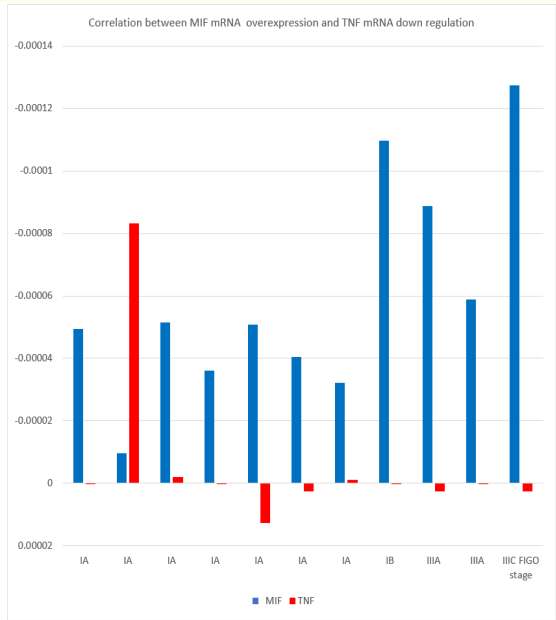

Figure 4: *In endometrial cancer samples, correlation between MIF mRNA over-expression and TNF mRNA down-regulation. MIF mRNA over expression was directly related to TNF mRNA (M1 marker) down-regulation (P < .001).*

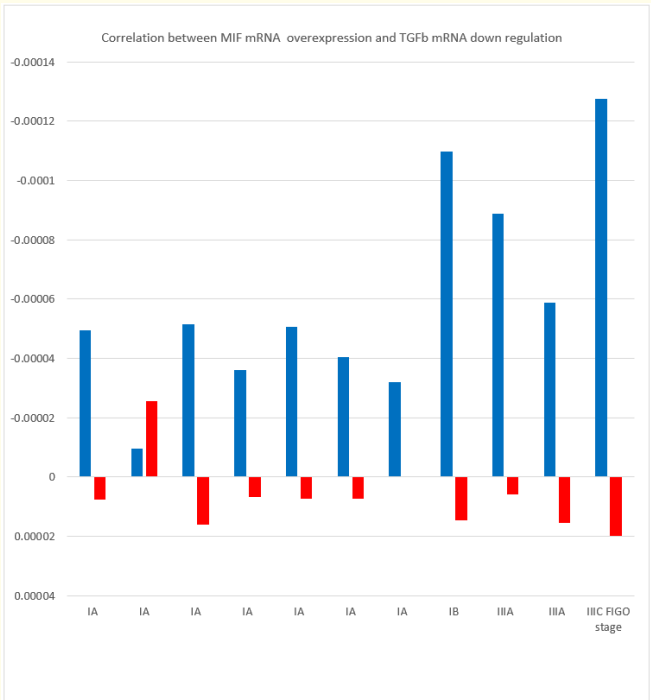

Figure 5: *In endometrial cancer samples, correlation between MIF mRNA over-expression and TGFβ1 mRNA down-regulation. MIF mRNA expression was inversely related to TGFβ1 mRNA expression (M2 marker) P < .001.*

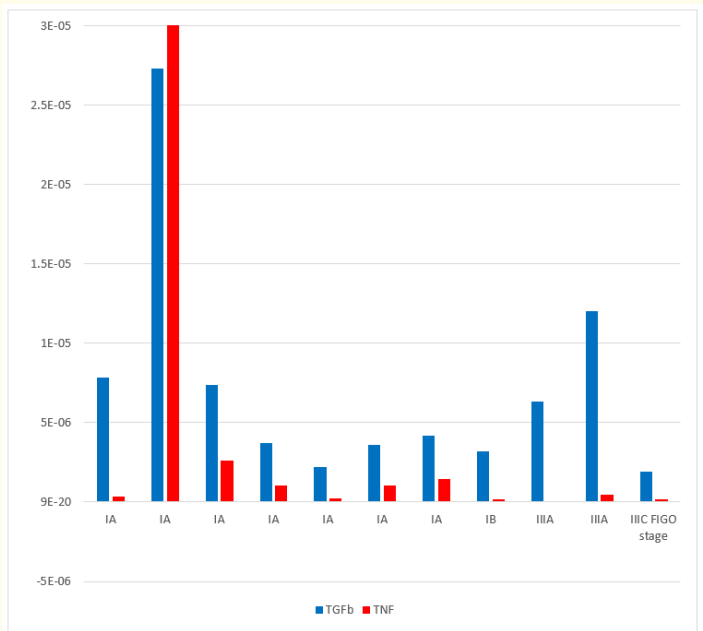

Figure 6: *In normal endometrial samples, we compared TGFβ1 mRNA expression versus TNF mRNA expression. In normal endometrium TGFb1 mRNA (marker of M2) was significantly over-expressed in comparison with TNF mRNA (marker of M1) (P = .003).*

