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Influence of Hypoxia Inducible Factors on the Immune Microenvironment in Ovarian Cancer

MARKUS DUECHLER[1], LUKASZ PECZEK[1], MARIA SZUBERT[2] and JACEK SUZIN[2]

[1]*Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, Poland;*
[2]*Department of Gynaecology and Obstetrics of the Medical University of Lodz, Clinical Department of Operative and Oncological Gynaecology, Lodz, Poland*

Abstract. *Background: Ovarian tumors remain immunogenic even at advanced stages, but cancer-induced immunosuppression abrogates immune surveillance. The composition of the immune microenvironment in ovarian tumors was characterized by analyzing selected immunosuppressive factors in specimens from cancer patients. The influence of the hypoxia inducible factors on the immune microenvironment was also addressed. Materials and Methods: Tumor tissue was collected from 21 ovarian cancer patients immediately following tumor excision during surgery. The mRNA expression of selected genes was quantified, and tumor infiltrating leukocytes were characterized by flow cytometry to identify regulatory T-cells, myeloid-derived suppressor cells, and type-2 macrophages. Results: Overall, a pronounced heterogeneity was found among the analyzed samples. Nevertheless, statistical analysis revealed that the expression of hypoxia inducible factors correlated with the transcription levels of several immunosuppressive molecules. Conclusion: The activity of hypoxia inducible factors contributes to cancer immunosuppression in ovarian cancer patients.*

Ovarian cancer accounts for approximately 4% of newly-diagnosed cancers in women. Despite recent progress in diagnosis and treatment, ovarian cancer still is responsible for about 5% of female deaths caused by malignant neoplasms (1). The 5-year survival rate of surgery followed by chemotherapy is below 50% (1, 2). This rather bad prognosis is mainly the consequence of late diagnosis – usually in advanced III/IV clinical stage – due to lack of initial symptoms. Moreover, intrinsic and acquired drug resistance induces a high relapse rate.

For some time, ovarian cancer was not considered highly immunogenic. The apparent lack of immunogenicity was explained by the clonal evolution of cancer cells resulting in cells devoid of immune-stimulating antigens (3). Meanwhile it became clear, that ovarian tumors remain immunogenic at advanced stages, and that it is the cancer-induced immunosuppression which mainly controls immune escape (4, 5). As more evidence is accumulating for an activated but suppressed immune response in ovarian cancer, therapies aiming at re-activation of an anticancer immune response gain attention as alternative or complementary treatment options (6).

Anticancer immunity is shut-down through direct cell-cell interactions between cancer cells and leukocytes and through soluble factors, altogether establishing an immunosuppressive environment for unimpeded cancer growth. There, the maturation of myeloid cells is inhibited while the development of regulatory T (T-reg)-cells is favoured. T-reg cells very efficiently block anticancer immune reactions (7) and the levels of T-reg cells in the peripheral blood of ovarian cancer patients have been correlated with disease relapse and poor prognosis (8, 9). The conversion of naive T-cells into T-reg cells is either promoted directly by tumor cells or by other suppressor cells which are attracted and instructed by the tumor. A major population among these tumor-associated immunosuppressive cells are myeloid-derived suppressor cells (MDSC) which include several sub-types like myeloid progenitor cells, immature dendritic cells and granulocytes (10, 11). Also macrophages are converted from an M1 type, capable of lysing tumor cells and presenting tumor-associated antigens, into M2- type suppressor cells, which exert tumor supportive activities promoting invasion and metastasis (12).

