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ORIGINAL ARTICLE

Interleukin 2 as a potential cancer marker in patients after kidney transplantation

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Abstract

Introduction. Transplant recipients have a significantly greater incidence of cancer, compared with the general population, who are referred to immunosuppressive therapy as an additional malignancy risk factor. Therefore, there is a need to search for an easy in clinical practice neoplasm predictor, especially for this group of patients.

Materials and methods. A group of 74 (43M and 31F; aged 46.8 ± 12 years) kidney transplant recipients was investigated in a three-year follow-up study. During the time of observation, 7 patients were diagnosed with neoplasm (7.4 ± 1.5 years after transplantation). A serum level of IL2 (ELISA test) and mRNA level of IL1beta, IL10 and TNFalfa in peripheral mononuclear blood cells – PBMCs (QRT – PCR method) were measured in every year of observation. *Analysis of variances and t-Student test were used in groups mean comparison:*

N – patients developing malignant neoplasm group (24 probes);
M – set of probes from patients with malignancies at the moment of diagnosis (11 probes);
P – set of probes from patients before developing malignant neoplasm (10 probes);
C – control group of healthy transplant recipients (31 probes).

Results. Among the analyzed agents, only serum IL2 level differed between the analyzed groups, with higher values in the M compared with the P group ($p<0.05$) and with C group ($p<0.01$). There were no differences neither between N and C or P and C groups ($p = 0.98$), nor any correlation between IL2 and IL1b, IL2 and TNFalfa.

Conclusions. The results may indicate that IL2 serum level might be consider as a useful late unspecific cancer marker, although larger studies should yield verification of this finding.

Key words

cancer marker, Interleukin 2, kidney transplantation

INTRODUCTION

It is well-known that solid organs transplant recipients are at higher risk for cancer development. Additionally, cancers in this group of patients grow more rapidly, occur earlier, and metastasize more widely compared with the general population [1]. In the kidney transplant population the incidence of cancer is increased two- to four- fold, which is, besides cardiovascular diseases, a major cause of morbidity [2]. After 10 years, the incidence of malignancy is around 20% in renal transplant recipients [3]. Analysis showed that the risk is three-fold in females, and two-fold in males, compared with the general population, and especially high, a rate ten- to twenty-fold higher for younger recipients [4]. Not all cancers are equally increased in the transplant population. There are greater increases in the incidence regarding kidney malignancies (up to fifteen-fold), and those tumours linked to oncovirus: Kaposi's sarcoma, lymphomas, skin cancer, the risk of which is increased more than 20- fold, compared to the general population. Common malignances, such as colon, lung, ovarian or gastric cancer were approximately two-fold higher, but the incidence of leukemia, bladder, testicular, liver and gynaecological tumours increased up to five-fold after renal transplantation [5].

The higher risk of malignancy after renal transplantation is the result of both conventional risk factors, such as genetic, immune or environmental, and those specific to transplant recipients, mainly immunosuppressive therapy and, in some cases, oncogenic viruses [1]. The type of immunosuppression, the dose, and the number of immunosuppressive drugs must also be considered as major risk factors [6]. Interestingly, some drugs promote carcinogenesis by mechanisms independent of their immunosuppressive effects [3], which is considered as an additional major risk factor for cancer development [1]. On the other hand, some immunosuppressive drugs, such as mTOR inhibitors (Rapamycin), play a protective role in cancer development [7]. The influence of major risk factors of cancer after renal transplantation, and significantly higher incidence of malignancy in these patients, demands the search for easy in clinical practice cancer markers, especially for this group of patients.

In the presented study, certain candidates for predictors of the malignancies development were of interest: Interleukin

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2 and 10 (IL2, IL10) as potentially anti-tumour agents; pro-inflammatory: Tumour Necrosis Factor alfa (TNFalfa) and Interleukin 1 beta (IL1beta);

Interleukin 2 (IL2) is a potentially strong anti-tumour factor. It has been found that IL2 can enhance the cytolytic activity of T lymphocytes and NK cells [8], and this ability has been exploited by adding this cytokine to a mixture of killed tumour cells and lymphocytes *in vitro*. It influences on the expression of proteins as porfirynes that are involved in the lytic activity of cytotoxic T-cells [9]. IL2 is also able to markedly diversify the range of target cells susceptible to cytotoxic T lymphocytes and NK cells. This HLA, unrestricted killing in response to IL2, is effected by lymphokine activated killer cells (LAKs) [10, 11]. Therefore, LAK cells and IL2 are currently used in anti-cancer therapy[8].

