Cytokine Measurements for Diagnosing and Characterizing Leukemoid Reactions and Immunohistochemical Validation of a Granulocyte Colony-Stimulating Factor and CXCL8-Producing Renal Cell Carcinoma

Biomarker Insights
Volume 13: 1–7

DOI: 10.1177/1177271918792246

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ABSTRACT

BACKGROUND: Various paraneoplastic syndromes are encountered in renal cell carcinomas. This case report illustrates that a paraneoplastic leukemoid reaction may precede the diagnosis of renal cell carcinoma and be explained by cytokine production from the cancer cells.

CASE PRESENTATIONS: A 64-year-old man was referred for hematology workup due to pronounced leukocytosis. While being evaluated for a possible hematologic malignancy as the cause, he was found to have a metastasized renal cell carcinoma, and hyperleukocytosis was classified as a leukemoid reaction. A multiplex panel for measurement of 25 serum cytokines/chemokines showed highly elevated levels of granulocyte colony-stimulating factor (G-CSF) and CXCL8 (C-X-C-motif chemokine ligand 8, previously known as interleukin [IL]-8). By immunohistochemistry it was shown that the renal carcinoma cells expressed both these cytokines. Two additional, consecutive patients with renal cell carcinoma with paraneoplastic leukocytosis also showed elevated serum levels of CXCL8, but not of G-CSF. Nonparametric statistical evaluation showed significantly higher serum concentrations of CXCL8, IL-6, IL-10, monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor, but lower interferon gamma (IFN-γ) and IL-1α, for the 3 renal cell carcinoma cases compared with healthy blood donors.

CONCLUSIONS: In suspected paraneoplastic leukocytosis, multiplex serum cytokine analyses may facilitate diagnosis and provide an understanding of the mechanisms for the reaction. In the index patient, combined G-CSF and CXCL8 protein expression by renal carcinoma cells was uniquely documented. A rapidly fatal course was detected in all 3 cases, congruent with the concept that autocrine/paracrine growth signaling in renal carcinoma cells may induce an aggressive tumor phenotype. Immune profiling studies could improve our understanding for possible targets when choosing therapies for patients with metastatic renal cell carcinoma.

KEYWORDS: chemokine, IL-6, IL-10, monocytosis, paraneoplastic leukocytosis, autocrine signaling, multiplex, inflammatory response, precision medicine, biomarker

RECEIVED: April 2, 2018. **ACCEPTED:** July 11, 2018.

TYPE: Case Report

FUNDING: The author(s) received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Background

Renal cell carcinoma (RCC) is unique among the genitourinary malignancies in that various paraneoplastic syndromes are found in 10% to 40% of the patients. Cytokine release by the tumor is the cause of many of the paraneoplastic conditions, including metabolic and hematologic disturbances.[1] Leukemoid reactions are suggested to be "side effects" of autocrine mechanisms used by tumor cells to stimulate their own growth. Tumor cell–produced cytokines (colony-stimulating factors and interleukins) bind to receptors on the same cells and stimulate their proliferation. Some of these cytokines are also hematopoietic growth factors and stimulate myeloid proliferation.[2] Modest neutrophilia and thrombocytosis occur in up to 20% of patients with RCC as part of a systemic inflammatory response.[3] Neutrophilia and thrombocytosis are included in the 6-factor prognostic model developed by the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) for the advanced disease.[4]

A leukemoid reaction is conventionally defined as a peripheral leukocyte count exceeding 50 000/μL, with a dominance of mature neutrophils, from reactive causes outside the bone marrow, such as infection or solid cancer.[2,5,6] Less pronounced

paraneoplastic leukocytosis is much more frequent.[2,7,8] Among specific causes of leukemoid reactions, granulocyte colony-stimulating factor (G-CSF)-producing tumors with mainly genitourinary, lung, skin, gastrointestinal, and oropharyngeal origins have been reported.[2] Twelve cases of G-CSF-producing RCC with leukemoid reactions have been reported, all from Asian countries.[9–11]

In this study, serum cytokine profiles of 3 consecutive patients with RCC and leukemoid reactions/paraneoplastic leukocytosis were investigated. All 3 patients had high CXCL8 levels, but only 1 had elevated G-CSF. In this patient, we could uniquely verify combined G-CSF and CXCL8 protein expression by the renal carcinoma cells using immunohistochemistry (IHC). High messenger RNA (mRNA) levels of these 2 cytokines in RCC were recently reported from tumor tissue in another case.[11] Our case illustrates that a leukemoid reaction may precede a diagnosis of RCC and might contribute to a rapidly progressive course, mandating diagnostic alertness with a multidisciplinary approach and timely treatment considerations.

