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Interleukin-27 is a potential marker for the onset of post-transplant malignancies

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ABSTRACT

Background. Malignancies represent the third leading cause of post-transplant mortality worldwide. The main challenge for transplant physicians is a timely diagnosis of this condition. The aim of the study was to identify a soluble diagnostic marker for monitoring the development of post-transplant malignancies.

Methods. This is a multicentre, observational, perspective, case–control study. We enrolled 47 patients with post-transplant solid neoplasia. As a control group we employed 106 transplant recipients without a history of neoplasia and matched them with cases for the main demographic and clinical features. We investigated the transcriptomic profiles of peripheral blood mononuclear cells from kidney graft recipients with and without post-transplant malignancies enrolled in two of the participating centres, randomly selected from the whole study population. Microarray results were confirmed by quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) in the remaining patients from the same transplant centres and validated in a further independent group enrolled in two different transplant centres.

Results. We identified 535 differentially expressed genes comparing patients with and without post-transplant malignancies (fold change $\geq$2.5; false discovery rate $<$5%). The cancer pathway was closely related to gene expression data, and one of the most down-regulated genes in this pathway was interleukin-27 (IL-27), a cytokine regulating anti-tumour immunity. Quantitative PCR and ELISA confirmed the microarray data. Interestingly, IL-27 plasma levels were able to discriminate patients with post-transplant neoplasia with a specificity of 80% and a sensitivity of 81%. This observation was confirmed in an independent set of patients from two different transplant centres.

Conclusions. Our data suggest that IL-27 may represent a potential immunological marker for the timely identification of post-transplant neoplasia.

Keywords: gene expression, immunology, interleukin-27, lymphomonocytes, post-transplant malignancies

INTRODUCTION

The improved immunosuppressive efficiency obtained in the last 3 decades in kidney transplantation has significantly improved graft survival [1]. In this scenario, the complications related to oversuppression of the immune system, including infection and malignancies, represent the main challenges in the management of kidney transplant patients. In particular, post-transplant malignancies represent the third leading cause of death in renal graft recipients in all the major national and international registries and their incidence is constantly increasing [2, 3]. Although the onset of neoplastic diseases in renal transplant patients may be related to the increased occurrence of viral infections or the direct effect of immunosuppressive drugs on specific cell populations, these causes alone cannot justify the high incidence of malignancies in this setting [3, 4]. On the other hand, the role of the immune system in

controlling tumour development and progression is widely accepted and might be even more relevant in the transplant scenario, although the cellular and molecular pathways underlying tumour escape from the immune response are still largely unclear. Several mechanisms have been suggested to modulate the immune response against neoplasia and a variety of cytokines, including transforming growth factor (TGF)-β, interleukin 10 (IL-10) and IL-27, have been proposed as the main mediators of these events [5].

De novo malignancies in renal transplant recipients are usually diagnosed at an advanced stage and show an aggressive behaviour [6, 7]. Data from the Israel Penn International Transplant Tumors' Registry (https://ipittr.uc.edu/) show that disease-specific survival is significantly worse among transplant recipients with solid cancer compared with non-transplant patients with the same malignancies. Multivariate analyses demonstrated that transplantation and cancer stage at diagnosis represent the most significant and negative independent survival predictors.

Timely diagnosis has a pivotal role in oncology and might be even more important in the post-transplant setting. Consequently the development of a specific and reliable soluble biomarker with the ability to uncover the changes in the immune response predisposing to development of post-transplant malignancies might improve early diagnosis, significantly reducing neoplasia-related morbidity and mortality. Thus the aim of this study was to identify such a marker through a transcriptomic-based approach.

MATERIALS AND METHODS

Patients

We performed a multicentre, observational, case–control study enrolling patients followed in the outpatient clinics of the four participating centres (Foggia, Bari, Padua and Verona in Italy). The study protocol was approved by the ethical committee of the coordinating centre (Prot. N. 670/CE) and was carried out according to the last adaptation of the Declaration of Helsinki. The clinical and research activities being reported are consistent with the principles of the Declaration of Istanbul as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism. Specifically, we included in the study, after obtaining written informed consents, 27 kidney transplant recipients with and 66 without *de novo* post-transplant malignancies, 34 immunocompetent subjects with malignancies and 29 healthy subjects followed in two of the participating centres (Bari and Foggia). All the groups were matched for the main demographic features, except for immunocompetent patients with malignancies that were older than other patients. The two transplant groups were additionally matched for graft function, transplantation vintage and immunosuppressive therapy (calcineurin inhibitors, mycophenolate mofetil and steroids). In order to evaluate the immunologic alterations associated with post-transplant neoplasia, we investigated the gene expression profile of peripheral blood mononuclear cells (PBMCs) by a microarray-based approach. To this end, in each study group we randomly selected eight patients (microarray group). The remaining patients were included in the testing group. To validate our observation, we enrolled a further cohort of transplant recipients (validation group) with ($n = 20$) and without ($n = 40$) post-transplant neoplasia, followed in two independent centres (Padua and Verona). Patients with post-transplant malignancies were enrolled at the diagnosis before any change in the immunosuppressive regimen. Malignancies in transplant recipients of the microarray groups included gastric adenocarcinoma ($n = 2$), renal clear cell carcinoma (RCC; $n = 2$), papillary thyroid cancer ($n = 2$), melanoma ($n = 1$) and squamous cell carcinoma (SCC; $n = 1$). In the testing group the malignancies affecting transplant recipients were RCC ($n = 2$), gastric adenocarcinoma ($n = 1$), lyposarcoma ($n = 1$), non-Hodgkin lymphoma (NHL; $n = 2$), SCC ($n = 4$), lung adenocarcinoma ($n = 1$), laryngeal SCC ($n = 2$), prostate adenocarcinoma ($n = 4$), hepatocarcinoma ($n = 1$) and bladder adenocarcinoma ($n = 1$). Finally, in the validation group, post-transplant neoplasia included RCC ($n = 2$), papillary thyroid carcinoma ($n = 1$), NHL ($n = 2$), Kaposi sarcoma ($n = 1$), SCC ($n = 4$), lung adenocarcinoma ($n = 2$), laryngeal SCC ($n = 1$), prostate adenocarcinoma ($n = 5$) and bladder adenocarcinoma ($n = 2$). Malignancies in the immunocompetent patients included in the microarray group were RCC ($n = 2$), prostate ($n = 4$) and bladder adenocarcinoma ($n = 2$), while the neoplastic disease affecting the immunocompetent patients of the testing set were RCC ($n = 6$), prostate ($n = 12$) and bladder adenocarcinoma ($n = 8$). Malignancies were diagnosed in all cases at an early stage.

