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Prognostic Histological and Immune Markers of Renal Cell Carcinoma

Tamás MAGYARLAKI,[1] István BUZOGÁNY,[2] László KAISER,[3] Farkas SÜKÖSD,[3] Róbert DÖBRÖNTE,[1] Barbara SIMON,[1] Attila FAZEKAS,[4] Judit NAGY[4]

[1] Department of Clinical Biochemistry, [2] Department of Urology, [3]Department of Pathology,[4]Nephrology Center, 2nd Department of Medicine, University of Pécs, Medical Faculty, Pécs, Hungary

Recent development on the fields of molecular genetics and immunology of human renal cell carcinoma (RCC) have resulted in more successful treatment of advanced and metastatic RCCs. Re-evaluation of the prognostic/predictive data aim the initial tumor staging of RCC patients to achieve better patient selection for immune and gene therapy. 125 RCC patients diagnosed according to the Heidelberg histological classification, graded, Robson staged, immune treated (Interferon-α + Vinblastin or Broncho-Waxom/Decaris) were followed-up clinically for 36 months. Tumor immunity markers by immunohistochemistry of tumor infiltrating lymphocytes (TIL) were detected by immunoperoxidase methods using monoclonal antibodies. Tumoral immune complexes (TIC) were visualized by fluorescent polyclonal antibodies. Histologically oncocytomas defined a better ($p<0.02$) and sarcomatous RCCs a worse ($p<0.01$) follow-up prognosis. Basically, the metastatic status (related with the stage and grade) determined the clinical outcome ($p<0.00002$) of the RCC patients. Tumoral immune complexes (TIC) were weak positive, while tumor infiltrating lymphocytes (TIL) weak negative predictors of the succes of Broncho-Waxom/Decaris immune therapy. Molecular genetic based histological classification, grade, stage and metastatic status parameters together with some tumor immunity parameters (TIL, TIC) can predict the success of immunotherapy of RCC patients. (Pathology Oncology Research Vol 7, No 2, 118–124, 2001)

Keywords: renal cell carcinoma, histology, immunohistochemistry, immunotherapy, clinicopathology

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Correspondence: Dr. Tamás MAGYARLAKI, Department of Clinical Biochemistry, University of Pécs, Medical Faculty, Pécs, Hungary, 13. Ifjúság útja, 7624 Pécs, Hungary; Tel/fax: (36) 72-536 120, E-mail: kellerm@apacs.pote.hu

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Abbreviations: RCC: renal cell carcinoma; onc: oncocytic; chr: chromophobe; pap: papillary; mixed: mixed type; NP: non-papillary; sarcomatous; met: metastasis; std: stage; grd: grade; VHL: von Hippel Lindau gene; TIL: tumor infiltrating lymphocytes; TIC: tumoral immune complexes; IL-2: interleukin-2; LAK: lymphokine activated killer cells; IFN-γ: interferon-γ; NK-cells: natural killer cells; MHC: major histocompatibility complex; IFN-α + VINBL therapy: Interferon-α2b (Intron-A) + Vinblastin treatment; B/D-therapy: BronchoWaxom/Decaris treatment; PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response.

Introduction

Renal cell carcinoma (RCC) is the most common adult form of malignancy in the human kidney. Early stages (Robson I-II) are successfully treated by surgery (radical or partial nephrectomy). Additionally, RCCs with vena cava tumor thrombi (Robson III) require thoracotomy and hypothermic circulatory arrest for succesful removal of the tumor. Therapy for metastatic disease (present in 20–30% of the patients at diagnosis), however, still remains inadequate. Solitary metastases are removable by surgery. The low overall chemotherapy response of the systemic metastatic RCCs (5–8%) has been increased by combination therapy with Interferon-α + Vinblastin up to 10–15% or IL-2 with ex vivo primed lymphocytes (LAK, TIL) upto 15–30% in randomized clincal trials.[2,3,6,14,30] New, more specific immunotherapies

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Table 1. **Therapeutic protocol, control procedures and evaluation criteria of the Interferon-α + Vinblasatin (IFN-α + VBL) treated patients see in "Materials and Methods")**

WEEKS	1	2	3	4	. . . 12
DAYS	1 2 3 4 5 6 7	1 2 3 4 5 6 7	1 2 3 4 5 6 7	1 2 3 4 5 6 7	1 2 3 4 5 6 7
IFN	□□□	□□□	□□□	□□□	□□□
VBL	▮			▮	▮
CONTROL	□	□	□	□	▯

□ Interferon 2b (Intron-A) 10 mill. IU sc.
▮ Vinblastin 0,1 mg/kg iv. inj.
□ Physical examination, laboratory tests, side effects general condition chest and abdominal CT, bone-scintigraphy, brain-scintigraphy (met.)

