Immunoexpression of lactoferrin in human sporadic renal cell carcinomas

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Abstract. By immunohistochemistry, lactoferrin (Lf) expression was retrospectively investigated in 40 formalin-fixed paraffin-embedded kidney samples, obtained at surgery from an equal number of patients. Histologically, 28 cases were clear cell carcinomas (CCC), 7 papillary carcinomas (PC) and 5 chromophobe carcinomas (CC). Ten specimens of unaffected renal parenchyma were utilized as tissue control. On 4-μm thick sections, the Lf immunoreactivity was revealed either by a rabbit polyclonal or mouse monoclonal anti-human Lf antisera; the quantification of Lf immunoreactivity was performed using an intensity-distribution (ID) score. A positive immunoreaction by both anti-Lf antibodies was found in 62.5% (25/40) of RCC, mainly evident and diffuse by monoclonal antiserum. The immunoreactivity was observed in the cytoplasmic boundary of neoplastic cells in CCC and PC, while in CC Lf showed a diffuse granular cytoplasmic localization. Moreover, significant differences in Lf ID score were found among CCC and non-CCC variants ($P<0.00001$), the former showed a lower score; no relationships between immunohistochemical data and the sex or age of patients, grade of RCC, stage of the disease or degree of terminal anemia were encountered. Normal unaffected tubular structures were positive for Lf; glomeruli were unstained. The reduced Lf immunoexpression in some CCC may be because of the down-regulation of Lf gene due to the frequent deletion of 3p regions reported in this RCC variant.

Introduction

Lactoferrin (Lf), an 80-kDa basic glycoprotein, is a member of the transferrin family of iron-binding protein, which was originally isolated from human milk (1). By radioimmunological and immunoenzymatic procedures, Lf has been detected in many biological fluids as well as in human fetal and adult tissues (2-7). By immunohistochemistry, the distribution of Lf has been analysed in normal human tissues such as stomach, kidney, lung, pancreas, liver and bone marrow (4). In human neoplastic conditions, the Lf immunoreactivity has been extensively investigated (8-20); in particular, a variable immunohistochemical pattern of this iron-binding protein has been reported in adenocarcinomas of the parotid gland (8), prostatic carcinomas (9), breast carcinomas (10,11), thyroid tumours (12,13,16,18), gastric adenomas and carcinomas (15), colorectal adenomas and carcinomas (17), gallbladder carcinomas (19), astrocytomas and multiforme glioblastomas (20), pigmented skin lesions (21) and endometrial carcinomas (22).

Under normal conditions, the tubular epithelium of the human kidney is stained for Lf, while glomeruli are non-reactive (4). In a small series (14), Lf has been detected by peroxidase and immunofluorescence in some cases of renal cell carcinomas and its presence has been related to the degree of anemia present in late phases of this neoplastic condition.

In the present study, by monoclonal and polyclonal antibodies, we have investigated the immunohistochemical distribution pattern of Lf in a retrospective series of sporadic renal cell carcinomas, in order to verify if differences in Lf immunoreactivity related to different antisera as well as relationships between Lf immunoexpression and clinico-pathological parameters.

Materials and methods

We investigated Lf immunoexpression in 40 sporadic renal cell carcinoma (RCC) samples (23 left and 17 right kidneys), taken from files of our Department, previously obtained at surgery from an equal number of patients (24 male, 16 female; age range 33-80 years, mean 62). Histologically, 28 cases were clear cell carcinomas (CCC), 7 papillary carcinomas (PC) and 5 chromophobe carcinomas (CC); moreover a sarcomatoid differentiation was encountered in two and one case of CCC and PC, respectively. The grading and staging of RCCs were performed by a 4-tiered system and TNM classification, respectively (23) (Table I). In addition, 10 specimens of unaffected renal parenchyma obtained at surgery or at autopsy were utilized as tissue control.

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Table I. Immunohistochemical detection of Lf in renal cell carcinoma specimens.