PBMC isolation and RNA extraction

At the time of enrolment, 20 mL of whole blood was harvested from all patients before any change in the immunosuppressive regimen and before any specific surgery or chemotherapy. PBMCs were isolated by density separation over a Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Total RNA was extracted automatically and qualitatively and quantitatively analysed through a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with good quality characterized by RIN >8 were used in the microarray experiment.

Microarray experiment

For transcriptomic profiling, labelled cRNA was generated using the Low Input Quick Amp Labeling kit, according to manufacturer's protocols (Agilent Technologies), from RNA samples of eight control transplant recipients, eight patients with post-transplant malignancies, eight immunocompetent patients with malignancies and eight healthy subjects. Gene expression data were obtained using Agilent Feature Extraction software. Results of the microarray experiments are available in Gene Expression Omnibus (accession numbers: for the eight control transplant patients, GSE51675; for the other patients, GSE94424). The differentially expressed genes were identified by applying a fold change ≥ 2.5 and a P-value < 0.05 after comparison of the two groups by *t*-test (moderated *t*-test). Permutation analysis was applied to reduce the false discovery rate. Results were statistically analysed using GeneSpring GX 12.5 software (Agilent Technologies) in order to identify genes differentially expressed and functionally analysed using the

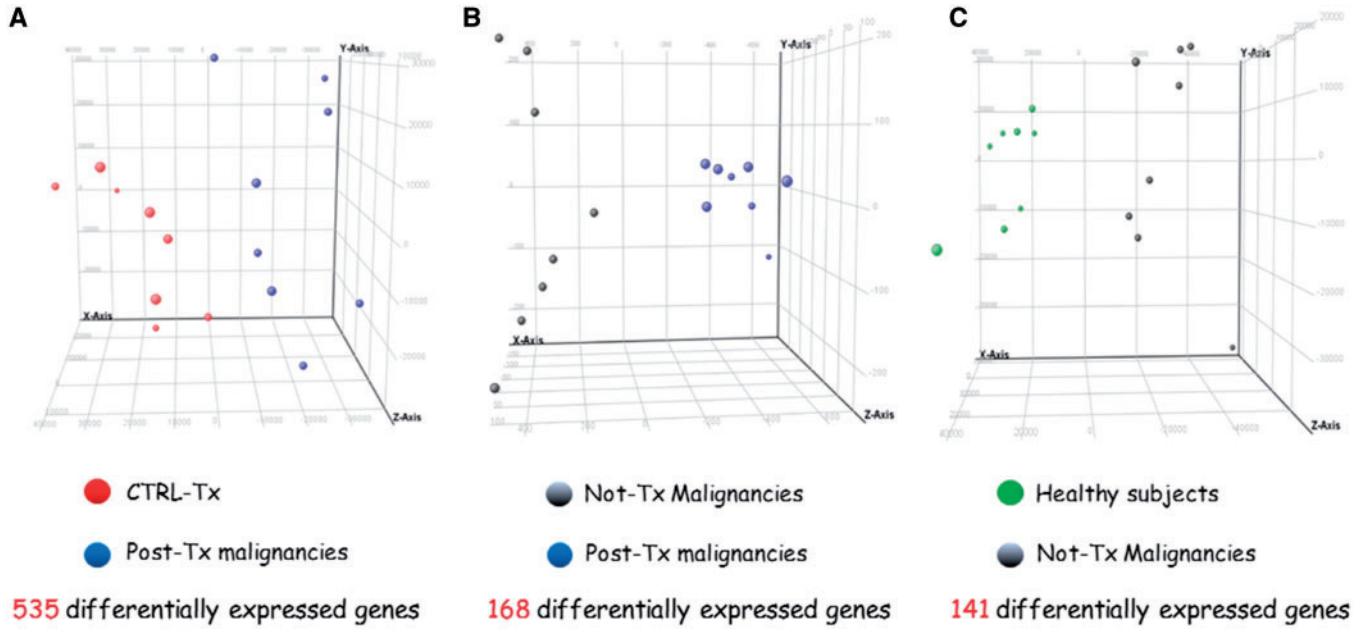

FIGURE 1: PCA of mRNAs discriminating the four patient categories. (**A**) PCA built on differentially expressed genes among post-transplant (post-Tx) malignancies ($n = 8$) and control transplant recipients (CTRL-Tx) ($n = 8$). (**B**) PCA built on differentially expressed genes among post-transplant malignancies ($n = 8$) and immunocompetent patients (non-Tx) with malignancies ($n = 8$). (**C**) PCA built on differentially expressed genes among immunocompetent malignancies ($n = 8$) and healthy subjects ($n = 8$).

Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com), which analyses the molecular and biological functions in which the differentially expressed genes are included.