Case Presentations

A 64-year-old Swedish man presented to the hospital after an episode of macroscopic hematuria. At initial urological evaluation, cystoscopy was normal and an abdominal ultrasound showed no sign of malignancy. The patient refused a computed tomographic (CT) scan at that time point. Plasma creatinine and blood counts were normal, with a leukocyte count of 8800 (reference 3500-8800/μL). Besides repeated episodes of hematuria, night sweats, weight loss, and nausea with occasional vomiting ensued. At renewed urological evaluation after 20 months, the patient accepted a referral for an abdominal CT scan. Meanwhile, at an evaluation of chronic cough, new blood tests revealed leukocytosis, with a leukocyte count of 48 700/μL. Hemoglobin was 123 g/L (reference 134-170) and platelets 276 000/μL (reference 145 000-350 000). Plasma creatinine was now slightly elevated, 114 μmol/L (reference 60-105).

Only 12 days later, at hematologic workup, the leukocyte count had increased to 79 400/μL. Repeated microscopic differentials thereafter showed 69% to 88% segmented and 6% to 24% band-formed neutrophils, 1% to 7% lymphocytes, 1% to 6% monocytes, 0% to 2% eosinophils, 0% to 1% basophils, 0% to 1% myelocytes, 0% to 1% metamyelocytes, and 0% to 1% blasts. The spleen was considered palpable under the left costal margin. A bone marrow examination showed close to maximal cellularity with increased, left-shifted myelopoiesis, but low percentages of blasts and promonocytes. Megakaryocytes were of varied maturity, some being hypolobulated, a feature frequently interpreted as dysplasia,[12] but the World Health Organization (WHO) 2016 diagnostic criteria for chronic myelomonocytic leukemia were not fulfilled due to less than 10% monocytes in peripheral blood.[13] Reticulin silver stain showed no increase in fibers. S-cobalamin was >1475 pmol/L

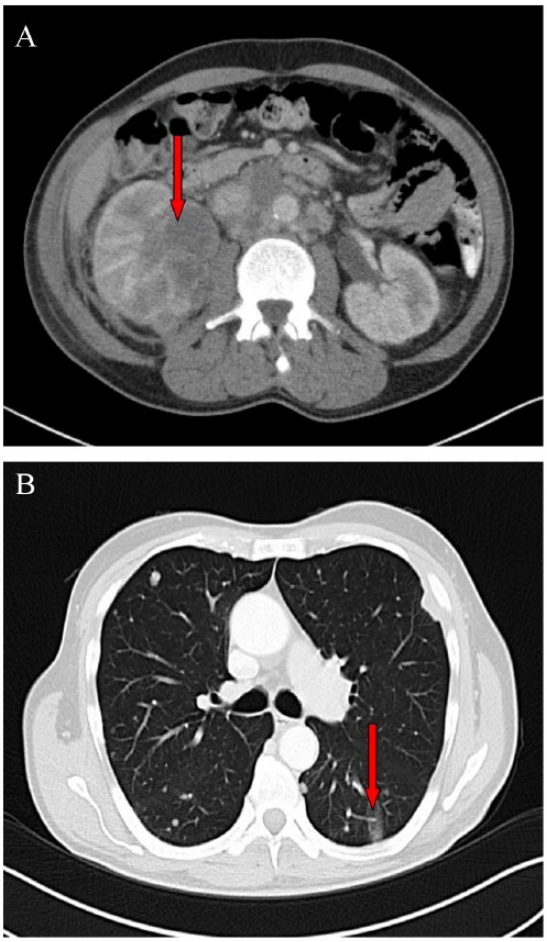

Figure 1. Computed tomography (CT) of (A) abdomen and (B) thorax at the time of diagnosis. CT of patient 1 showed (A) a large right-sided renal tumor 7.5 cm × 5 cm × 5 cm in the kidneys upper and middle parts (see arrow) and (B) bilateral multiple rounded suspect metastases in the lungs, up to 2.5 cm in diameter (see arrow).