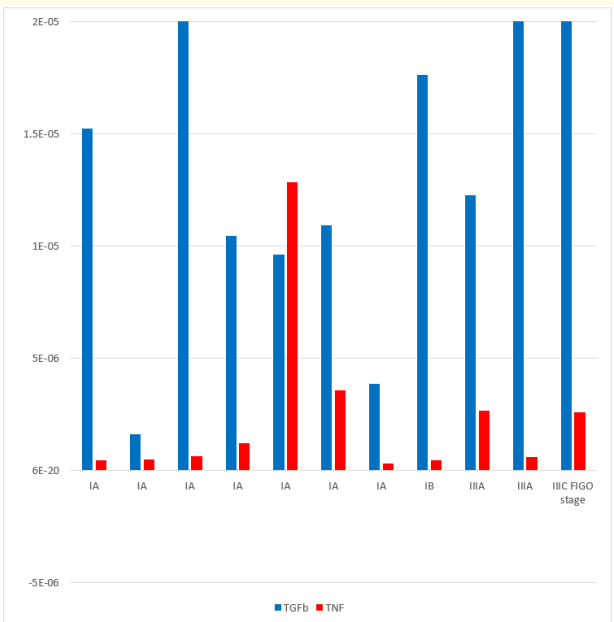

Figure 7: *In endometrial cancer samples, we compared TGFβ1 mRNA expression versus TNF mRNA expression. In endometrial cancer samples TGFb1 mRNA (marker of M2) was over-expressed in comparison with TNF mRNA (marker of M1) but this differences was not statistically significative (P = NS).*

In Literature no one compared TGFb1 and TNF in endometrial cancer fresh tissues samples as a marker of TAMS. As expected in our series, included mainly early stage of disease and low risk histotype (Table 1), considering TGFb1 as a marker of M2 and TNF as a marker of M1, we might assume that in endometrial cancer microenvironment, compared with normal endometrium counterpart, there is a down-regulation of both M1 and expecially M2 mediated by MIF. These data are consistent with previous observation in Literature that both TNF and TGFb1 are reported to enhance tumor- progression and poor prognosis [14-17]. In addition, our finding regarding over-expression of MIF ($P < .001$) in endometrial cancer versus normal endometrium, is in line with the results previously reported by Bando in breast cancer, an hormone dependent tumor, where up-regulation of MIF was related to a good prognosis [11]. These result could be explicited by assuming that M1 in a first moment can interfere with tumor growth, but in a second one it could favor tumor invasion. Indeed, in a previous analysis of tumor growth kinetic in the endometrial cancer xenograft model, Dumas indicated that M1 greatly reduced, while M2 increased tumor size respectively when compared to control [18]. However, more specifically, *in vitro* assays Dumas indicated that M2 increased cell viability but failed to promote cell invasion while M1 in contrast, decreased cell survival but in the other hand greatly increased cell invasion [18]. On one side, in addition we reported in normal endometrial samples (adjacent to endometrial cancer), an over expression of TGFb1 versus TNF ($P = .003$) that could be interpreted as a prevalence of M2 over M1 in the normal microenvironment where endometrial cancer developed and where the M2 higher density could have possibly favored tumor progression and invasion. On the other side, in endometrial cancer samples the difference between M1 and M2, expressed by TNF and TGFb1, was not yet significant and this could be explained probably by a relative increase of M1 over M2 as an attempt to favor invasion. In literature, increased TNF serum concentration has been reported in patients with endometrial carcinoma significantly related to a higher risk of endometrial cancer [19-21]. We found a down regulation of TNF and this result is in line with Literature considering that our study population included mainly early stage of disease and low risk histotypes. More in detail, our finding of M2 prevalence over M1 in endometrial cancer, confirm the data reported by Kelly showing higher density of M2 in endometrial hyperplasia (median 29 M2/HPF) compared with endometrial type 1 (median 12.5 M2/HPF) and type 2 cancer (median 11.5 M2/HPF) [22].

Conclusion

In NE, the prevalence of M2 over M1 (P.003) might favor tumor progression and invasion. In EC this difference is not yet significant: probably expression of relative increase of M1 in the tumor microenvironment that could be interpreted as an attempt to invasiveness. In EC, MIF mRNA expression seems down-regulate all TAMS, but especially M2 and can reinforce the data that its over expression is related to better prognosis.

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