Real-time polymerase chain reaction (PCR)

The results gathered by microarray were validated in the testing group by quantitative real-time polymerase chain reaction (PCR) on the *IL27* gene and on two additional potential biomarkers, the phospholipase A2 group IID (*PLA2G2D*) gene and the multimerin 2 (*MMRN2*) gene. Reverse transcription of total RNA (500 ng) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Relative quantification was obtained as previously described [8] by a comparative C_t method using 18s rRNA as a stably expressed endogenous reference gene. The following Taqman Gene Expression Assays (Applied Biosystems) were employed: Hs00377366_m1 (*IL27*), Hs00226971_m1 (*MMRN2*), Hs01572940_m1 (*PLA2G2D*) and Hs99999901 (18s rRNA). The quantitative PCR was carried out with the Light-Cycler Real-Time PCR system (Roche, Basel, Switzerland) with 5 µL TaqMan Universal PCR Master Mix in a 10-µL reaction volume. The optimized thermal cycling conditions were 95°C for 10 min, 40 cycles at 95°C for 15 s each and 60°C for 60 s.

ELISA

Plasma levels of IL-27 were measured in the testing and in the independent validation groups using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The colour reaction was measured as optical density 450 nm units on a multimode microplate reader (PerkinElmer 2030, VictorX3). The level of detection of the assay is 156 pg/mL. Intrapatient variability was 7.6 ± 1.5%.

Statistical analysis

Microarray statistical analysis was performed as previously described [8, 9]. Data are presented as mean ± SD or median and interquartile range and compared by analysis of variance or Wilcoxon test, as appropriate. P-values <0.05 were considered statistically significant.

RESULTS

We investigated, through a microarray-based approach, the gene expression profiles of PBMCs from kidney graft recipients with and without post-transplant malignancies and in two further control groups of immunocompetent patients with or without a diagnosis of neoplasia.

The main clinical and demographic features of the study population are summarized in Table 1. According to independent statistical algorithms, applying a fold change ≥2.5 and a false discovery rate <5%, we identified 535 mapped genes differentially expressed between kidney graft recipients with and without malignancies (Supplementary data, Table S1), 141 genes when comparing immunocompetent subjects with and without neoplasia (Supplementary data, Table S2) and 27 genes between kidney graft recipients and immunocompetent patients with and without malignancies (Supplementary data, Table S3). The principal component analysis (PCA), based on the microarray data, revealed that gene expression profiles of the different groups of patients allowed their different spatial clusterization (Figure 1A–C).

The functional analysis generated by the IPA software of the main diseases and biological functions in which the

Table 1. Demographic and clinical characteristics of the study population

Demographic and clinical characteristics	Microarray group (training group)					Testing group					Independent validation group		
	Not Tx malignancies	Post-Tx malignancies	CTRL Tx	Healthy subjects	P-value	Not Tx malignancies	Post-Tx malignancies	CTRL Tx	Healthy subjects	P-value	Post Tx malignancies	CTRL Tx	P-value
Patients, *n*	8	8	8	8		26	19	58	21		20	40	
Gender (M/F), *n*/*n*	6/2	6/2	5/3	5/3	n.s.	22/4	15/4	39/19	18/3	n.s.	15/5	24/16	n.s.
Age (years), mean ± SD	65.0 ± 4.0	59.8 ± 8.0	52.0 ± 8.9	53.5 ± 4.6	n.s.	72.9 ± 9.6	59.6 ± 11.2	51.6 ± 12.4	47.1 ± 11.8	0.03[a]	57.9 ± 14.6	53.1 ± 6.9	n.s.
Tx vintage (months), mean ± SD		109.2 ± 48.0	80.4 ± 8.4		n.s.		119.3 ± 78.1	102 ± 60		n.s.	115.2 ± 96.1	103.5 ± 63	n.s.
Cyclosporine, *n*		5/8	1/8				11/20	10/58			9/20	23/40	
Tacrolimus, *n*		3/8	7/8				7/20	31/58			11/20	17/40	
mTOR inhibitor, *n*		0/8	0/8				1/20	7/58			0/20	0/20	

[a]Not-Tx malignancies versus other.
CTRL Tx, control transplant recipients; post-Tx, post-transplant recipients; n.s., not significant.

differentially expressed genes were included showed that cancer was one of the most significant pathways closely related to gene expression data (post-transplant malignancies versus control transplant group: genes, $n = 400$; P-value range 1.18×10^{-2} – 4.6×10^{-5}) (Figure 2A). Among the top canonical pathways gathering the differentially expressed genes in patients with post-transplant neoplasia compared with control kidney transplant recipients, there were signalling pathways influencing the innate and adaptive immune response (ERK-MAPK and PI3K-Akt) and directly involved in the development of cancer (Figure 2B).

We then applied the IPA biomarker filter to the data set that included the differentially expressed genes between post-transplant malignancies and control transplant patients and we identified 239 potential biomarkers measurable in the blood (Table 2). When we restricted our analysis to soluble mediators secreted by immune cells, we ended up with 22 molecules, with *IL27* being the most down-regulated gene (Supplementary data, Table S4). Interestingly, the analysis of the functional networks generated by IPA in post-transplant neoplasia versus the control transplant group revealed that *IL27* was included in network number 12 of 25 (IPA score = 19), with antimicrobial response, inflammatory response and cellular movement as the top disease and bio-functions assigned to the network (Figure 2C). We then investigated *IL27* mRNA abundance in PBMCs of transplant and immunocompetent patients included in the testing set ($n = 19$ post-transplant neoplasia, $n = 21$ control graft recipients, $n = 25$ immunocompetent subjects with neoplasia and $n = 21$ healthy subjects). *IL27* gene expression in kidney graft recipients with post-transplant malignancies was significantly lower than in transplanted patients without a history of neoplasia, confirming the microarray data (Figure 3A). We also investigated the gene expression of two further potential biomarkers to validate the results of our microarray study, listed as the second and the third most down-regulated genes in Supplementary data, Table S4. The gene expression of both *PLA2G2D* and *MMRN2* evaluated by quantitative real-time PCR ($n = 8$/group) confirmed the microarray findings (Figure 3B and C). Since Macedo *et al.* [10] reported that inhibition of mammalian target of rapamycin (mTOR) can induce IL-27 expression in cultured dendritic cells (DCs) and mTOR inhibition has been shown to reduce the development of post-transplant neoplasia, we investigated *IL27* gene expression in kidney graft recipients without a history of malignancy with ($n = 8$) or without ($n = 8$) an mTOR inhibitor in their immunosuppressive regimen. Interestingly, these patients, despite similar demographic and clinical features (Table 3), presented a significantly higher *IL27* gene expression (Figure 3D).