Accumulating evidence indicates that hypoxia contributes to cancer immune suppression as well (13-16). Hypoxia, the lack of sufficient oxygen supply, is a common feature of solid tumors and aggravates the disease in different ways. Hypoxia is well-known to increase resistance to chemotherapy and

Correspondence to: Markus Duechler, Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, 112, Sienkiewicza Street 90-363 Lodz. Tel: +48 426803272, Fax: +48 426815483, e-mail: mduchler@cbmm.lodz.pl

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radiotherapy, and to stimulate the release of angiogenic factors which induce the growth of new blood vessels (17). Tumor hypoxia also promotes epithelial-mesenchymal transition (EMT) and metastasis formation (18-21), and regulates the frequency of tumor-initiating cells (22). Hypoxic conditions activate the hypoxia-inducible factors (HIFs), the master transcription factors governing the cellular response to low oxygen tension. HIFs (mainly HIF-1 and HIF-2) are heterodimeric proteins that consist of a tightly regulated α-subunit and a constitutively expressed β-subunit. The α-subunits are rapidly degraded in normoxic conditions but are stabilized under hypoxia. Alternatively, increased HIF-1α protein and mRNA levels can be stimulated under normoxic conditions by growth factor signaling (23-25) which is frequently constitutively activated in ovarian tumors (26, 27).

Several immunosuppressive factors are direct HIF target genes such as VEGF (vascular endothelial growth factor), which exerts immunosuppressive functions in addition to its angiogenic activity (28). Hypoxia regulates the maturation and function of MDSC and constitutively activated HIF-1 in advanced tumors maintains elevated MDSC levels (29). MDSC express several enzymes involved in immunosuppression, such as indoleamine 2,3-dioxygenase-1 (IDO-1) which catalyzes the rate-limiting step of tryptophan metabolism. Up-regulation of IDO-1 inhibits T-cell activation and proliferation by tryptophan deprivation (30). MDSC also produce immunosuppressive cytokines, such as TGF-β (transforming growth factor-β) and IL-10 (interleukin-10). TGF-β together with IL-6 and IL-8 was found to frequently contribute to the establishment of the immunosuppressive environment in ovarian cancers (31).

IL-10 and TGF-β are also secreted by M2 type tumor-associated macrophages (TAM) which are attracted into hypoxic areas (32-34). In ovarian cancer, the presence of TAMs was inversely associated with relapse-free survival (35). Also T-reg cells are recruited into the hypoxic tumor through the chemokine ligand-28 (CCL28) (36). The conversion of naive into regulatory T-cells is controlled by the transcription factor FoxP3 which is up-regulated in hypoxia through HIF-1 (37). One of the FoxP3 target genes is heme oxygenase-1 (*HO-1*) (38) which is essential for T-reg-mediated immune suppression (39). Another transcription factor important in cancer immunosuppression is STAT-3 (40) which controls the expression of several immunosuppressive molecules and regulates the expansion and survival of MDSC (41). STAT-3 interacts with HIF-1α degradation prolonging the half-life of HIF-1α in solid tumor cells (42) suggesting that the functional interaction of STAT-3 with HIF-1 aggravates immune suppression (14).

We were interested to characterize the immunosuppressive microenvironment in ovarian cancer to evaluate the possible impact of HIFs. To address these questions, the mRNA of selected HIF target genes (cytokines, enzymes, transcription factors) were quantified in tumor tissue specimens after ovarian cancer surgery, and tumor-infiltrating leukocytes were characterized. Our analysis aimed at yielding a picture of the tolerogenic interactions between cancer cells and their immune microenvironment to visualize the immunosuppressive status and to possibly identify a ‘hypoxic signature’ in samples with elevated HIF expression. The obtained data were analyzed by statistical methods to identify sub-groups with common expression patterns and associations with clinical features.

Materials and Methods

Patient samples. Twenty-one patients with an average age of 56.1±14.1 years were included in the study. All patients had undergone primary surgery for ovarian cancer at the Clinical Department of Operative and Oncological Gynaecology, Medical University of Lodz. The study was approved by a local ethical commission and conducted according to the Helsinki declaration of ethical principles for medical research. Information about clinical parameters including staging, tumor size and ascites are shown in Table I.