Case	Sex	Age	Kidney	Histotype	Grade	TNM stage	Lf staining distribution	Lf staining intensity	Lf ID score
1	F	73	Sn	CCC	1	I	0	0	0
2	M	80	Dx	CCC	3	I	0	0	0
3	M	68	Sn	CCC	4	III	0	0	0
4	F	61	Dx	CCC	2	III	0	0	0
5	F	58	Sn	CCC	3	I	0	0	0
6	F	64	Dx	CCC	3	I	0	0	0
7	M	44	Sn	CCC	2	IV	0	0	0
8	F	62	Sn	CCC	2	III	0	0	0
9	M	69	Sn	CCC	4	II	0	0	0
10	M	67	Sn	CCC	2	I	0	0	0
11	F	59	Sn	CCC	2	III	0	0	0
12	M	45	Sn	CCC	1	I	0	0	0
13	M	65	Dx	CCC	2	II	0	0	0
14	F	58	Sn	CCC	2	III	0	0	0
15	M	72	Sn	CCC	2	I	0	0	0
16	M	74	Sn	CCC	3	II	1	1	1
17	F	60	Sn	CCC	3	III	1	1	1
18	M	52	Dx	CCC	1	I	1	1	1
19	F	62	Dx	CCC	1	I	1	1	1
20	M	60	Sn	CCC	3	III	1	1	1
21	F	63	Dx	CCC	2	I	1	1	1
22	M	71	Sn	CCC	2	II	1	1	1
23	M	70	Sn	CCC	3	III	1	1	1
24	M	72	Sn	CCC	2	III	1	1	1
25	F	58	Dx	CCC	3	I	1	1	1
26	M	73	Sn	CCC	1	III	1	1	1
27	F	41	Dx	CCC	2	I	1	2	2
28	M	75	Sn	CCC[a]	3	IV	1	1	1
29	M	51	Dx	PC	2	II	2	1	2
30	M	60	Sn	PC	1	I	2	1	2
31	M	52	Dx	PC	1	II	2	1	2
32	M	55	Sn	PC	2	I	2	1	2
33	M	66	Dx	PC	4	I	2	1	2
34	F	56	Sn	PC[a]	4	I	2	2	4
35	F	78	Dx	PC[a]	3	III	2	2	4
36	F	33	Sn	CC	1	I	1	2	2
37	M	61	Dx	CC	3	I	2	1	2
38	M	60	Dx	CC	2	I	2	1	2
39	M	86	Dx	CC	2	II	2	2	4
40	F	35	Dx	CC	3	II	2	2	4

[a]Cases with sarcomatoid differentiation. CCC, clear cell carcinoma; PC, papillary carcinoma; CC, chromophobe carcinoma.

All samples were fixed in 10% neutral formalin for 24 h at room temperature (RT) and then embedded in paraffin at 56°C. From each tissue block, three serial 4-μm-thick sections were cut and mounted on silane-coated glasses, then dewaxed in xylene and rehydrated in graded ethanols. One section was subjected to haematoxylin and eosin (H&E) stain, the others were utilized for the immunohistochemical analysis. Antigen retrieval, by heating slides placed in 0.01 M citrate buffer pH 6.0 in a microwave oven for 3 cycles x 5 min, was performed before adding primary antibodies. For the immuno-

histochemical study, sections were treated in a moist chamber: i) with 0.1% H_2O_2 in methanol to block the intrinsic peroxidase activity (30 min at RT); ii) with normal sheep serum to prevent unspecific adherence of serum proteins; iii) with polyclonal (rabbit anti-human lactoferrin, DakoCytomation, Denmark 1:300; overnight at 4˚C) as well as monoclonal (Clone 1A1; Biodesign International, USA; w.d. 1:75; 60 min at RT) primary antibodies anti-human Lf; iv) with sheep anti-rabbit or anti-mouse immunoglobulin antiserum (Behring Institute; w.d. 1:25; 30 min at RT); and v) with rabbit or mouse anti-horseradish peroxidase-antiperoxidase complexes (DakoCytomation; w.d. 1:25; 30 min at RT). For the demonstration of peroxidase activity the sections were incubated in darkness (24) for 10 min with 3-3' diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA), in the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate-buffered saline (PBS). The nuclear counterstaining was performed by Mayer's haemalum.