▯ CR Negative control: end of the therapy.
PD Positive control: New combination therapy: continued or stopped.
SD The therapy is continued.
PR The therapy is continued.

and gene therapy methods are being introduced to improve on the outcome for patients with high risk and metastatic RCCs.[7,10,15,27,28]

Molecular genetics and immunology of RCC is one of the most successful fields of oncology today. Two decades of molecular genetic research of the von Hippel-Lindau (VHL) gene[8,15,19,24,27] in VHL-syndrome[25] and sporadic renal cell carcinoma[17,30] and new results in other subtypes of RCC (oncocytoma,[20] papillary[21] and chromophobe RCC[9]) have resulted in a new – molecular genetic based – histological classification.[16] The Heidelberg classification complemented with the classical clinicopathological staging/grading data separates distinct tumor subtypes and tumor transformation stages, predicting the outcome and the success of therapy in RCC patients.[16,30] Prognostic immunological markers are also available for immunotherapy. The predictive value of HLA expression on tumor cells,[3,5] tumor infiltrating macrophages[3], tumor infiltrating lymphocytes (TIL)[13,14,22,29] and tumoral immune complexes (TIC)[22,23] are under evaluation in clinicopathological studies. New findings in the basic research of tumor immunity in RCC[1,11,12] facilitate the introduction of more specific immunotherapy modalities in to clinical practice.[2,7,10,28,31] The present study statistically compares the prognostic value of histology, classical clinicopathological data (grade, stage, metastasis) and immune markers (TIL, TIC) in a follow-up of 125 nephrectomized RCC patients with or without immunotherapy.

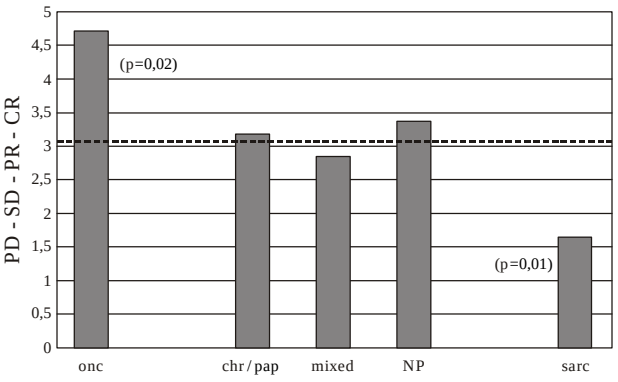

Figure 1. The diagram y-axis shows the index of clinical status (PD=progressive disease/1/; SD=stable disease/2/, PR=partial response/3/; CR=complete response up to 18 months/4/ and more than 18 months/5/). The x-axis represents the Heidelberg histological subgroups of 125 RCC patients followed-up for 36 months (onc=oncocytic/8/; chr/pap=chromophobe and papillary/6/; mixed=mixed type/9/, NP=non-papillary/84/, sarcomatous/18/). Oncocytomas have significant better, sarcomatous type a worse prognosis. Dotted line shows the average of all RCCs.

Materials and Methods

Patients

Native tumor tissues with adjacent renal parenchyma were obtained from 125 resected kidneys of RCC patients. The histology according to the Heidelberg classification[16] revealed a subtype distribution (common clear cell, 68%; sarcomatous, 14%; mixed, 7%; chromophobe, 2%; papillary, 4%; renal oncytoma, 5%); pathological grade (I-II, 73%; III-IV, 27%); stage (Std I-II, 41%; III-IV, 59%) and metastatic cases (28%) comparable with those in the literature.[30]

At the Department of Urology – in the period of 1991–1998 – 37 patients out of the 125 RCC-related nephrectomy cases receired standard postoperative Interferon-α2b (Intron-A) + Vinblastin *(IFN-α + VINBL)* combination immunotherapy (stage III and IV patients)[6] (*Table 1*).