Since we were looking for a potential soluble biomarker, we investigated IL-27 plasma levels by ELISA. IL-27 plasma concentrations were significantly lower in kidney graft recipients with post-transplant malignancies then in any other study groups included in the testing set ($n = 19$ post-transplant neoplasia, $n = 37$ control graft recipients, $n = 26$ not-transplanted malignancies and $n = 21$ healthy subjects) (Figure 4A). Finally, we applied ROC curve analysis to evaluate the sensitivity and specificity of IL-27 as marker of post-transplant neoplasia. The

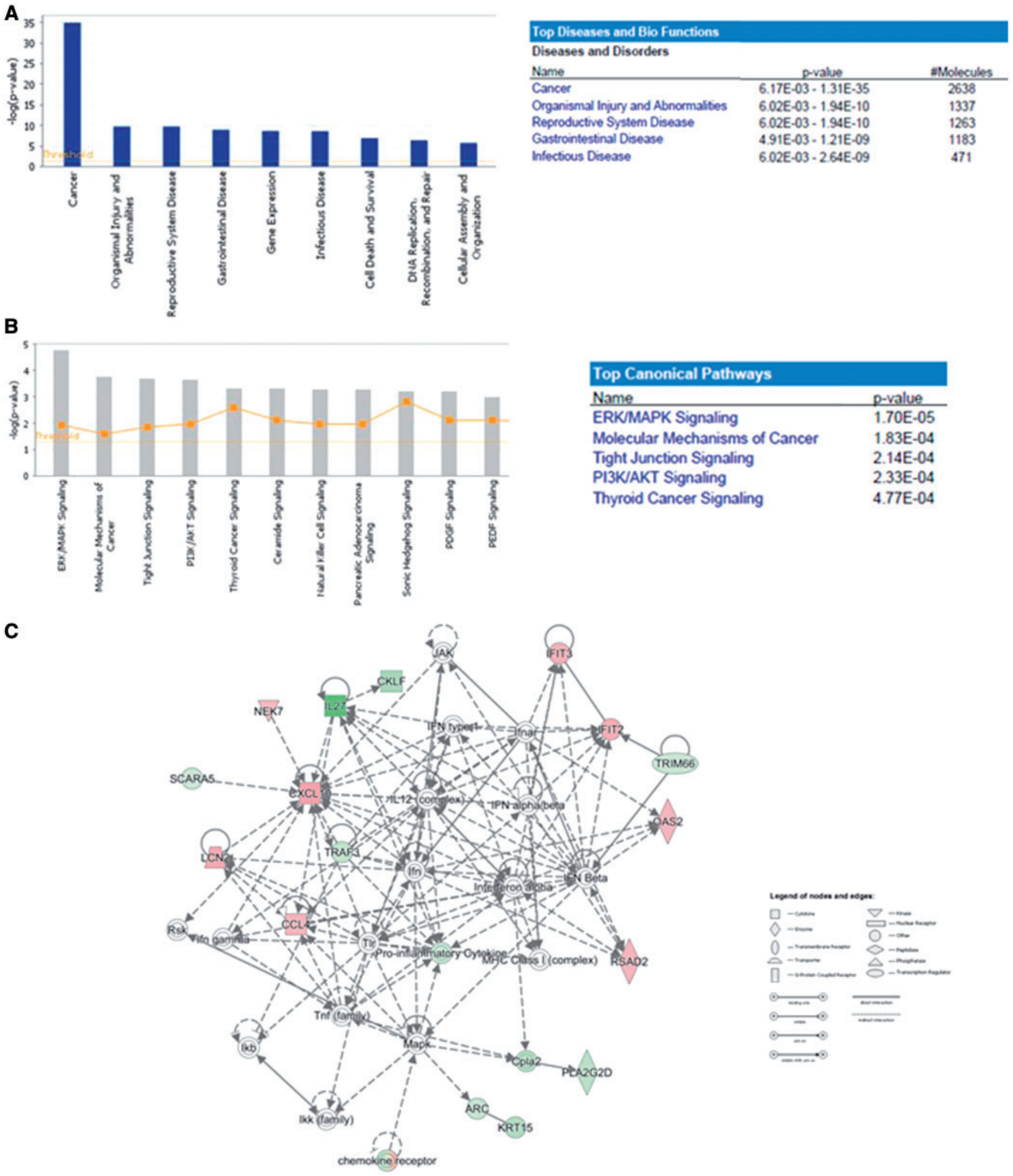

FIGURE 2: Functional analysis using the IPA software of the differentially expressed genes among the four patient categories. (**A**) Main diseases and biological functions of differently expressed genes among post-transplant malignancies versus control transplant recipients (left panel). Table on the right indicates the P-value and the number of molecules included in the top diseases and biological functions. (**B**) Main canonical pathways in which the differently expressed genes among post-transplant malignancies versus control transplant recipients (left panel) were included. Table on the right indicates the P-value and the number of molecules included in the top canonical pathways. (**C**) Functional network including IL-27 in post-transplant malignancy patients versus control transplant recipients. Down-regulated genes are depicted in green while the up-regulated genes are indicated in red with the significance of that regulation represented by the colour grade.