(reference 140-650). Bone marrow metaphase chromosomes were normal. Fluorescent in situ hybridization analysis for translocation 9;22 and polymerase chain reaction for *BCR-ABL* (fusion of the *ABL* gene on chromosome 9 to the *BCR* gene on chromosome 22) were negative, ruling out chronic myeloid leukemia. *JAK2* mutation analysis showed no V617F mutation and thus gave no proof of a myeloproliferative neoplasm.

Because no hematological malignancy was demonstrated, a leukemoid reaction due to a solid tumor was suspected. Cautious oral cytotoxic treatment with hydroxyurea, 500 mg daily, was started to halt the rapidly increasing leukocytosis. The patient was shortly thereafter hospitalized with severe nausea and weakness. Whereas a gastroscopy was normal, the planned CT scan showed in the upper and middle part of the right kidney a large renal tumor, 7.5 cm × 5 cm × 5 cm (Figure 1A), with overgrowth into the perirenal fat, possibly the right liver lobe, around the inferior vena cava and aorta, and with continuity to enlarged necrotic lymph nodes along major retroperitoneal vessels. The spleen measured 15.5 cm × 14.5 cm × 7 cm. Bilateral multiple

Table 1. Serum cytokine/chemokine analysis results in controls and 3 patients with renal cell carcinoma (RCC) with leukemoid reactions/paraneoplastic leukocytosis, including statistics with Mann-Whitney test.

	CONTROL SUBJECTS			PATIENTS WITH RCC			*P* VALUE
	N	MEAN ± SD, PG/ML	5TH-95TH PERCENTILE	PATIENT 1, PG/ML	PATIENT 2, PG/ML	PATIENT 3, PG/ML	*P* VALUE RCC VS C
Eotaxin/CCL11	102	76.1 ± 48.4	20–142	41.9	70.8	53.4	.44
G-CSF	19	8.8 ± 10.9	0–27	**1688**	16	6	.16
GM-CSF	105	22.8 ± 44.8	0–97	1.1	0	0	.34
IFN-α2	16	14.6 ± 27.2	0–54	0	16.3	6	1.00
IFN-γ	108	33.0 ± 37.4	0–100	0	0	0	**.02 DOWN**
IL-1α	102	42.7 ± 81.7	0–213	0	0	0	**.03 DOWN**
IL-1β	22	1.4 ± 3.7	0–11	0	2.4	**16.6**	.15
IL-2	22	1.6 ± 4.1	0–7	0	0	0	.66
IL-3	16	0.9 ± 3.5	0–3	0	0	0	.88
IL-4	108	2.3 ± 11	0–7	0	0	0	.76
IL-5	22	0.0 ± 0.0	0–0	0	1.5	0	.40
IL-6	108	9.1 ± 13.7	0–36	32.2	34.0	**48.3**	**.002 UP**
IL-7	22	3.4 ± 6.1	0–19	0	11.3	3.5	.50
IL-8/CXCL8	108	8.6 ± 7.6	0–22	**352**	**1093**	**89.5**	**.000 UP**
IL-10	108	9.4 ± 11.6	0–31	**60.8**	**48.7**	**34.2**	**.000 UP**
IL-12 (p40)	102	26.7 ± 48.9	0–126	36.7	0.5	0	.93
IL-12 (p70)	22	2.5 ± 6.7	0–7	0	1.8	3.8	.50
IL-13	22	0.5 ± 1.4	0–4	0	1.2	0	.72
IL-15	17	1.1 ± 2.6	0–6	0	**11.9**	2.14	.22
IL-17	102	7.5 ± 10.3	0–23	0	0	0	.10
IP-10/CXCL10	102	539.0 ± 441.2	184–1244	280	289	727	.72
MCP-1/CCL2	108	258.7 ± 172.1	101–642	408	350	346	**.04 UP**
MIP-1β/CCL4	16	31.1 ± 14.1	12–51	26.9	**65.5**	**55.8**	.20
TNF-α/TNF	22	3.6 ± 4.7	0–14	12.4	**23.8**	**18.9**	**.003 UP**
TNF-β	16	3.3 ± 7.1	0–18	0	1.0	0.2	.56

The leukocyte counts at sampling were 91 700/μL (patient 1), 119 100/μL (patient 2), and 35 200/μL (patient 3). Patient values above the 95% percentile of the control values, and significant *P* values, are shown in bold.

rounded suspect metastases were seen in the lungs, up to 2.5 cm in diameter (Figure 1B).