Tissue processing. Immediately after surgery a small piece of tumor tissue (20-50 mm^3) was submerged in RNAlater (Ambion/Life Technologies, Carlsbad, CA, USA) and transported on ice to the laboratory where it was stored at –20˚C until RNA isolation. A second tissue piece of about 0.3-0.8 cm^3 was put into HBSS-containing penicillin, streptavidin and fungizone and kept on ice until further processing. This tissue was minced with a scalpel and digested in HBSS/antibiotics with collagenase IV (Sigma-Aldrich, Poland) at 1 mg/ml for 1-1.5 h at 37˚C. The obtained suspension was passed through a cell strainer (pore size 40 μm). Erythrocytes were lysed for one minute at room temperature in erythrocyte lysing solution (BD Biosciences/Immunogen, Chorzow, Poland). After an additional washing step in staining buffer (PBS, 1% BSA, 0.1% azide) the cell suspension was used for cell characterisation.

RNA isolation and Quantitative Reverse Transcription PCR. For RNA isolation, the specimens were manually homogenized in a lysing reagent (TriPure Isolation Reagent, Roche Diagnostics, Mannheim, Germany) and total RNA was isolated according to standard protocols. The mRNA levels of *HIF-1α, HIF-2α, IL-10, VEGF-A, FoxP3, TGF-β1, HO-1, STAT-3, IDO-1* were evaluated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the LC RNA amplification kit SYBR Green I, and a LightCycler Instrument 1.0 (Roche Diagnostics). The target gene expression levels were related to the house-keeping reference gene TATA-box binding protein (TBP). All primers used have been published elsewhere (43). The amplification products were analysed by the thermal dissociation method.

Cell characterization by flow cytometry. Antibodies for flow cytometry were purchased from BD Biosciences, except for APC-labelled anti-FoxP3 which was from eBioscience (Bender MedSystems, Vienna, Austria). For the characterisation of various kinds of immune suppressor cells the following combinations of directly-labelled antibodies were used: T-reg cells, CD4-CD25-FoxP3, CD3-CD8-CD28, CD8-CD28-FoxP3, and CD3-IL-10; the T-cell subset characterised by CD3-CD4-CD25 was also assessed; myeloid-derived suppressor cells (MDSC), CD11b-CD14-HLA-DR and CD11b-CD15-HLA-DR; and type 2 macrophages, CD14-

CD40-CD163. Antibodies detecting CD45 were included for a clear separation of leukocytes from other cell types. B cells and NK cells were characterised by staining of CD19 and CD56, respectively. Appropriate isotype controls were included in each experiment. For intracellular stainings (FoxP3, IL-10) cells were fixed with 4% formaldehyde solution and permeabilized with 0.2% Nonidet P-40. Flow cytometric data acquisition was performed on a FACS-Calibur and CellQuest Pro was used for data analysis (BD Biosciences). Due to the limited amount of tissue each experiment was conducted only once.

Statistical analysis. The normality of the data distribution was analyzed by the Shapiro-Wilks' W-test. The relation of two sets of parameters was calculated with Spearman coefficient. Differences of the median values between two independent groups of data were calculated using the non-parametric Mann–Whitney *U*-test. For the cluster analysis the Euclidean distance was measured and the Ward's method was chosen as amalgamation rule. All statistical analyses were done using Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA). A value of $p<0.05$ was considered statistically significant.

Results

Highly variable degree and composition of tumor infiltrating leukocytes (TIL). TIL were characterized by flow cytometry. Antibody combinations were chosen for the characterisation of various kinds of T-reg cells, MDSC- and M2-type macrophages. Median values and ranges of the relative leukocyte fractions obtained in the 21 ovarian cancer samples are shown in Table II. The only suppressor cell type found in all patients was the T-reg cell subset characterized by $CD3^+CD8^+CD28^-$ expression. The relative numbers of TIL (CD45+) and of most cellular subsets were distributed over a wide range indicating a high variability of immune cell infiltration among the examined tumor samples.