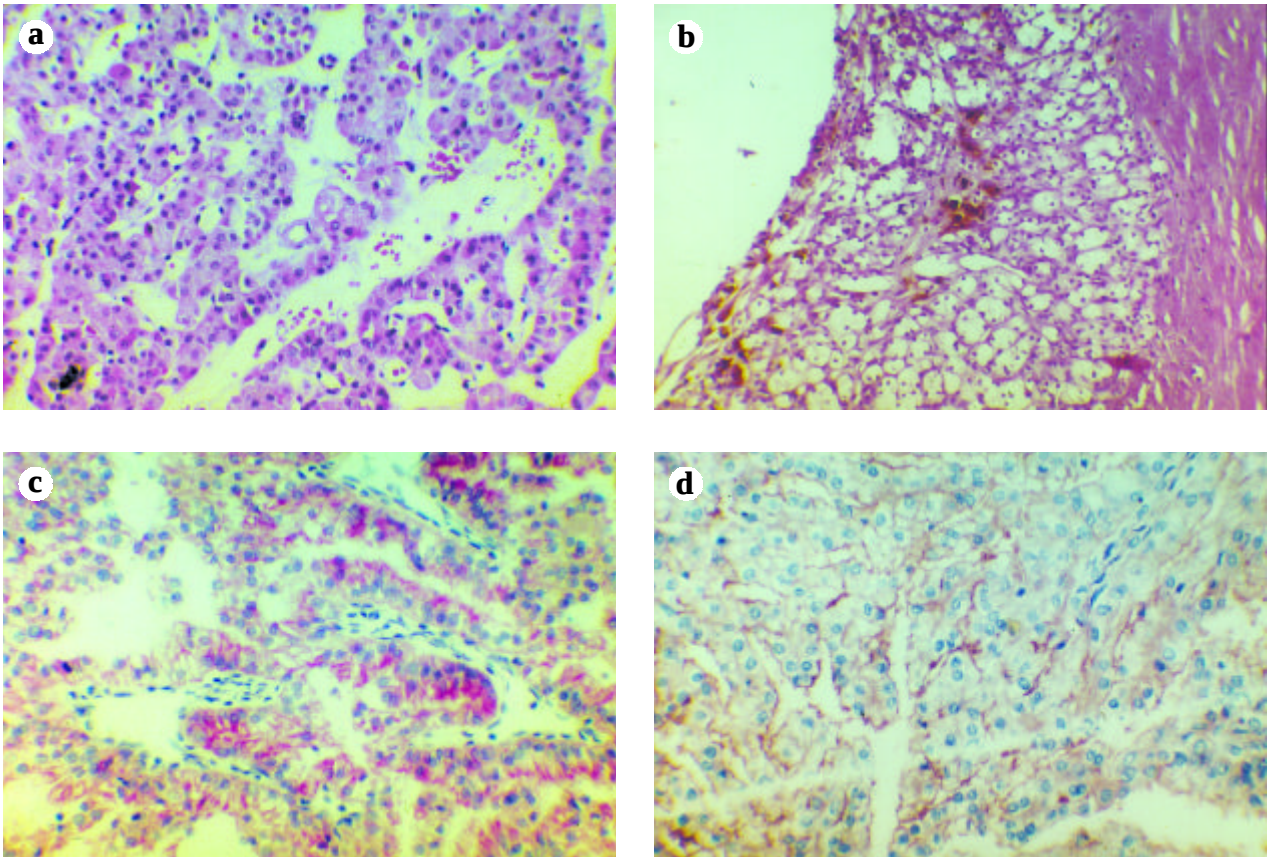

Figure 2a. Formol/paraffin slide of a well differentiated renal oncocytoma with a typical acinar growth pattern and granular cytoplasm. Haematoxilyn-eosin. 250x. – 2b. A cystic tumor cell wall of a non-papillary, "common" type renal cell carcinoma with typical "clear cell" morphology, fibrous "pseudocapsule" and haemosiderin ladden macrophages. Haematoxilyn-eosin. 250x. – 2c. Cytokeratin staining of a "pure" papillary renal cell carcinoma. Papillary growth pattern of the tumor with uniform small nuclei and an intense red cytoplasmic intermediate filament staining is visible. Anti-human cytokeratin. Alkaline posphatase-anti alkaline phosphatase staining. 400x. – 2d. PNA (Peanut Lectin) staining of a chromophobe renal cell carcinoma. Irregular tumor nuclei in a solid growing pattern. Diffuse surface PNA positivity. ABC-immunoperoxidase method. 400x.