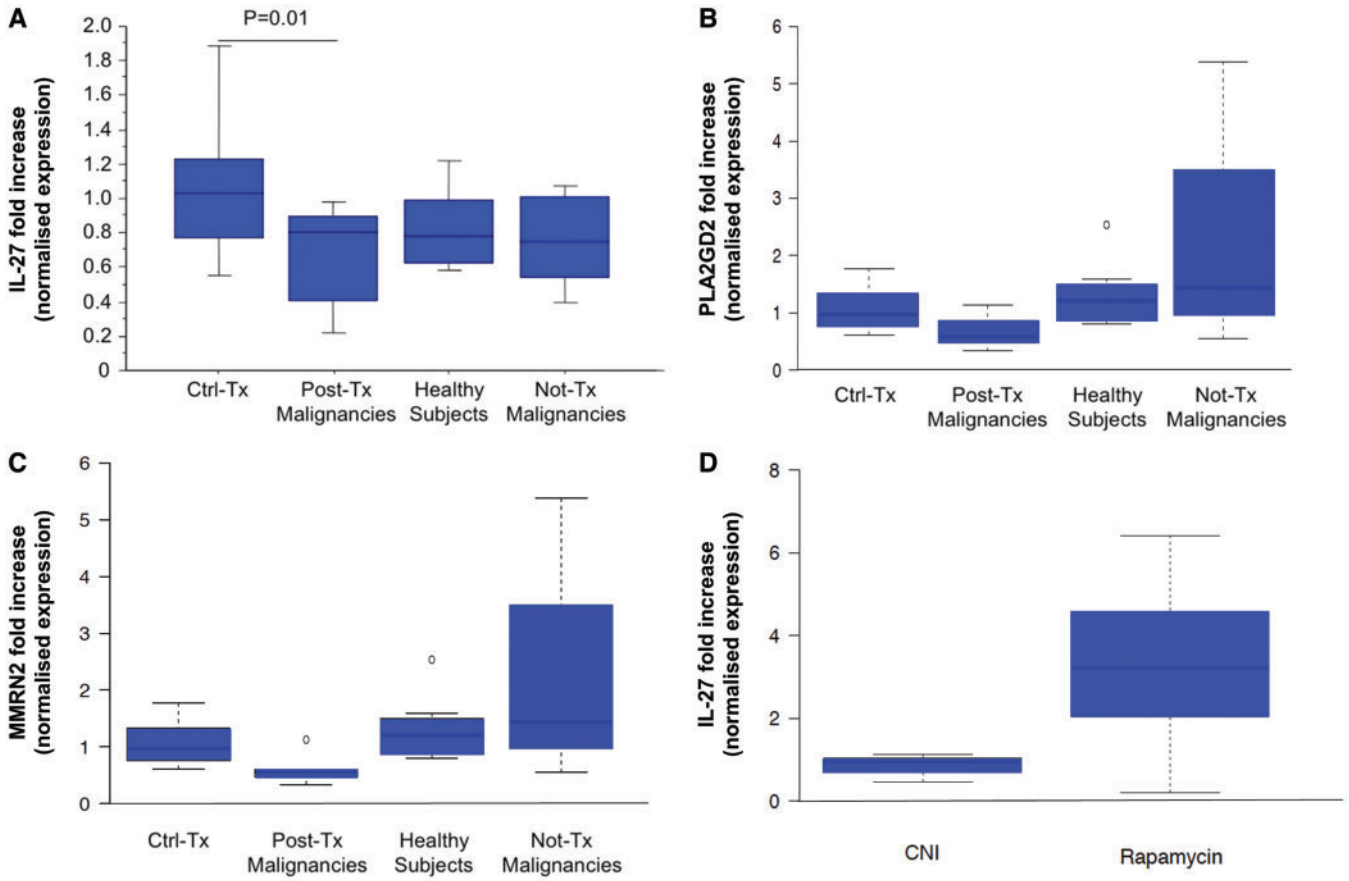

FIGURE 3: Analysis of (**A**) *IL27*, (**B**) *PLA2G2D* and (**C**) *MMRN2* gene expression. Quantitative real-time PCR of *IL27*, *PLA2G2D* and *MMRN2* abundance. Expression levels were evaluated in PBMCs isolated from the testing set of patients composed by post-transplant (post-Tx) malignancies ($n = 19$), control transplant recipients (CTRL Tx; $n = 21$), immunocompetent patients with malignancies (not-Tx malignancies; $n = 25$) and healthy subjects ($n = 21$). (**D**) In addition, *IL27* gene expression was evaluated in transplant recipients with ($n = 8$) and without (CNI; $n = 8$) rapamycin in their immunosuppressive regimen. Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers.

AUC was 0.880 and the value of plasma IL-27 of 1.175 ng/mL allowed discrimination of patients with post-transplant neoplasia with a specificity of 80% and a sensitivity of 81% (Figure 4B). To confirm our observation, we investigated IL-27 plasma levels in an independent cohort of patients ($n = 20$ post-transplant neoplasia, $n = 40$ control graft recipients). The main clinical and demographic features of this further group of patients are summarized in Table 1 and do not differ significantly from those of the two other groups. As shown in Figure 4C, IL-27 plasma levels of patients with post-transplant malignancies were significantly lower than in control transplant recipients.

DISCUSSION

In the present study we identified, using a transcriptomic approach, a potential immunological marker associated with the development of post-transplant neoplasia. The novelty of our study resides in both the methodological approach and the identification of a single molecule, IL-27, characterized by a significant reduction in its expression and secretion by circulating immune cells in patients with post-transplant malignancies.