Tumour Necrosis Factor alfa (TNFalfa) and Interleukin 1 beta (IL1beta) – with few other cytokines (IL6, IL23) and the cells releasing them, are considered as dominant tumour-promoting forces of the immune system [12]. TNF itself is a critical inflammatory mediator and plays an essential role in models of carcinogenesis, acting through the induction of genes encoding NF-kB–dependent anti-apoptotic molecules [13, 14]. TNF-α has also been proposed to contribute to tumour initiation, to enhance tumour progression, include promotion of angiogenesis and metastasis, as well as impairment of immune surveillance by strongly suppressing many T cell responses and the cytotoxic activity of activated macrophages [13, 15]. IL-1β can activate NF-κB in a manner similar to TNF [12].

Interleukin 10 (IL-10) – may be considered as primarily anti-tumour cytokine, preventing inflammation and tumour angiogenesis, because of its NF-κB inhibition, hinter angiogenesis within the tumour micro-environment, and is essential for the proper down-regulation of inflammation[12]. On the other hand, IL-10 can help to promote tumour development by activating STAT3 taking a part in cell proliferation and survival [13].

MATERIALS AND METHOD

The aim of the presented study was to search for a specific marker for the identification of patients at high risk of developing *de novo* malignancies for renal transplant recipients. Among the potential candidates for markers, interleukin 2 serum levels, the mRNA expression of IL1beta and IL10 in PBMCs were measured in renal transplant recipients who developed cancer (tested group) without malignancy (control group) during three years of follow-up.

A group of 74 all kidney-only transplant recipients in a three years follow-up study were investigated (43 M and 31F; aged 46.8 ± 12 years) of various duration after transplantation (mean 7.3, ± 2.3 years). All patients have taken immunosuppressive therapy according to a three drugs scheme, including: cyclosporine or tacrolimus, prednisone and mycophenolan mofetil or sodium. Rapamicine was taken by 6 patients at high malignancy risk or with a cancer history. Each patient had been undergoing complex clinical control (minimum ultrasound examination of the abdomen, heart and neck) and additional laboratory tests in every year of observation. During the time of observation, 7 patients were diagnosed with neoplasm (4 males and 3 females, aged 55.3 +/- 13.3 years, average 7.4 +/- 1.5 years after transplantation). The subjects were diagnosed with different kinds of solid tumours: cancer of kidney, cutis, coli, ovary, early stages of carcinoma of the urinary bladder and uteri, and in one case, metastases to lung and liver and additional case of myeldysplastic syndrome (MDS).

A serum level of IL2 and mRNA expression of TNF alfa, IL1beta and IL10 in mononuclear blood cells were measured in every year of observation. The results were analyzed in groups: N – patients developing malignant neoplasms (7 subjects, 24 probes), and C – control group of healthy transplant recipients after excluding various acute or chronic concomitant diseases that may influence serum cytokine level, and were observed during the time of observation (16 subjects, 31 probes). The obtained data was also analysed in different configurations: M – set of probes from patients with malignancies at the moment of diagnosis and/or in the state of tumor present in their bodies (7 subjects, 11 probes); P – pre-neoplasm group, set of samples from patients before developing a malignant neoplasm (5 subjects, 10 probes); C– the same as the above control group of healthy transplant recipients.

The study was approved by the Bioethics Committee of the Medical University in Katowice, Silesia, Poland, in accordance with the Declaration of Helsinki regarding medical research involving human subjects. The study and its purpose was explained to each participant or their legal guardian, who gave their informed written consent.