Broncho-Waxom/Decaris *(B/D-therapy)* patients (11 cases) had Robson-I or II stage with an increased risk for progression: tumor diameter >7 cm, high grade (III–IV) and/or concomitant systemic disease (diabetes mellitus, autoimmune disease, other tumors). Antitumor doses of Decaris (3x50 mg/day) may cause agranulocytosis, so the peripheral blood and liver function with electrolytes were tested weekly and every 3 months, respectively.

Comparing the therapeutic responses between the different patient groups and their controls (35 stage III and IV plus 42 stage I–II patients, respectively) four standard categories were established after an average clinical follow-up of 36 [2–64] months: *Complete Response* = no residual/recurrent tumor or metastasis; *Partial Response* = more than 50% reduction of the tumor or metastasis; *Stable Disease* = less than 50% more than 25% reduction in primary or metastatic neoplasma; *Progressive Disease* = less than 25% tumor reduction, new metastatic leasion(s). Low overall response rate (CR+PR) to chemotherapy (7%) was increased by *IFN*-α + *VINBL* combination therapy to 15% comparable the literature[6,30] but no survival advantage 36 months follow-up have been observed. The same long-term follow-up result (no survival advantage over the stage I-II controls) was observed in *B/D-therapy* patients but all (n=11) responded well (10 CR+1PR) to immunotherapy.

Immunohistochemistry

Staining was performed on aceton-fixed cryostat sections. In a direct technique, fluoresecent isothiocyanate (FITC)-labeled polyclonal rabbit antihuman IgG, IgA, IgM and complement (C1q, C3) antibodies (DAKO, diluted 1:10) were applied[22] The ABC-immunoperoxidase method demonstrated tumor infiltrating lymphocytes (UCHL-1 for "memory T lymphocytes", Mac 387 for monocytes, CD45RB for B lymphocytes), the beta-chain of MHC-I (beta-2-microglobulin), HLA-DR alpha-

Table 2. **Follow-up correlations in different histology groups (met/std/grd/TIL/TIC correlations with the clin. status)**

	Total RCCs (exc.ROCs)	*Pap/chr*	*Mixed*	*NP*	*Sarcomatous*
	(n = 117)	(n = 6)	(n = 9)	(n = 84)	(n = 18)
*met**	–0.71	–0,96	–0,64	–0,75	[–0.38]
std.	–0.32	low / negative correlations			
grd.	–0.29				
TIL	+0.19	low / positive correlations			
TIC	+0.18				
	r-values				

*p=0.05

Linear correlation (r-values) of the indices of met=metastasis (MNo=1; Vo=2; MN1=3; MN2/3=4), std=stage (I,II,III,IV), grd=grade (1,2,3,4), TIL="tumor infiltrating lymphocytes" (1,2,3,4) and TIC="tumoral immune complexes" (1,2,3,4) with the clinical status indices (PD=progressive disease/1/; SD=stable disease/2/, PR=partial response/3/, CR=complete response upto 18 months/4/ and more than 18 monts/5/). These values are calculated in all renal cell carcinomas (Total RCCs) and in different histological subgroups of RCC (Pap/chr=papillary and chromophobe; Mixed=mixed; NP=non-papillary and sarcomatous types).

chain of the MHC-II (DAKO monoclonals, dilution 1:50) and the cytokeratin intermediate filament positivity (DAKO anti-cytokeratin, dilution 1:50). A secondary biotinylated anti-mouse immunoglobulin (DAKO, 1:200) and as a third step peroxidase conjugated streptavidin-biotin (SABC, Amersham, 1:100) reagents were used. Biotinylated PNA (Peannut agglutinin lectin, 1:100, Sigma) binding was also demonstrated by the SABC reagent. Neuraminidase pretreatment (from Vibrio cholerae, 2 U/l in 0.1 mM acetate buffer, pH:5.3, Serva) for 20 minutes at 37 °C was applied on PNA-stained slides.[23]

Evaluation of immunohistochemistry

Immunohistochemical staining was microscopically graded as described before in detail.[23] Briefly, tumor tissues RCC cases with mild, focal or negative staining, were from assessed as negative, in contrary to cases with moderate to strong diffuse positivity categorized as positive. Tumor infiltrating lymphocytes were counted in 20 microscopic fields of an 0.05 mm^2 eyepiece graticule at x200 magnification. Cases with marginal immune cell infiltration or intratumoral TIL below 100 cells/mm^2 were assessed as negative. In contrary, RCC cases with TIL present exclusively in the tumor area and exceeding 100 cells/mm^2 were positive.