This post-transplant complication has increased in the last 20 years and represents a serious issue, significantly affecting patients' survival considering the poor outcome of malignancies in the transplant setting [11, 12]. Thus an early diagnosis of this condition has a paramount clinical relevance [11, 12]. The link between immune suppression and neoplasia has been clearly defined, although only recently a number of studies have suggested the cellular mechanisms underlying this connection [12]. Hope *et al.* [13] demonstrated, in a population of patients with post-transplant neoplasia dimensionally similar to the one used in our study, that the presence of an increased number of circulating regulatory T cells and class-switched memory B cells was associated with the development of skin malignancies. In a following study, the same authors demonstrated that patients who develop a malignancy after transplantation are characterized by poor natural killer (NK) function [14].

Since the immune system plays a central role in the development of post-transplant neoplasia and circulating immune cells (T, B and NK cells) present several relevant changes in their phenotype in patients with this condition, these cells may represent the ideal target to search for a clinically relevant biomarker. This observation led us to investigate, through a transcriptomic approach, the molecular changes in circulating immune cells

Table 2. Biomarkers measurable in the blood differentially expressed in post-transplant malignancies versus control transplant groups

Gene symbol	Entrez gene ID	Exp fold change
LAMA4	3910	−20.578
SLC7A11	23657	−13.193
IL27	246778	−10.888
ITGA9	3680	−8.498
MMRN2	79812	−7.102
RORC	6097	−6.652
CACNA1C	775	−6.171
BTN2A1	11120	−5.827
GLTSCR1	29998	−5.021
ADRA1D	146	−4.822
KRT15	3866	−4.742
GSX1	219409	−4.722
CHAT	1103	−4.631
SCN3A	6328	−4.59
CD2AP	23607	−4.545
CDC42	998	−4.45
LAMA1	284217	−4.37
CAND1	55832	−4.18
SSTR3	6753	−4.176
EDNRB	1910	−4.129
PLA2G2D	26279	−4.048
BCL10	8915	−3.976
CLN8	2055	−3.744
TRAF3	7187	−3.689
UTP15	84135	−3.646
KLF6	1316	−3.594
ICMT	23463	−3.576
GNG2	54331	−3.552
CSH1/CSH2	1443	−3.525
CLEC4M	10332	−3.485
SCARB1	949	−3.477
CMIP	80790	−3.454
RBMX	27316	−3.443
KCNE1	3753	−3.436
LPAR3	23566	−3.375
RAB4A	5867	−3.281
C4BPB	725	−3.255
SYT3	84258	−3.209
PROKR1	10887	−3.195
CTSZ	1522	−3.117
RPS12	6206	−3.097
HAUS3	79441	−3.008
KRT85	3891	−3.002
HGS	9146	−2.966
SLC4A5	57835	−2.963
SMC5	23137	−2.959
RPN1	6184	−2.939
SYNGR4	23546	−2.938
INHBC	3626	−2.935
SFPQ	6421	−2.928
EFNA2	1943	−2.927
RPS15A	6210	−2.909
CNTNAP2	26047	−2.886
C2CD4C	126567	−2.828
ERN1	2081	−2.812
SDC2	6383	−2.806
NR3C2	4306	−2.794
ANGPTL2	23452	−2.746
PEX10	5192	−2.735
DUT	1854	−2.732
PDE4C	5143	−2.723
TBC1D12	23232	−2.705
KCNB1	3745	−2.691
PPY	5539	−2.665
EFNB3	1949	−2.633
EIF5B	9669	−2.613
ANO8	57719	−2.612
RTN4RL1	146760	−2.606
MYO10	4651	−2.595
RAPGEF3	10411	−2.58
HELZ2	85441	−2.556
GFRA2	2675	−2.555
GFRA3	2676	−2.54
DRC7	84229	−2.538
PALD1	27143	−2.525
RAB7B	338382	−2.52
NGF	4803	−2.515
PDE4D	5144	2.565
OAS2	4939	2.578
ACKR3	57007	2.594
ANKRD20A4	441430	2.595
IFIT3	3437	2.795
ASPM	259266	2.816
NPTX1	4884	2.827
BICD2	23299	2.835
CA1	759	2.952
XK	7504	2.983
CXCR2	3579	2.999
KIR2DL4	3805	3.012
CXCR1	3577	3.417
PTPRF	5792	3.485
DEFA3	1668	3.912
RHOB	388	4.19
KCNJ2	3759	4.939
ALAS2	212	6.329
HBB	3043	12.922

associated with the development of post-transplant malignancies. The transcriptomic profile was able to clearly separate patients with and without post-transplant malignancies. Since a soluble biomarker is better suited for clinical monitoring, we concentrated our attention on genes coding for protein released by immune cells. In this context *IL27* was the most down-regulated gene in our analysis. IL-27 is an IL-12 family cytokine chiefly produced by antigen-presenting cells, including DCs and monocytes/macrophages. This cytokine has been shown to present a significant anti-neoplastic activity since it boosts anti-tumour immunity through the modulation of NK cell activity and it exerts a potent direct anti-angiogenic and anti-metastatic action [15, 16]. It should be noted that Hope *et al.* [14] recently reported that low NK cell function is associated with and may even predict the development of post-transplant neoplasia. In addition, IL-27 may induce cytotoxic T lymphocyte generation by inducing expression of the transcription factor T-bet and the cytotoxic effector molecules granzyme B and perforin [17–19]. Finally, it has been shown that IL-27 might play a key role in an anti-neoplastic immune response by modulating the T-helper 1 (Th1)/Th2 bias and the generation of regulatory T cells (Treg). Indeed, in a murine neuroblastoma model, IL-27 inhibits IL-2-induced Treg expansion within neoplasia while promoting, at the same time, a Th1-driven anti-neoplastic immune response [19]. Interestingly, Hope *et al.* [13] reported that higher Treg