Tissues. Venous blood samples were collected into EDTA containing tubes, and a 7.5 ml sample from each patient was centrifuged on a Ficoll-Conray gradient immediately after blood collection. Additionally, a 5ml serum sample was collected for further ELISA test assay.

mRNA extraction from tissue specimens. Total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, Calif). RNA extracts were treated with DNase I (MBI Fermentas, Vilnius, Lithuania), according to manufacturer's instructions. The quality of extracts was checked electrophoretically using 0.8% agarose gel stained with ethidium bromide. The results were analyzed and recorded using the gel documentation system 1D Bas-Sys (Biotech-Fisher, Perth, Australia). Total RNA concentration was determined by spectrophotometric measurement in 5-µl capillary tubes using the Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK).

Real-Time QRT-PCR Assay. Quantification of IL1B, IL10, TNFα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was achieved by means of the Opticon™ DNA Engine Continuous Fluorescence Detector (MJ Research, Watertown, MA, USA). RT-PCR was based on the TaqMan fluorogenic detection system (QuantiTect Probe RT-PCR Kit, QIAGEN, Valencia, CA, USA), using a fluorogenic oligonucleotide probe designed to hybridize to the specific target sequence (TaqMan Gene Expression Assays, PE Applied Biosystems). The TaqMan probes were labeled at the 5' end with the fluorescent reporter dye FAM (6-carboxyfluorescein) (R), and at the 3' end with the quencher dye TAMRA (6-carboxytetramethylrhodamine) (Q).

The thermal profile for one-step RT-PCR was as follows: reverse transcription at 50˚C for 30 min, denaturation at 95˚C for 15 min and then 40 cycles of amplification for 15s at 94˚C and 1 min at 60˚C followed by terminal elongation for 10 min at 72˚. The point at which the PCR product is first detected above a fixed threshold – termed cycle threshold (Ct) – was determined for each sample, and the average Ct of triplicate samples was calculated. All samples were tested in triplicate. GAPDH was included in order to monitor the QRT-PCR efficiency. RT-PCR products were separated on 6% polyacrylamide gels and visualized with silver salts.

Quantification of expression of target genes. To quantify the results obtained by RT-PCR for IL1B, IL10, TNFα and GAPDH, the standard curve method was used. Commercially available standards of β-actin cDNA (TaqMan® DNA Template Reagent Kit, PE Applied Biosystems, Inc., Foster, CA, USA) were used at five different concentrations (0.6, 1.2, 3.0, 6.0, and 12.0 ng/µl), in order to simultaneously detect the expression profile of each investigated gene. Values of copy numbers for standards were calculated based on the relationship: 1 ng of DNA=333 genome equivalents (PE Applied Biosystems). Amplification plots for each dilution of the commercially available standard template were used to determine Ct values. A standard curve was generated by plotting Ct values against the log of the known amount of β-actin cDNA copy numbers. Correlation coefficients for standard curves ranged from 0.988 – 0.995, indicating a high degree of confidence for measurement of the copy number of molecules in each sample. The copy numbers of analyzed mRNAs were calculated from the linear regression of the standard curve.

Measurement of serum level of target proteins. Interleukin-2 values were assayed with an enzyme-linked immunosorbent assay – ELISA (Human Il-2 High Sensivity ELISA with Signal Amplification, eBioscience) in the serum of patients and control subjects, according to the manufacturer's instructions.

Statistic analysis. *Data obtained were presented as mean ± standard deviation. Normality of data distribution was tested with the Shapiro-Wilk test. In the case of non-normal distribution, data were normalized with logarithmic transformation. Analysis of variances (with Tukey post-hoc test) and t-Student test were used in groups mean comparison. Homogeneity of variances was tested with the Levene test. Correlations were calculated with the Spearman correlation coefficient. All calculation was performed with Statistica 9.0 Software, and p<0.05 was set as statistically significant.*

RESULTS

The serum level of IL2 was similar in both the N –neoplasm and C – control group, but comparing those factors in various groups (M – group of patients with malignancies, P – preneoplasm group, and C– control group) statistically significant differences were found in the serum level of IL2 between M (mean 3.29 pg/ml, SD 1.36) and P (mean 1.01 pg/ml, SD 0.54) group (p<0.05) and between M and C (mean 1.15 pg/ml, SD 1.00) group (p<0.01). There was no differences between P and C group (p=0.9) (Fig. 1). IL1 beta, IL10 and TNFalfa mRNA level in PBMCs did not differentiate in any of the examined groups (Figs. 2, 3, 4). Despite the supposed influence of IL2 on the expression level of other pro-inflammatory cytokines, there were no statistically significant correlation between IL2 and IL1beta, or IL2 and TNFalfa.