Expression of HIF-1α, HIF-2α and their target genes. The mRNA levels of *HIF-1α* and HIF-2α, as well as of seven potential HIF target genes with known immunosuppressive function were assessed by one-step qRT-PCR (Table III). The mRNA expression of the transcription factors *FoxP3* and *STAT-3*, the enzymes *HO-1* and *IDO-1*, as well as the cytokines *IL-10*, *VEGF-A*, and *TGF-β1* was measured. The results of qRT-PCR analysis were related to the house-keeping gene product TATA-box binding protein (*e.g. HIF-1α/TBP*) for statistical analysis. These relative expression levels are shown in Table III. The mRNA expression of most HIF target genes was detected in all samples. A relatively elevated expression of *HIF-1α* (>2-fold higher than the expression of the house-keeping gene) was found in about 25% of the samples.

Hierarchical clustering and correlation analysis reveals a 'hypoxic signature'. For each patient, 18 values were assessed which characterized part of the immunosuppressive microenvironment. Pattern recognition analysis using hierarchical clustering with the combined data for mRNA expression and TIL numbers did not reveal any clearly separated subgroups. Clustering based on the mRNA expression data alone brought out a small group of samples (6 out of 21, Figure 1A) with an elevated expression of *HO-1, IDO-1, IL-10, VEGF-A* and *STAT-3* mRNAs (Figure 1B). Also the expression levels of HIF-1α and HIF-2α were increased in this subgroup although not with statistical significance (Figure 1B). Further analysis showed that the small subgroup with relatively elevated expression of HIF target genes had a reduced number of infiltrating $CD4^+CD25^+FoxP3^+$ T-reg cells ($p=0.043$), but a higher number of B-cells ($p=0.036$).

The data sets obtained from the determination of TIL cell type frequencies and gene expression were analyzed pairwise. This analysis resulted in a number of significant associations as shown in Tables IV and V. For *HIF-1α* mRNA expression positive correlations were found with the mRNA levels of *HIF-2α, Stat3, IDO-1* and *VEGF-A*, and with the relative number of $CD8^+CD28^-FoxP3^+$ cells. *HIF-2α* mRNA expression correlated positively with *STAT-3* mRNA levels, and *STAT-3* expression was connected to IDO-1 and VEGF-A expression. The number of *IL-10*-expressing T-reg cells was associated with *VEGF-A* and *IL-10* mRNA levels. More positive

Table I. *Characteristics of study patients*.

Number of patients	21
Age (years)	
Mean±SD	56.1±14.1
Tumor size (cm)	
Range (median)	5-30 (11)
Body/mass index	
Mean±SD	22.3±4.3
Number of children	
Range (median)	0-7 (1)
Ascites (ml)	
Range (median)	0-7,000 (850)
Ca-125 (U/ml serum)	
Range (median)	10.7-4,768 (266)
Clinical stage	
Borderline	5
Stage 1	5
Stage 2	1
Stage 3	9
Histological grading	
G0	5
G1	0
G2	6
G3	7
Histology	
Serous	4
Mucinous	2
Endometrioid	7
Other/not determined	8
Subtype: adenocarcinoma	8

Table II. *Relative percentages of tumor-infiltrating leukocytes based on the expression of characteristic markers. The percentage of immunosuppressive cell subsets was related either to the percentage of CD45-positive cells to obtain relative leukocyte fractions, or to the total T cell (CD3-positive) fractions in case of Treg cell subsets. Percentages are given as the range and the median values.*