Table 3. Demographic and clinical characteristics of the patients with (rapamycin) and without (CNI) an mTOR inhibitor in their immunosuppressive regimen

Demographic and clinical characteristics	CNI	Rapamycin	P-value
Patients, *n*	8	8	
Gender (M/F), *n*/*n*	6/2	6/2	n.s.
Age (years), mean ± SD	65.0 ± 4.0	59.8 ± 8.0	n.s.
Transplant vintage (months), mean ± SD	56.5 ± 36.8	61.4 ± 41.3	n.s.
Cyclosporine, *n*	0/8	0/8	
Tacrolimus, *n*	8/8	0/8	
mTOR inhibitors, *n*	0/8	8/8	

proportions and numbers were independent risk factors for the development of post-transplant malignancies.

Another important anti-neoplastic role of IL-27 relies on its anti-angiogenic effects. IL-27 significantly inhibits tumour growth in severe combined immunodeficiency mice by inducing endothelial cell expression of anti-angiogenic factors, including interferon (IFN)-inducible protein 10 (IP-10) and monokine induced by IFN-γ (MIG) [20]. IL-27 strongly inhibits tumour growth of primary multiple myeloma cells by inhibiting angiogenesis [16]. Molecular analysis of cultured myeloma cells treated *in vitro* with IL-27 demonstrated a significant down-regulation of a wide panel of pro-angiogenic mediators, including matrix metalloproteinase-9, TGF-β and vascular endothelial growth factor, along with increased expression of the angiostatic chemokines IP-10 and MIG [20]. In addition, an anti-proliferative and anti-angiogenic effect of IL-27 was also observed in other haematological malignancies, including acute myeloid leukaemia, B-cell lymphoma and lymphoblastic leukaemia [21–23].

IL-27 has been shown to induce IL-10 and this cytokine may mediate in part the IL-27 anti-neoplastic effect [24]. Although the role played by IL-10 in tumour immunity is controversial, an increasing body of evidence supports a key role for this cytokine in the priming of anti-tumour immune responses. Indeed, IL-10 null mice present a significantly reduced anti-tumour response and a markedly higher tumour incidence and growth [25]. However, IL27RA null mice developing a neoplasia did not show any significant difference in IL-10 expression or secretion by immune cells [26].

The use of mTOR inhibitors has been associated with a reduced incidence of post-transplant neoplasia both in registry studies [27] and in randomized controlled clinical trials [28]. Interestingly, Macedo *et al.* [10] demonstrated that rapamycin significantly induces the expression of IL-27 by monocyte-derived DCs. Rapamycin-treated DCs cross talk with allogeneic NK cells via IL-27 and IL-12 and cause NK cells to produce IFN-γ [10]. Thus, in consideration of our data, it is conceivable that at least a part of the anti-neoplastic effect of mTOR inhibition may depend upon IL-27 induction.

One of the strengths of our work is the timing of patients' enrolment and transcriptomic evaluation. Indeed, PBMC harvesting in patients with post-transplant malignancies was performed at the time of diagnosis before any change in the immunosuppressive regimen and before any specific surgery or chemotherapy.

The two main weaknesses of the study are the sample size and the lack of information on the ability of IL-27 to predict the