One could speculate that this was a leukemoid reaction due to a cytokine-producing RCC. A preselected multiplex 25 cytokine/chemokine analysis kit (at leukocyte count 91 700/μL) was therefore offered and revealed serum G-CSF >60× the 95th upper normal percentile, and among the interleukins, CXCL8 was >15× and IL-10 almost 2× the 95th percentile. IL-6 and tumor necrosis factor (TNF) were close to the 95th upper percentile. IL-12 (p40) and monocyte chemoattractant protein 1 (MCP-1) were high normal, whereas other cytokines (including granulocyte-macrophage colony-stimulating factor [GM-CSF]) and chemokines were low normal (see patient 1 in Table 1). Needle biopsies from the kidney revealed partially necrotic tumor tissue with cells of low differentiation, having abundant eosinophilic cytoplasm and large rounded nuclei with nucleoli. Using IHC, a positive reaction with vimentin, and a faint focal reaction with CD10, together with negative reactions with cytokeratins 7 and 20 gave the diagnosis clear-cell renal carcinoma with high-grade atypia (Fuhrman grade 4).

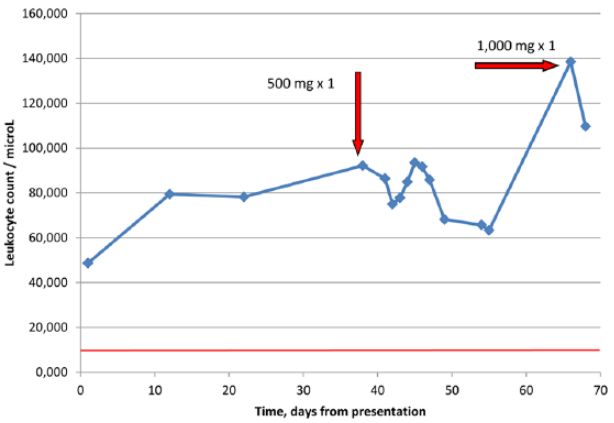

Figure 2. Leukocyte counts related to time from hematological presentation and treatment with hydroxyurea (patient 1). The *X*-axis represents time in days and the *Y*-axis represents leukocyte counts (/μL). The blue line represents the trend in the leukocyte count. The red line represents the upper limit of the reference interval for the leukocyte count (8800/μL). The initial dose of hydroxyurea was 500 mg daily, later increased to 1000 mg daily (see arrows).

In view of the dismal prognosis, predicted by the advanced tumor stage and low differentiation grade, only palliative care was given, including continuing with hydroxyurea against the leukocytosis which resulted in a stabilizing effect during the first month. Thereafter, the dose was incremented to 1000 mg daily when the leukocyte count had risen to 139 000/μL, soon after which monitoring of laboratory values was stopped due to a generally worsened condition (Figure 2). In addition to prominent neutrophilia, an absolute monocytosis ranging from 2100 to 3800/μL (reference 100-1000), basophilia with maximum 690/μL (reference 0-200), and slight eosinophilia with maximum 720/μL (reference 0-600) were observed.

The patient died within 2 years from the first episode of hematuria and within 1 month from the diagnostic renal biopsy. The G-CSF and CXCL8 expressions of the renal carcinoma cells were later confirmed by IHC (Figure 3A and B, with method descriptions below).

Two additional, consecutive patients with RCC with leukemoid reactions/paraneoplastic leukocytosis (maximum leukocyte counts 131 500/35 200/μL) investigated in our institution also had absolute neutrophilia, monocytosis (maximum 2600/2300/μL), and high/relatively high serum levels of CXCL8, IL-6, IL-10, MCP-1, and TNF similar to the first patient, but no increased levels of G-CSF (see patients 2 and 3 in Table 1). All 3 cases had undetectable IFN-γ and IL-1α. The concentrations of the abovementioned 5 upregulated and 2 downregulated molecules were all significantly different ($P < .05$) for the 3 patients with RCC compared with healthy controls (Table 1). Cytokine measurements were performed only once per patient. Patient 2 had a left-sided renal tumor measuring 12.5 cm × 8 cm × 8 cm and suspected liver and lung metastases. He died while hospitalized before a planned diagnostic renal biopsy. Patient 3 was nephrectomized for a 11 cm × 8 cm × 7.5 cm right-sided renal tumor with a radiologically suspected nearby