Cell type	Markers	Relative leukocyte fraction	
		Range (%)	Median
Leukocytes (rel. to total cell number)	$CD45^+$	1.45-53.0	6.33
T cells (rel. to total cell number)	$CD3^+$	0.30-30.0	1.52
B cells (rel. to $CD45^+$)	$CD19^+$	0.18-17.0	1.36
NK cells (rel. to $CD45^+$)	$CD56^+$	0-36.0	0.99
T cell subset (rel. to $CD3^+$)	$CD3^+/CD4^+/CD25^+$	0-10.0	1.33
Treg cells (rel. to $CD3^+$)	$CD4^+/CD25^+/FoxP3^+$	0-4.67	1.69
	$CD3^+/CD8^+/CD28^-$	6.67-71.5	34.4
	$CD8^+/CD28^-$/FoxP3+	0-26.3	2.93
	CD3+/IL-10^+	0-4.67	0.30
MDSC (rel. to $CD45^+$)	$CD11b^+/CD14^+/HLA\text{-}DR^-$	0-39.1	2.07
MDSC (rel. to $CD45^+$)	$CD11b^+/CD15^+/HLA\text{-}DR^-$	0-27.4	0.51
M2 macrophages (rel. to $CD45^+$)	$CD14^+/CD40^+/CD163^+$	0-22.0	0.27

Table III. *Relative expression levels of 9 genes determined in 21 ovarian cancer samples by the ΔCt method. The ratio of the expression of the tested genes is shown in relation to TBP expression.*

Gene	Median (n-fold)	Range (n-fold)
HIF-1α	1.05	0.15-9.45
HIF-2α	0.003	0.0003-0.031
IL-10	0.05	0.001-1.71
VEGF-A	1.61	0.06-21.37
TGF-β1	0.007	0.0015-0.05
HO-1	0.27	0.00-2.30
IDO-1	0.05	0.00-1.57
FoxP3	0.07	0.02-0.31
STAT-3	0.50	0.01-3.23

Table IV. *Significant correlations were detected by pairwise association analysis of mRNA expression levels. Correlations were calculated with the Spearman coefficient.*

mRNA Expression	mRNA Expression	*p*-Value
HIF-1α	HIF-2α	0.003
HIF-1α	STAT-3	0.017
HIF-1α	IDO-1	0.014
HIF-1α	VEGF-A	0.003
HIF-2α	STAT-3	0.042
HIF-2α	VEGF-A	0.061
HO-1	IL-10	<0.0001
STAT-3	IDO-1	0.003
STAT-3	VEGF-A	0.001
IDO-1	VEGF-A	0.001

correlations were found between the mRNA expression values for *IL-10* and *HO-1*, as well as *IDO-1* and *VEGF-A*. An association of *HIF-2α* with *VEGF-A* expression was also observed but did not reach statistical significance (p=0.061).

Several parameters determined in the clinic (including tumor grade, clinical stage, tumor size, age, *etc.*) (Table I) were also included in the statistical analyses (Table V). Cancer cell grading based on scores for proliferation and differentiation revealed that approximately one third of patients was grade 2, and another third was grade 3. G3 patients showed a significant higher age than patients in lower stages. Also the *HIF-2α* mRNA expression was higher in samples from older patients. Furthermore, the volume of ascites fluid was found to correlate with *HIF-1α* mRNA expression. The histological classification of ovarian cancer knows 4 major subtypes, serous (30-70%), endometrioid (10-20%), mucinous (5-20%), clear cell (3-10%), as well as undifferentiated ones (1%). Due to the relatively small number of patients involved in our study, the sample number for most subgroups was too small to be involved in any any meaningful statistical analyses. However, tumor tissues characterized as adenocarcinoma (8 out of 21 samples) showed a positive association with the expression of VEGF-A (Table

Table V. *Associations of mRNA expression levels with relative cell type numbers and clinical features. The Spearman coefficient or the Mann–Whitney U-test were employed for the calculation of the significance of the correlations.*

mRNA Expression	Second parameter	*p*-Value
HIF-1α	$CD8^+CD28^-FoxP3^+$	0.044
IL-10	$CD3^+$IL-10^+	0.013
VEGF-A	$CD3^+$IL-10^+	0.003
TGF-β	$CD3^+CD4^+CD25^+$	0.004
HIF-1α	volume of ascites fluid	0.034
HIF-2α	age	0.027
VEGF-A	adenocarcinoma	0.018