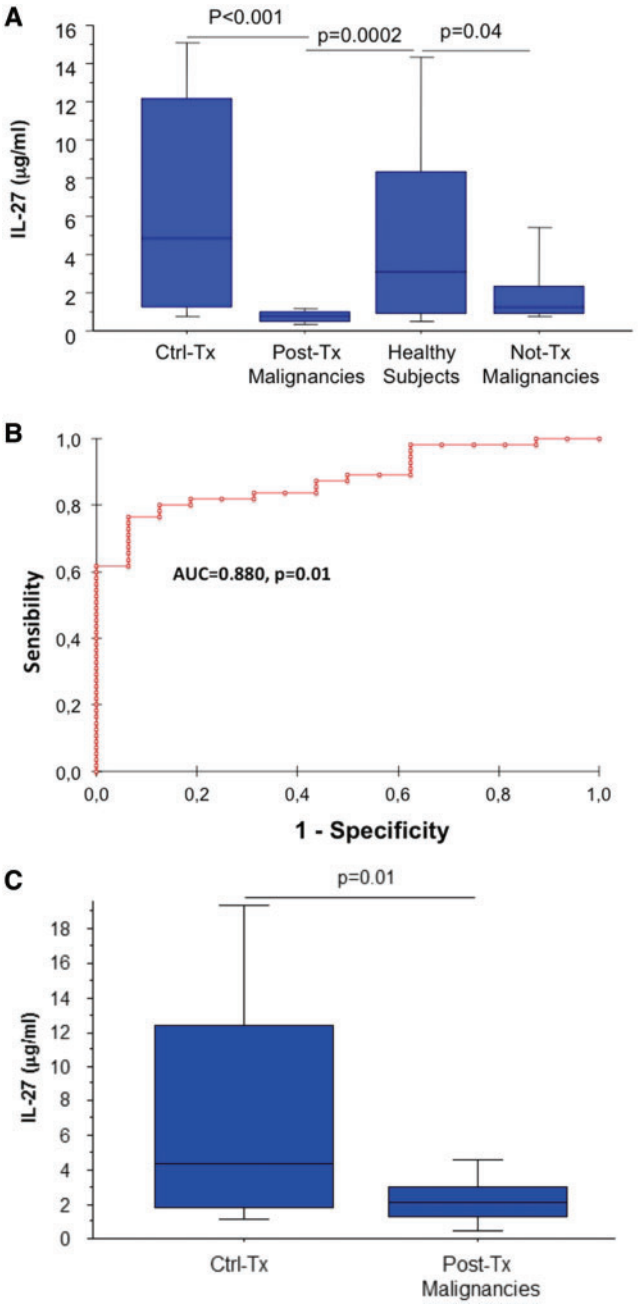

FIGURE 4: Analysis of IL-27 plasma levels. (**A**) IL-27 plasma levels evaluated by ELISA in the testing set of patients composed of post-transplant (post-Tx) malignancies ($n = 19$), control transplant recipients (Ctrl-Tx; $n = 37$), immunocompetent patients with malignancies (not-Tx malignancies; $n = 26$) and healthy subjects ($n = 21$). Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers. (**B**) ROC curve analysis to evaluate the sensitivity and specificity of IL-27 as a marker of post-transplant neoplasia. (**C**) IL-27 plasma levels in an independent validation set of patients composed of post-transplant (post-Tx) malignancies ($n = 20$) and control transplant recipients (Ctrl-Tx; $n = 40$). Data are expressed as median and 25th and 75th percentiles in boxes and 5th and 95th percentiles as whiskers.

development of post-transplant neoplasia. We tried to overcome the first issue through the use of two independent groups to validate the results obtained in the microarray study. On the other hand, after demonstrating the association of low IL-27 plasma levels with post-transplant neoplasia, it is now feasible to design a larger study to investigate its ability to predict the development of a neoplastic disease after transplantation. In this setting it would be interesting to evaluate whether low IL-27 plasma levels appear after transplantation in patients developing cancer or are already present at the time of the transplant. The inclusion in our study population of several different neoplastic diseases may represent either a strength or a weakness. Indeed, on the one hand, we can suggest that the modulation we observed in IL-27 expression is not the feature of a particular cancer, but a common characteristic of post-transplant neoplasia, while on the other hand, the presence of several neoplastic diseases, adding a further variable, may weaken our analysis.

In addition, since we included in our study all consecutive malignancies observed in our centres, it was unavoidable to include a significant number of prostate cancers, a cancer not specifically increased in transplant recipients but still one of the most common in the general population. Although this kind of neoplastic disease has been thought to be mainly hormone related, there is now clear evidence of the importance of the immune response in this setting [29]. Indeed, immune therapy has been shown to be effective in prostate cancer and the only approved DC-based vaccine is available for this specific neoplasia [29].

This study suggests for the first time, using a high throughput approach, specific molecular changes in the immune cells of patients developing post-transplant malignancies and indicates that IL-27 is a key player in this scenario. This cytokine might represent a useful tool to identify solid organ cancer development after kidney transplantation and can be simply detected at peripheral level. These findings may have important implications to fully understand the impact of immune system modulation in the development of post-transplant neoplasia and represent the 'proof-of-concept' basis to design a larger observational cohort study in kidney transplant recipients to investigate the use of IL-27 as a diagnostic and/or a predictive marker of post-transplant neoplasia.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

P.P., F.R., G.S. and G.G. were responsible for conception and design of the study, analysis and interpretation of data, drafting the article and final approval of the version to be published. G.Z. and M.A. were responsible for the design of the study, analysis and interpretation of data, revising the manuscript; providing intellectual content of critical importance to the work described and final approval of the version to be published. S.S., B.I., L.F., G.C., P.D. and L.C. were responsible for interpretation of data, revising the manuscript, providing intellectual content of critical importance to the work described and final approval of the version to be published. G.C., A.L., P.R., L.G. and M.B. were responsible for revising the manuscript, providing intellectual content of critical importance to the work described and final approval of the version to be published.

CONFLICT OF INTEREST STATEMENT

The authors of this manuscript have no conflicts of interest to disclose. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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An integrative approach for the assessment of peritubular capillaritis extent and score in low-grade microvascular inflammation—associations with transplant glomerulopathy and graft loss

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ABSTRACT

Background. Peritubular capillaritis (ptc), reported by the ptc score, is a major feature of kidney allograft rejection and microvascular inflammation (MVI). MVI sum scores (ptc + glomerulitis score $\geq$2) are accepted diagnostic surrogates of human leucocyte antigen (HLA)–antibody interaction. However, low-grade inflammation is common and ptc scores (number of leucocytes/capillary) may not mirror all aspects of ptc morphology. Recently we observed a relationship of the diffuse extent of ptc (inflammation of >50% of the renal cortex) with graft loss and significantly higher donor-specific antibody levels, suggesting potential inclusion of diffuse ptc as an additional surrogate of antibody–antigen interaction.

Methods. We sought to assess how a combination of ptc score and extent in low-grade inflammation (ptc1) affects transplant glomerulopathy (TG) and graft loss risk. Patients ($n = 616$) were assessed for MVI in first indication biopsies. Cases with a ptc score of 1 but diffuse extent ($ptc1_{diffuse}$, g-score = 0, $n = 26$) were considered additional surrogates of HLA–antibody interaction and compared with MVI $\geq$2 and MVI <2.

Results. The $ptc1_{diffuse}$ and MVI score $\geq$2 subjects had worse graft survival (42% and 59%) compared with an MVI score <2 (70%) ($P = 0.002$). The incorporation of $ptc1_{diffuse}$ in the MVI score $\geq$2 increased the receiver operating characteristics curve for TG [area under the curve (AUC) 0.602; $P = 0.008$] compared with a Banff MVI score $\geq$2 (AUC 0.56; $P = 0.12$); cases with baseline TG were excluded. In multivariate analysis, $ptc1_{diffuse}$ remained independently related to TG (odds ratio 3.89; $P = 0.008$) and graft loss (hazard ratio 2.64; $P = 0.001$) even after inclusion of all rejection episodes.