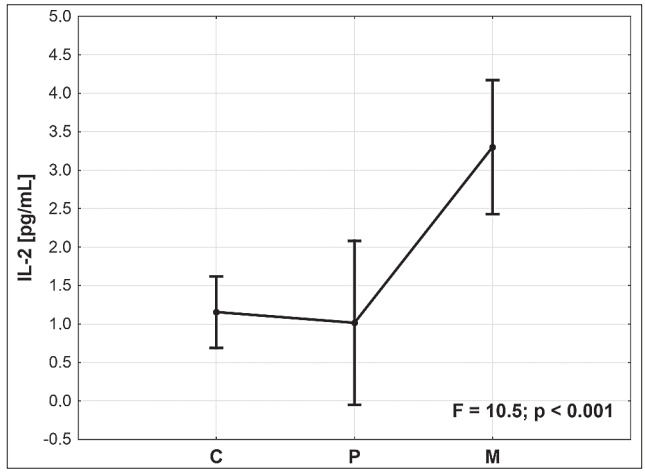
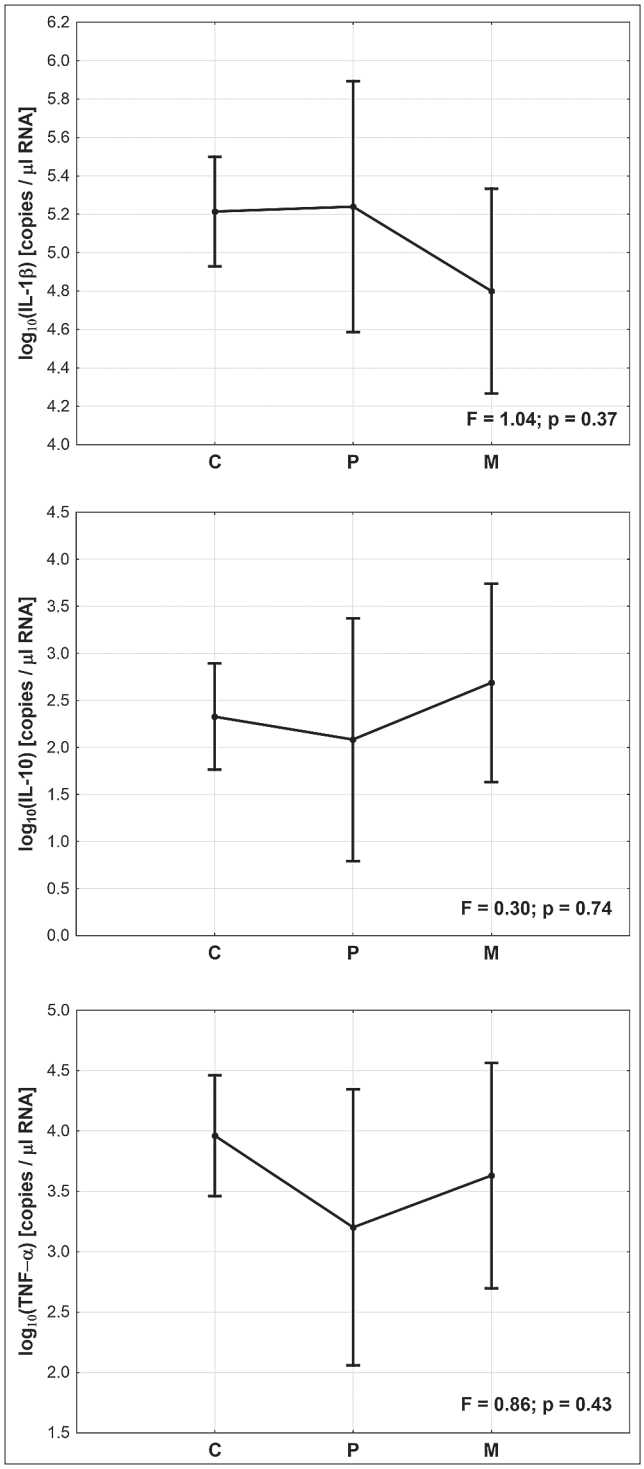