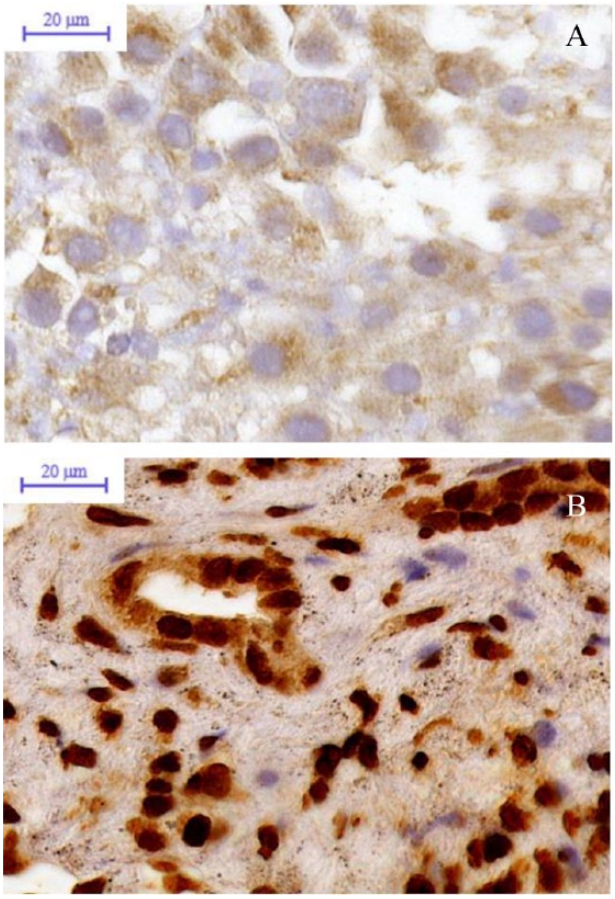

Figure 3. Renal biopsy immunohistochemistry results by microscopy. (A) G-CSF expression (brown) and (B) CXCL8 expression (brown) in the renal carcinoma cells. Original magnification ×100, patient 1.

8 cm × 3 cm × 2 cm lymph node conglomerate. Histology of the tumor revealed low differentiation (Fuhrman grade 4) and growth into the renal vein. Immunohistochemistry of the tumor was performed but did not include analysis of G-CSF or CXCL8. The leukocyte count decreased in the early postoperative period. However, the patient received no systemic antitumoral treatment and died 6 weeks postoperatively.

Methods

Multiplex cytokine measurements

Preselected serum cytokines (including G-CSF and several interleukins) and chemokines were measured with a 25-plex Milliplex human kit, as available for clinical applications at the Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden. The multiplex procedure was performed according to the manufacturer's instructions (Millipore Corporation, St. Louis, MO, USA). Sample analysis was performed on a Luminex 200 platform (Luminex, Austin, TX, USA) using Milliplex Analyst Software (Millipore Corporation). The reference material consisted of serum samples from healthy adult female and male blood donors (n = 16-108) used also in an earlier study.[14] Blood donors in Stockholm

County Council are requested to give a generalized consent, with the possibility to opt out, that their blood can be used in ethically approved research and determinations of laboratory reference values.

IHC for G-CSF and CXCL8

Immunohistochemistry for assessment of G-CSF and CXCL8 expression in the renal tumor from patient 1 was performed on formalin-fixed paraffin-embedded tissue blocks. The biopsies used in this study were taken at the time of diagnosis. The blocks were sectioned into 4 µm and pretreated using PT Link (Dako, Glostrup, Denmark) with EnVisionTM FLEX Target Retrieval Solution High pH (Dako) containing Tris/EDTA buffer at pH 9.5. All washing was performed with EnVision FLEX Wash Buffer pH 7.75 (Dako). Staining was performed according to the manufacturer's protocol (Dako). The samples were incubated with either the primary mouse monoclonal anti-G-CSF antibody (Santa Cruz Biotechnology Cat# sc-53292, RRID:AB_629553, clone 3D1, Dallas, TX, USA) at dilution 1:50 or the primary rabbit polyclonal anti-CXCL8 antibody (Abcam Cat# ab7747, RRID:AB_306040, Cambridge, UK) at dilution 1:100 for 30 minutes. The secondary antibody incubation time was 15 minutes. All detection reagents were from the EnVision FLEX series by Dako (secondary antibodies, EnVision FLEX HRP, and EnVision FLEX Substrate Buffer). The slides were counterstained with hematoxylin (EnVision FLEX Hematoxylin), dehydrated, and then mounted using an automated Tissue-Tek (Sakura, Finetek Europe B.V., Zoeterwoude, The Netherlands). Pancreas and tonsil tissue were used as positive controls for G-CSF and CXCL8, respectively.