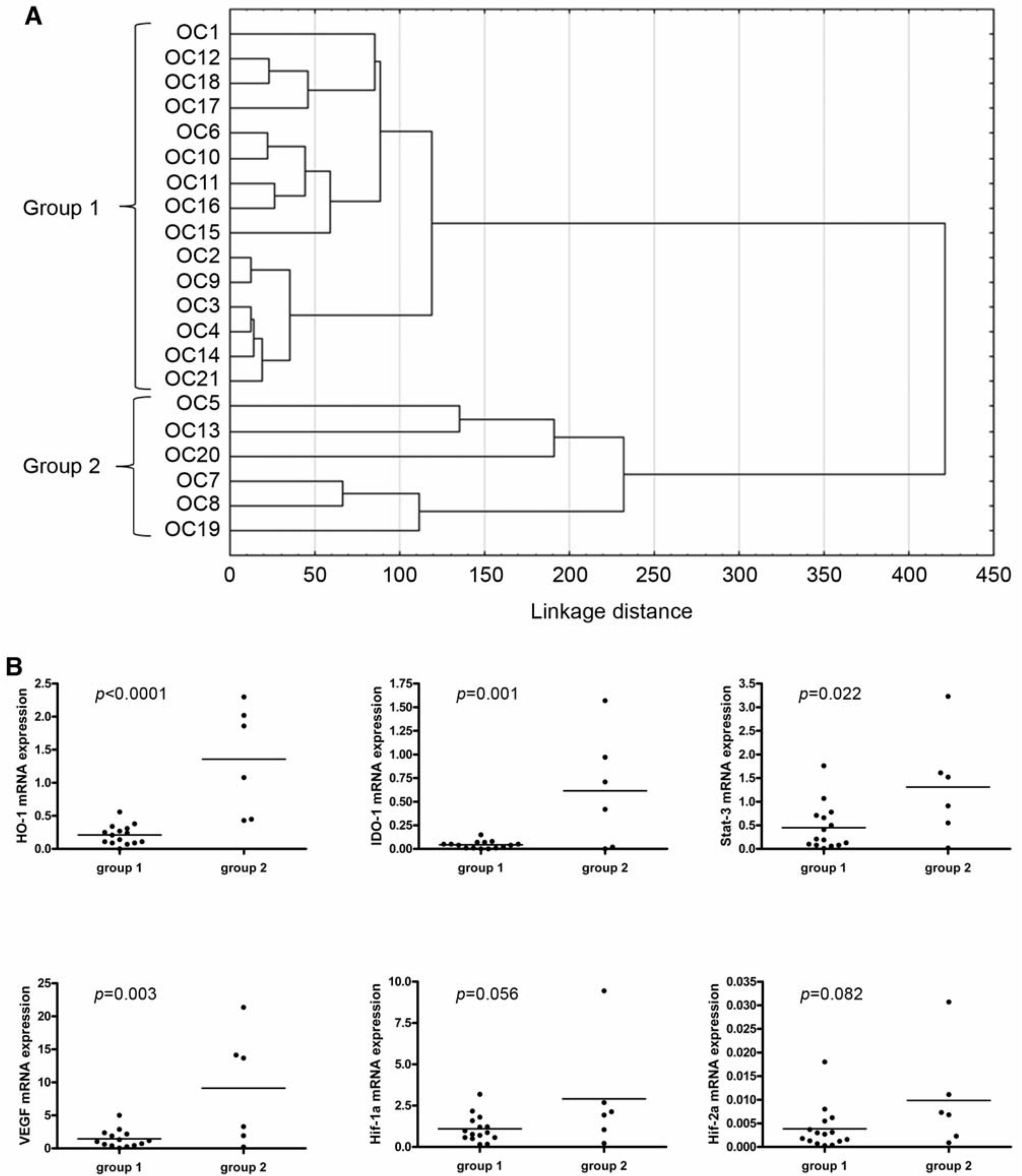

Figure 1. *Hierarchical tree plot based on the relative mRNA expression data. A: The results of the qRT-PCR analysis were related to the house-keeping gene product TATA-box binding protein. The resulting relative expression levels were subjected to cluster analysis using Euclidean distance as a distance measurement method. The Ward's method was chosen as amalgamation rule. B: Comparison of expression levels of HO-1, IDO-1, IL-10, VEGF-A, STAT-3, HIF-1α and HIF-2α in the two subgroups derived from the cluster analysis.*