To test the specificity of Lf immunostaining in order to deny the possibility of non-specific reaction, serial sections of each affected renal specimen were tested by replacing the specific antiserum by either PBS, normal rabbit serum or absorbing with excess of purified human Lf from human liver and spleen (Sigma Chemical Co.) as well as with pre-absorbed primary antibody: the results obtained were negative. Moreover, the Lf immunoreactivity demonstrated in granules of polymorphonuclear neutrophils present inside lesions was utilized as positive control. Finally, in order to test the inter-run variability of Lf staining, the same Lf-positive parotid sample was utilized in every run.

Immunostained sections were estimated by light microscopy using a x20 and x40 objective lens and x10 eyepiece. Two pathologists using a double-headed microscope performed the assessment of Lf immunostained sections on a consensus basis. The percentage of stained neoplastic epithelial cells (staining distribution) was graded as follows: 0 (no staining); 1 (>0-5%); 2 (>5-50%); and 3 (>50%). In addition, the staining intensity (weak, 1; moderate, 2; strong, 3) was also taken into consideration. Successively, Lf intensity-distribution (ID) score was calculated by multiplying, for each case, the staining intensity by the staining distribution, similarly to that reported (25,26).

The possible correlations between immunohistochemical data, reported as Lf ID-score, and clinicopathological data of renal specimens were investigated using non-parametric methods (Mann-Whitney U test; Kruskal-Wallis H test). A probability (P) value <0.05 was considered statistically significant. Data were analysed using the SPSS package version 6.1.3 (SPSS Inc., Chicago, IL).

Results

A positive immunoreaction with monoclonal as well as polyclonal anti-Lf antibodies was found in 62.5% (25/40) of RCC. The immunoreactivity was mainly observed in the cytoplasm of neoplastic renal cells, although an occasional nuclear immunolocalization was encountered. The Lf immunostaining was mainly evident and diffuse utilizing the monoclonal antibody (Fig. 1a), while the polyclonal one showed a more focal and slight positivity in neoplastic elements present in corresponding serial sections (Fig. 1b). The pattern of positivity was different in RCC variants. In CCC (Fig. 1a), PC (Fig. 2) as well as in sarcomatoid component (Fig. 3) Lf immunostaining was mainly evident at the cytoplasmic