Statistical analyses

Mean values ± SD as well as 5% to 95% percentiles for the serum cytokine/chemokine concentrations in the healthy controls were calculated and supplied. Differences in distributions between RCC and healthy controls were analyzed by the Mann-Whitney nonparametric test due to few patient samples and nonnormal distributions. The resulting exact *P* values, with <.05 considered statistically significant, were only shown in Table 1 due to a focus in this report on individual RCC cases rather than group-level data. The software IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, NY, USA) was used for the computations.

Discussion and Conclusions

Combined G-CSF and CXCL8 mRNA overexpression by an RCC has recently been reported in one case,[11] and these cytokines, on the protein level, were also overexpressed in our index patient as determined by highly elevated serum levels as well as distinctly positive immunostaining of tumor cells in the patients' renal biopsy. Not only was neutrophilia prominent in this patient but also monocytosis, basophilia, and slight eosinophilia were found. These features are well-known effects of G-CSF in the treatment setting. However, the 2 additionally investigated patients with RCC of our study with leukemoid reactions/paraneoplastic leukocytosis also presented with monocytosis and high serum levels of CXCL8, IL-6, IL-10, MCP-1, TNF, and low IFN-γ and IL-1α similar to the first patient, but without increased serum levels of G-CSF (Table 1, patients 2 and 3).

As already mentioned, 12 earlier published cases of G-CSF–producing RCC with leukemoid reactions were all from Asian countries.[9–11] A report from the Netherlands found IL-6 and CXCL8 but not G-CSF production by an RCC with a leukemoid reaction.[3] Among other cytokines, high GM-CSF serum levels were found associated with paraneoplastic hypereosinophilia in renal cell[15] and other cancers.[2] Recent studies have evaluated the IHC expression of G-CSF,[16] GM-CSF,[17] M-CSF,[18] and M-CSF receptor[19] in RCC and found high expression to have a negative prognostic influence.

For other urological cancers, a systematic review restricted to the Japanese population revealed that G-CSF overproduction was reported in 46 cases of upper urinary tract carcinoma, with the primary location being the renal pelvis, ureter, or both.[20] Of these 46 cases, 63% had positive IHC staining for G-CSF. The other 37% showed high levels of serum G-CSF associated with leukocytosis which was not related to infection or blood disease.[20] In another study from Japan, serum G-CSF levels were measured in 141 patients with bladder cancer and found elevated in 9.2%, compared with less than 5% of 21 patients with RCC.[21] Autocrine stimulation due to concomitant expression of G-CSF and functional G-CSF receptors was demonstrated in bladder cancer cells.[22] A recent case report (from the United States) of a female patient with bladder cancer and a serum G-CSF level 10× the upper limit of normal also reviewed several biological aspects, and attempts to target autocrine/paracrine G-CSF signaling in bladder cancer were proposed.[23]

Mobilization of leukocytes from bone marrow into blood is one recognized effect of CXCL8.[24,25] A contribution of tumor CXCL8 expression (and secretion) to the leukemoid reaction of our patient is therefore suggested. Increased CXCL8 expression by metastasizing RCC is a recognized phenomenon and is related to a poor prognosis.[26–30] Increased IL-6, CXCL8, and IL-10 serum levels correlate with increased C-reactive protein.[29]

High serum levels of IL-6 are also related to leukocytosis, monocytosis, thrombocytosis,[30] and a poor prognosis in patients with metastasized RCC[29–31] as well as to the clinical benefit of pazopanib treatment.[31] It was shown that an integrated approach of combined markers for regulatory T cells and serum cytokines/chemokines (IL-6, CXCL8, VEGF (vascular endothelial growth factor), EGF (epidermal growth factor), HGF (hepatocyte growth factor), CXCL10 [C-X-C-motif chemokine ligand 10], CXCL11 [C-X-C-motif chemokine

ligand 11]) would provide an informative host immunity-related prognostic profile for RCC.[32] An in vitro study of human RCC cell lines showed that TNF induced both chemokine receptors and their ligands leading to progressive features.[33] In total, the knowledge and understanding of links between inflammation, cytokines, and cancer accumulate rapidly.[34,35]