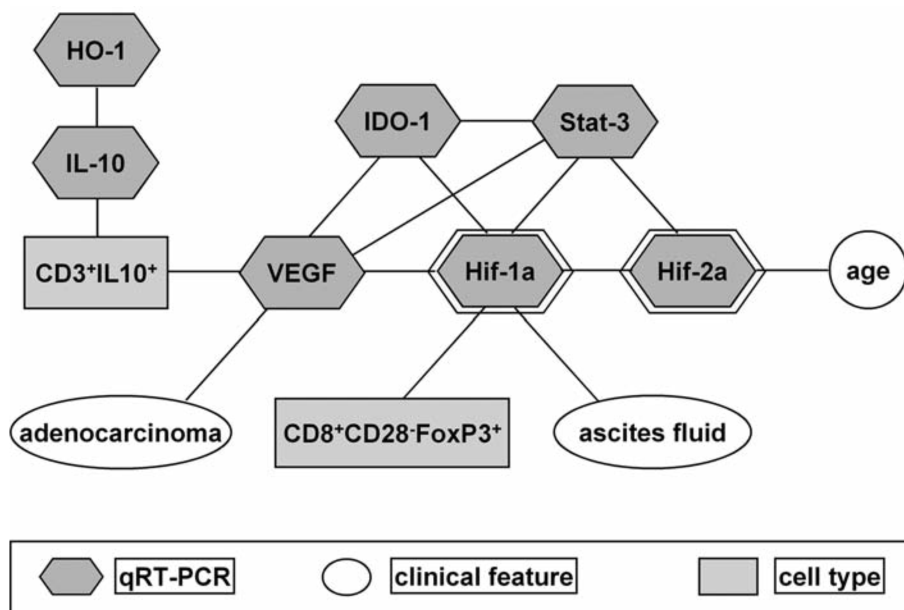

Figure 2. *Schematic representation of positive correlations between the parameters assessed in the study. Comparison of single parameter sets (e.g. $CD3^+/CD8^+/CD28^-$ T-reg cells versus HIF-1α mRNA expression) was performed by Spearman coefficient analysis, while median values from defined sub-groups (e.g. VEGF mRNA expression in adenocarcinoma samples) were compared by the non-parametric Mann–Whitney U-test.*

V) and a reduced relative number of $CD3^+CD8^+CD28^-$ T-reg cells (p=0.024). The statistically significant positive correlations are schematically summarized in Figure 2.

Discussion

A large number of immunosuppressive cells and factors is involved in the induction and maintenance of tolerance which is required for the establishment of malignant tumors. The components of the immune microenvironment constitute an immunoregulatory network whose actual composition may have profound influence on disease outcome (44). HIFs and STAT-3 are among the key transcription factors controlling the expression of several tolerogenic factors in the tumor tissue (16). To obtain some insight into the immune signature of individual ovarian tumors the relative amount of selected suppressor cell types and tolerogenic factors was determined together with *HIF* and *STAT-3* mRNA expression in 21 ovarian cancer tissue samples. The tissue was preserved right at the surgery to obtain an actual picture of the cancer immune environment in the patients. mRNA degradation was kept at a minimal level by immediately submerging the excised tumor tissue in RNA-preserving solution. To avoid mRNA degradation due to tissue manipulation, gene expression was evaluated for the whole tissue as obtained from surgery without separation of specific cell types. Consequently, the cell type from which a given mRNA was derived remained unknown.