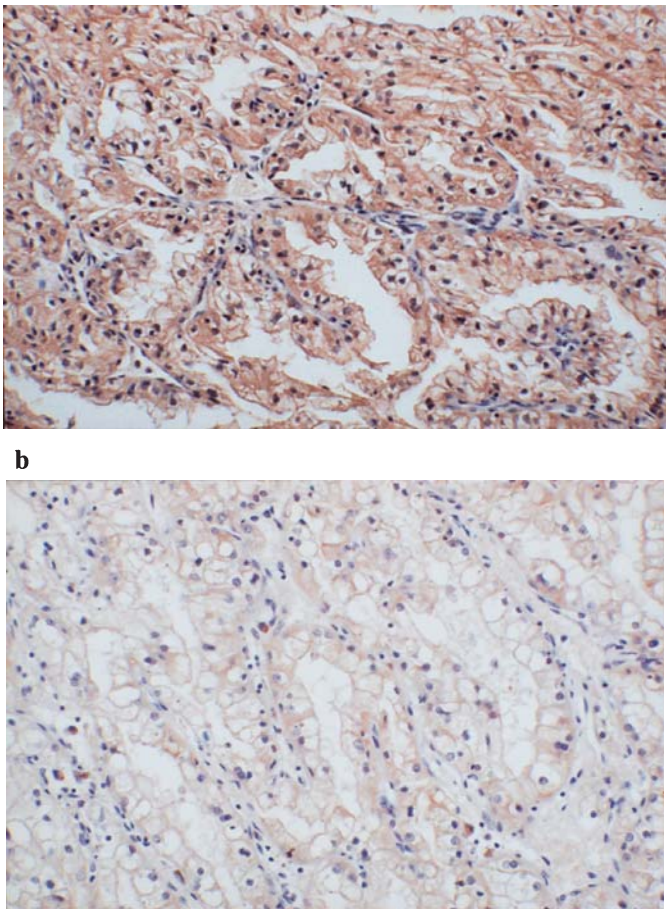

Figure 1. Lf immunoreactivity: (a), evident cytoplasmic positivity was found at the periphery of neoplastic elements in clear cell variant by Lf monoclonal antiserum; (b), slight staining was appreciable in the same elements by Lf polyclonal antiserum (immunoperoxidase, Mayer's haemalum counterstain).

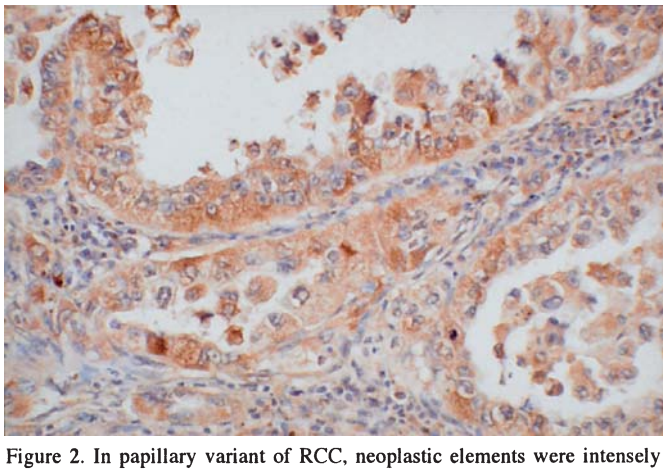

Figure 2. In papillary variant of RCC, neoplastic elements were intensely stained for Lf (immunoperoxidase, Mayer's haemalum counterstain).

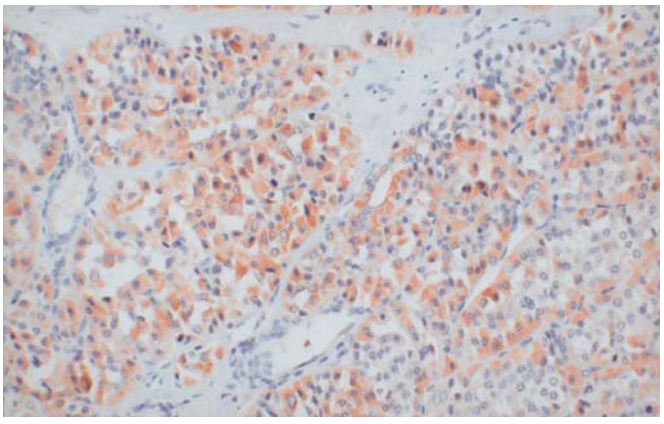

Figure 3. Cromophobe Lf positively-stained cells were found in direct contact with negative ones (immunoperoxidase, Mayer's haemalum counterstain).