Statistical methods

In order to perform statistical comparisons on semiquantitavely registered data different clinicopathological indices were created for: *1*) metastasis (MNo=1; Vo=2; MN1=3; MN2/3=4), stage (I=1,II=2,III=3,IV=4) and grade (1,2,3,4); *2*) tumor infiltrating lymphocytes /TIL:-/1/; +/2/; ++/3/; +++/4/ and tumoral immune complexes /TIC/ indices (-/1/; complement only/2/, immunoglobulins only/3/; complement+immunoglobulins/4/; paraneoplastic nephropathy/5/), /3/ for the comparison of the clinical condition of the patients (PD=progressive disease/1/;SD=stable disease/2/, PR=partial response/3/, CR=complete response upto 18 months/4/ and more than 18 monts/5/).

Each indices were calculated in each individual cases and summed in the statistically compared patient groups. Student-t test and linear correlation statistical program package of Microsoft Office Excel 7.0 under Microsoft Windows 95 was used.

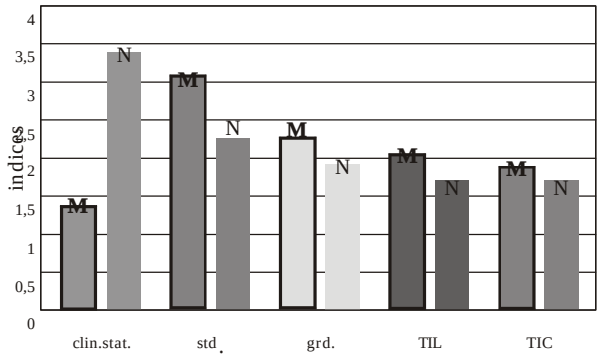

Figure 3. Prognostic parameters (clinical status, std, grd, TIL and TIC indices) are compared in metastatic and non-metastatic renal cell carcinoma (RCC) cases. Significant differences are reflected in p-values by student-t-test.

Results

The statistical results of the follow-up of 125 renal cell carcinoma (RCC) patients in different histological subgroups are shown in *Figure 1*. The mean of the follow-up index was significantly higher (p=0.02) in oncocytomas, and lower (p=0.01) in the sarcomatous subtype than that of the average of all RCCs. On the one hand, this finding is related with the fact that oncocytomas have a good clinical prognosis. On the other hand, sarcomatous tumors respond purely to therapy. No significant differences with chromophobe, papillary, mixed and non-papillary (clear cell) RCC cases were found. The general linear correlation of the follow-up outcome with the histology (oncocytic>other RCCs>sarcomatous) was, however, strong in the total RCC population (r=–0,6671, n=125) and in non-immune treated patients (r=–0,7378, n=77) [data not shown].The typical histological and immunohistochemical patterns of the main RCC subtypes are shown in *Figure 2a,b,c,d.*

The correlation of the clinical follow-up results with the clinicopathological parameters (*met*=metastasis, *std*=stage, *grd*=grade, *TIL*="tumor infiltrating lymphocytes", *TIC* ="tumoral immune complexes") are shown in *Table 2*. The clinical status (expressing the quality of the response to therapy as a follow-up index) showed strong negative correlations with the presence of metastasis in all RCCs (except the sarcomatous subtype). Descending (negative or positive) correlation with other clinicopathological parameters (std, grd, TIL, TIC) were observed.

Prognostic parameters (clinical status, std, grd, TIL,TIC) were statistically compared in the metastatic and non-metastatic RCC cases in *Figure 3*. Metastatic RCCs per se had a significant lower mean follow-up index (clinical status) with higher average stage and grade as typical signs of their more aggressive clinical behaviour compared to that of the non-metastatic cases. No significant differences in tumor immunity parameters (TIL, TIC) were found between these two groups. The typical immunohistochemical features of tumor infiltrating lymphocytes (TIL) and tumoral immune complexes (TIC) are shown in *Figure 4a and 4b* respectively.