The numbers of tumor-infiltrating leukocytes showed a big variation in the analyzed samples ranging from less than 2% to more than 50% of the total cell composition. A similar broad distribution range was found for the relative numbers of single immune suppressor cell types supporting the view of a very heterogeneous immune microenvironment in ovarian cancer patients. This heterogeneity could complicate immune therapies and might contribute to low response rates. Highly variable ranges were also observed for the mRNA expression levels, although all samples expressed several HIF target genes at least to some extent. However, this observation must be qualified as the obtained relative mRNA levels were not fully-matchable between the samples, the inherent variability of the cell composition in the tumors allowed only for semi-quantitative comparisons. Bearing these limitations in mind, the relative numbers of cell types and gene expression levels were used for statistical analysis. Hierarchical clustering was employed to identify subgroups of patient samples with a common immune signature. The mRNA level clustering revealed a small group with relatively higher expression of HIF target genes with known immunosuppressive activity. These samples also showed elevated HIF-1α and HIF-2α expression demonstrating that the HIF mRNA levels correlated well with the expression of its target genes. The co-regulation of HIF activity with the mRNA levels of *HIF-1α* and *HIF-2α* was also observed in the pairwise analysis of the studied mRNA expression data. HIF activity is regulated *via* protein stability during changes in oxygen availability with rapid

degradation of HIF proteins in normoxic conditions and stabilization in hypoxia. Alternatively, growth factor signalling *via* activated Ras/Mek/MAPK and PI3K/Akt/mTOR pathways can stabilize HIF proteins in normoxia (23, 24). Basically, HIF activity was measured through expression analysis of their target genes. The association of the mRNA levels of HIFs with the expression of their target genes suggests that HIF proteins are, at least partially, stabilized *via* growth factor signalling in ovarian cancer samples. Tumor growth beyond the diameter of 5 mm requires the establishment of blood supply. As all tumors in our study had a diameter larger than 5 cm, they had passed the phase of angiogenesis induction and intra-tumoral hypoxia was not an obligatory feature in the collected tissue specimens.

HIF-1α expression also correlated with the relative number of tumor-infiltrating T-reg cells characterized by co-expression of CD8 and FoxP3, but lacking expression of the co-stimulatory receptor CD28. This association confirmed the already described up-regulation of FoxP3 by HIF-1α (37). Not surprisingly, the accumulation of $CD3^{+}IL\text{-}10^{+}$ T-reg cells was accompanied by higher levels of *IL-10* mRNA expression suggesting that these cells significantly contributed to the overall IL-10 production. Also VEGF-A was elevated in tissue samples with higher numbers of $CD3^{+}IL\text{-}10^{+}$ T-reg cells. These results are in line with a recently published study describing significant co-expression of VEGF and IL-10 assessed by immunohistochemical analysis of ovarian cancer tissue samples (45). *VEGF-A* mRNA expression was also relatively elevated in our samples characterized as adenocarcinomas. VEGF was suggested as an independent prognostic factor for ovarian cancer and high levels in plasma or ascites fluid inversely correlate with survival (46). Furthermore, VEGF was shown to induce ascites by increasing the peritoneal permeability (47). We found a positive association between *HIF-1α* mRNA expression and the volume of ascites fluid. As VEGF is one of the major target genes of HIF-1α our results suggest that HIF1α mediates ascites accumulation through VEGF production. Two parameters were found to be associated with older age, namely high grade cancers (G3) and *HIF-2α* mRNA expression. A similar connection of cancer grade and age has been observed previously (48). In conclusion, the association of HIF mRNA expression with the mRNA levels of HIF target genes suggests that in ovarian cancer HIF activity is at least partially upregulated by growth factor signalling. Nuclear expression of HIF-1α was suggested as independent prognostic factor for ovarian cancer (49). Relatively elevated expression levels of several HIF target genes exerting immunosuppressive functions was observed in about one quarter of the examined samples indicating a subgroup of patients in which targeting HIF might be considered to weaken cancer immunosuppression.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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