As G-CSF is expressed by normal monocytes, monocytosis might contribute to increased serum G-CSF, but serum G-CSF was elevated only in our first patient who had strong expression by the RCC cells, suggesting that monocytosis was probably not a major source. Generally, in solid cancer, excessive cytokine generation, sometimes causing leukemoid reactions, may emanate from cancer cells or from cells reacting to the malignancy.[2,7,36] By crosstalk mechanisms, myeloproliferation in cancer can be important for tumor progression and has been proposed as a new target of therapy.[7] An association specifically between absolute monocytosis and poor prognosis has been found for a diversity of malignancies.[8,37–40] In RCC, adverse prognostic influences of low ratios of blood lymphocytes/monocytes[41] and lymphocytes/neutrophils[42] have been highlighted.

In summary, a pattern of neutrophilia and monocytosis together with high serum levels of CXCL8, IL-6, IL-10, MCP-1, and TNF, but low IFN-γ and IL-1α, was found in 3 consecutive patients with RCC and leukemoid reactions/paraneoplastic leukocytosis. One of the patients also showed a highly elevated serum level of G-CSF. To our knowledge, co-expression of G-CSF and CXCL8 by RCC cells in a patient with leukemoid reaction was here verified by IHC for the first time, whereas high mRNA levels of these 2 cytokines in RCC tissue were shown in a recently reported case exhibiting high serum levels of CXCL8 (1260 pg/mL), IL-6 (474 pg/mL), and G-CSF (143 pg/mL).[11] Gene expression analysis was not performed in our patients, but upregulated RCC gene transcription could be a mechanism behind their elevated cytokine expressions. A broad stimulation of pro-inflammatory genes such as *IL-6, CXCL8*, and *MCP-1* through nuclear factor-κB signaling is common in cancer,[11,43] but further molecular studies are necessary to explain variability of cytokine expression combinations among individual cases and different tumor types with leukemoid reactions.[2,35]

As suggested for bladder cancer, autocrine cytokine signaling loops may become future therapeutic targets even for other cancers with leukemoid reactions.[23] There is a need for innovative treatments for RCC with paraneoplastic leukocytosis because of the advanced stage at diagnosis, aggressive tumor phenotype, and poor survival with conventional therapies. Reliable prognostic and predictive markers should facilitate decision making for an accurate individualized treatment approach for metastasized RCC.[35,44,45] Aspects of quality of life must be taken into consideration. On another side of the RCC disease spectrum, searches for early detection biomarkers should be prioritized[35,45,46] to aid timely actions against the "emperor of all maladies"[47] for greater curability.

Although the index patient of our study had hematuria early in his disease course, a leukemoid reaction preceded the diagnosis of RCC, giving rise to suspicion of a hematological malignancy. Overall, the consecutive series of 3 cases indicates the value of multiplex serum cytokine measurements for diagnosis and understanding mechanisms, provided accessibility and reliability in the clinical context.[48] For future studies, new high-throughput immunoassay platforms, which enable simultaneous and accurate measurements of large numbers of proteins with high sensitivity and specificity, may be recommended for efficient biomarker identification.[44,49–52] Furthermore, multi-omics approaches are warranted for attainment of deeper biological insights to facilitate precision-based and personalized treatments, for improved outcomes.[53]

Acknowledgements

The authors are grateful to Elizabeth Ishizaki and Per Marits at the Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden, for analyzing the cytokines/chemokines and valuable advice, respectively.

Author Contributions

MÅ conceptualized the study, collected data, and drafted the manuscript. WT and MGK performed the histological and IHC examinations and contributed to the manuscript. OL contributed to data acquisition and interpretation. JP performed critical review of the manuscript. PL contributed to data acquisition and performed critical review of the manuscript. All authors read and approved the final manuscript.

Disclosures and Ethics

Blood sampling from patients for cytokine analyses was performed to aid diagnosis, after oral consent. Formal consent was not required for these laboratory investigations as they were available in clinical routine. Written informed consent for patient information and images to be published was provided by legally authorized representatives of all 3 patients. Copies of the written consents are available for review by the Editor-in-Chief of this journal. The data and materials that support the findings of this study are available from the corresponding author on reasonable request.

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