The follow-up results in differently treated patient groups are shown in *Figure 5* and *Table 3*. In *Figure 5* the clinical status and metastasic indices of the immunotherapy groups *(IFN-α + VINBL and B/D therapy)* seemed to be statistically comparable with their stage matched tumor nephrectomy controls (stage III–IV and stage I–II controls, respectively). *Table 3* shows, that follow-up correlations with metastasis are dominant in both immunotherapy patients' groups comparable with their controls. At the same time a significant negative TIL and positive TIC follow-up correlation was found exclusivelly in the *B/D therapy* group compared with its controls (stage I–II patients). This means that the presence of "tumor infiltrating lymphocytes" (TIL) may negatively and "tumoral immune complexes" (TIC) positively influence the succes of *B/D therapy*. Weak TIL/TIC correlations were found in the *IFN-α + VINBL therapy* group unsignificantly different from its stage III–IV controls.

Discussion

Recent development in the fields of molecular genetics[9,17,21,25] and immunology[1,11,12,18,26] in renal cell carcinoma (RCC) facilitated the introduction of a new histological classification based on molecular biology (Heidelberg classification)[16] and new immunotherapy protocols.[2,7,10,28,31] Selecting the patients with advanced and/or metastatic RCC for an adequate therapy still remains problematic. Re-evaluation of the prognostic/predictive value of the histology,[16] classical clinicopathological data (stage, grade,

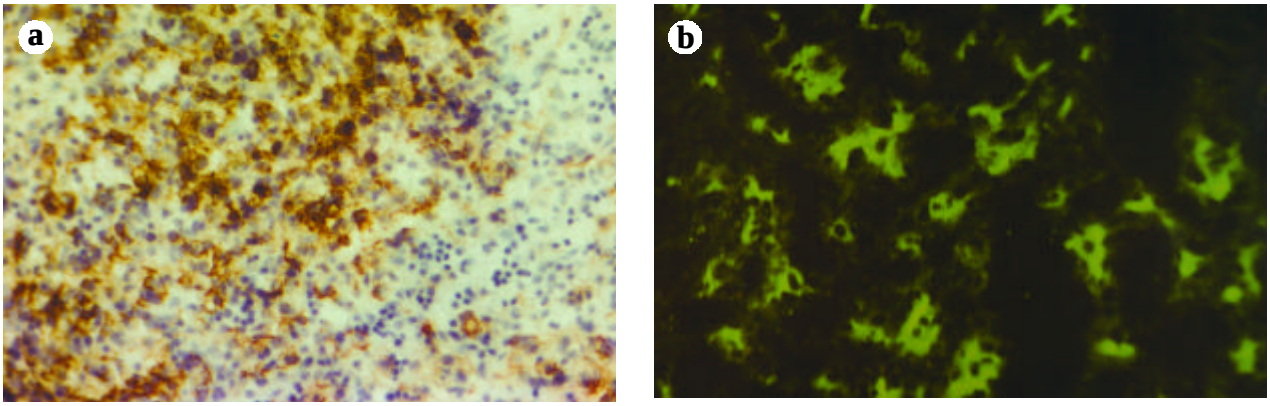

Figure 4a. HLA-DR postivity of a non-papillary renal cell carcinoma. Many tumor cells and some of the tumor infiltrating lympocytes (TIL) are positive. ABC-immunoperoxidase staining. 250x. – 4b. Fluorescent isothiocyanate (FITC) labeled anti-human IgA positivity of a non-papillary renal cell carcinoma. Green colour of the tumor cell clusters at a dark bacground. Direct fluorescent staining. X400.

Table 3. **Follow-up correlations in differently treated groups**

Patient groups	*IFN-alpha therapy (n=37)*		*Contr. III-IV (n=35)*	*B/D-therapy (n=11)*		*Contr. I-II (n=42)*
met	–0.5558	*NS*	–0.5764	–0.7455	*NS*	–0.8341
TIL	–0.2171	*NS*	+0.1327	–0.5258	*	+0.2310
TIC	–0.1417	*NS*	+0.0182	+0.5590	**	+0.1815
			r-values			

*p = 0.06; **p = 0.2

Linear correlations of the met = metastasis, TIL = "tumor infiltrating lymphocytes" and TIC = "tumoral immune complexes" indices with the clinical status indices in differently treated patient groups. Significance was calculated by a student-t-test.

metastasis)[30] compared with predictive immunological factors (tumor infiltrating lymphocytes = TIL, tumoral immune complexes = TIC, HLA-expression)[3,5,12,14,22,29] may result better patient selection for succesful adjuvant or supportive immunotherapies. The present clinicopathological follow-up study of our RCC patients statistically compares the clinical value of potentially predictive factors. All RCCs were nephrectomized, reclassified on the basis of the Heidelberg classification and treated or not with Interferon-+Vinblastin *(IFN-α +VINBL)* or Broncho-Waxom/Decaris *(B/D)* therapy according to their Robson stage, grade and metastatic status.