Figure 1. Serum level of IL2 (pg/ml) in M – set of probes from patients with malignancies at the moment of diagnosis and/or in state of tumour present in their bodies; P – preneoplasm group, and in C – control group of healthy transplant recipients

DISCUSSION

Prevention of graft rejection after kidney transplantation depends on chronic treatment with immune-suppressive agents. However, impaired immune functions in renal recipients result not only in a higher incidence of cancer development, but may also disturb the cytokine blood level compared with its concentration observed in the general population. Therefore, searching for a useful clinical cancer risk marker is a big challenge in those group of patients because of the unpredictable direct change in the levels of potential agents.

In renal transplant recipients who are undergoing immune-suppressive therapy and have been developing cancer, a decreased serum concentration level was expected of the anti-tumour agent IL2, compared with the control subjects (also after renal transplantation), which might be considered as a higher risk state of carcinogenesis in those patients. The results obtained did not confirm this hypothesis of the general predisposition of some individuals to developing cancer depending on decreased serum IL2 level. Instead, a significant increasing of IL2 serum concentration was observed at the moment of neoplasm diagnosis, which might be a reaction of the immune system on neoplasm presence, and could be a promising cancer marker candidate. However, the IL2 serum level was similar in blood samples of those patients included before cancer diagnosis (pre-neoplasm group) and with control samples of renal recipients, excluding the usefulness of this agent as a predictor of high neoplasm risk. Interestingly, the IL2 serum concentration was rather lower than expected values. This may indicate a general lower concentration level of this cytokine in the transplant recipients population and influence their predisposition towards cancer developmental.



Figures 2–4. IL1 beta, IL10 and TNFalfa mRNA level in PBMCs in M – set of probes from patients with malignancies at the moment of diagnosis and/or in the state of tumour present in their bodies; P – preneoplasm group, and in C – control group of healthy transplant recipients

Recent data show that IL2 and IL2 receptor beta (IL2R) play key role in immune modulation and that genetic variants of IL2RB, but not IL2, may be associated with the development of acute rejection episodes after renal transplantation [16]. According to the knowledge that binding of IL-2 to its receptor activates a pathway in which the mTOR is stimulated [17], the obtained results may confirm a potential influence of immunosuppressive therapy on IL2 serum level in those groups of patients. However, there are limitations in use of IL2 as a specific cancer marker, taking under consideration the acting of IL2 and IL2R in the final step in T cell mediated renal allograft rejection [18], and the patients treatment with anti-IL-2 receptor antibody as basiliximab or daclizumab [17].

mRNA expression in PBMCs of pro-inflammatory TNFalfa and IL1beta, which are considered as important factors involved in the initiation, proliferation, angiogenesis and metastasis of various types of cancers [13], did not differentiate cancer patients in the prsented study. TNFalfa and IL1beta transcription levels were also of interest because of reports that IL2 stimulates the production of both IL-1alfa and IL1 beta by human PBMCs [19], and induces the production of TNFalfa and beta [20]. No correlation was found between IL2 serum level and TNFalfa and IL1beta transcripts level. Similarly, mRNA level of anti-inflammatory IL10 that is believed to play a complex role in the development and survival of cancer cells [12], did not appear to be a good candidate for a cancer marker in this study.

CONCLUSIONS

Among the studied agents, only IL2 was differentiated between the patient groups in the presented study. Paradoxically, to the primary hypothesis, the obtained results may indicate that the IL2 serum level could be considered as a useful, late, unspecific cancer marker, but not an early predictor of malignancy development risk. Therefore, patients with a high instead of low IL2 serum level might be rather required to be under complex clinical control with active searching for neoplasm in additional diagnostic tests. Larger studies should yield verification of this finding.

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