The clinical follow-up of different histological subgroups of RCC revealed that oncocytomas (ROC placed on new molecular genetic grounds[20]) had excellent outcome, while the sarcomatous transformation (associated with p53 inactivation, tumor progression and therapy resistance[16,21]) represented a particularly pure prognosis. Few cases of chromophobe and papillary RCCs were included in our study to get conclusions. We refer to other clinicopathological studies.[9,21]

Statistical analysis of the predictivity of the classical *clinicopathological data* (metastatic status, stage, grade) is difficult: 1) these data are "ab ovo"used in patients' preselection for immunotherapy, 2) they are closely interrelated with each other, 3) strongly influence the clinical outcome (hiding the potential effects of other factors). In our study, the metastatic status was used as a "statistical etalon" to measure the predictivity of other factors. On the one hand, a good follow-up correlation with the metastasis was found in our histology subgroups (except with the sarcomatous subtype), which was much weaker with the grade and stage. At the same time, however, the stage and grade was strongly predictive in metastatic versus non-metastatic cases. These findings are in accordance with that of the literature.[6,30] On the other hand, tumor immunity parameters (TIL, TIC) in our study were not in any correlation with that of the "classical" clinicopathological data (metastases, grade, stage). This relationships are contraversial also in the literature.[2,3,5,14,18,23]

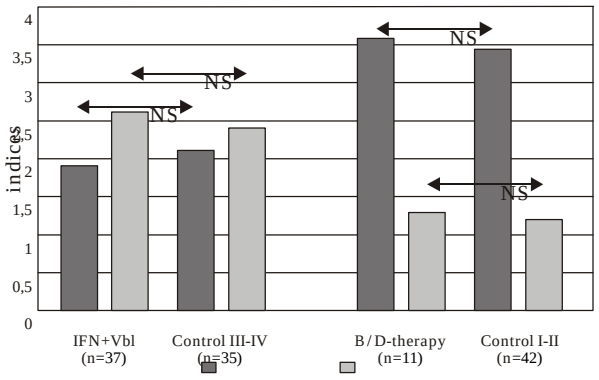

Figure 5. The indices of clinical follow-up status (clin.stat.) and metastases (met) are compared in differently treated patients'' groups (IFN-α+VINBL and B/D v.s. their controls).

Predictivity of the tumor immunity parameters (TIL, TIC) was tested also on immunotherapy groups *(IFN-α +VINBL and B/D)* compared with their stage/metastasis-matched controls. Despite the fact, that TIL,[11-13,19] NK-cells,[14,29] monocytes[3] and TIC[14,29] are frequently detectable in RCC, their predictivity is linked to special therapeutic groups and modalities. For example, while the presence of NK-cells is a positive predictive factor for IFN-γ therapy,[14,29] conversely monocytes in the tumor negatively influence the outcome of IL-2 treated metastatic RCC cases.[3] In some clinical trials the predictivity of the tumor immunity parameters is controversial.[2,7] In our study, we had an opportunity to test the predictivity of TIL and TIC in two protocols *(IFN-α+VINBL* or *B/D-treated* RCCs). According to previous data[6,30] the IFN-α seemed to act through non-immunological mechanisms (or through cytokines). There was very week follow-up correlation with the presence of TIL or TIC in our RCC cases. In contrast, *B/D-treated* patients showed a positive therapy-response correlation with the presence of TIC (humoral mechanisms may have been involved). Negative predictivity of the TIL, at the same time suggested that local cellular antitumor immunity may disturb the systemic effects of the *B/D-therapy* (supposably through an inhibition of

the humoral [type-2] response by the type-1 differentiation pattern of T-helper TIL lymphocytes[1]). As a conclusion, potentially predictive tumor immunity markers must be selected according to the therapeutic modalities applied.

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