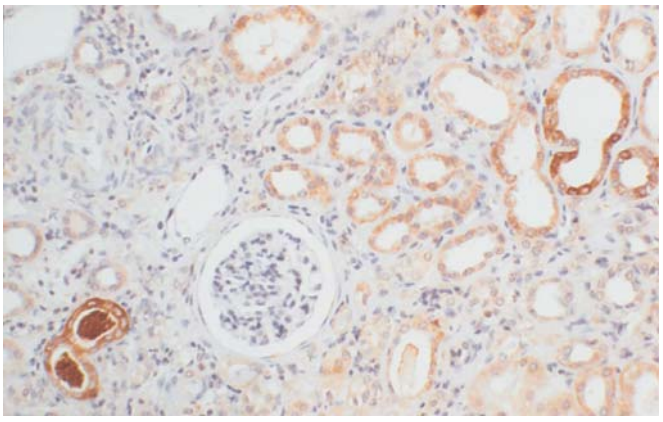

Figure 5. Tubular structures were reactive with Lf antisera, while glomeruli were unstained (immunoperoxidase, Mayer's haemalum counterstain).

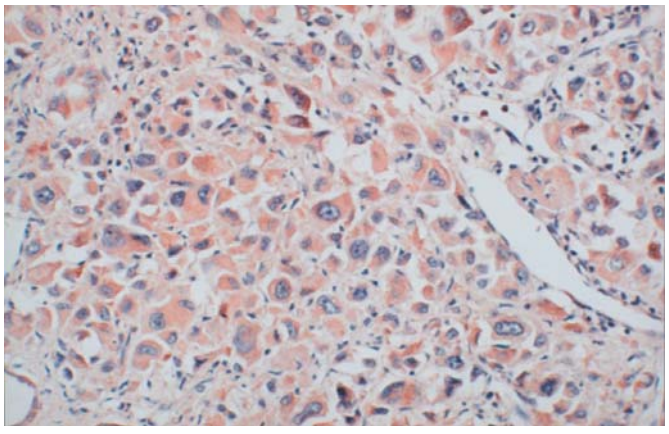

Figure 4. The Lf immunoreactivity was diffusely revealed in sarcomatoid atypical cells by monoclonal antiserum (immunoperoxidase, Mayer's haemalum counterstain).

boundary of neoplastic cells. In CC variant, Lf showed a diffuse granular cytoplasmic immunolocalization, with intensely stained positive cromophobe elements in direct contact with negative ones (Fig. 4).

Immunohistochemical data obtained by Lf monoclonal antibody and related to clinicopathological characteristics of RCC are given in Table I. Lf immunoexpression was different in histological variants of RCC; 13/28 (46.4%) cases of CCC showed immunoreactivity with an ID score ranging from 0 to 2 (median value 0), while all cases of PC and CC were positive with an ID score ranging from 2 to 4 (median value 2). When all examined RCC were analyzed by Mann-Whitney U test, significant differences in Lf ID score were found among CCC (mean rank 14.64) and non-CCC (mean rank 34.17) variants ($P<0.00001$); moreover, no relationships between immunohistochemical data and the sex or age of patients, grade of RCC, stage of the disease as well as degree of terminal anemia were encountered.

Normal unaffected tubular structures (Fig. 5), as well as polymorphonuclear neutrophils or monocytes were positive for Lf; by contrast, glomeruli were unstained (Fig. 5).

Discussion

In the present study we report Lf immunostaining in RCC utilizing different antibodies, either polyclonal or monoclonal, Lf was detected in the cytoplasm of 25/40 RCC (62.5%) with a variable ID score, although an occasional nuclear immunolocalization was also found. The site of Lf immunoreactivity both in the nucleus and cytoplasm is not surprising, since this secretory protein has been already detected in the nucleus, mainly in nucleoli, and it has been thought to be involved in ribosomal biogenesis (27,28). On the other hand, after its transport into the nucleus, Lf is able to bind to specific DNA sequences, activating transcription (28). Furthermore, the pattern of Lf positivity was different in clear cell, papillary or cromophobe RCC variants; neoplastic clear cells exhibited a Lf immunolocalization mainly evident at the cytoplasmic boundary, while immunoreactive cromophobe elements were found in direct contact with negative ones showing a diffuse granular Lf cyto-plasmic distribution. Although the Lf immunostaining in RCC was appreciable with both antisera, the highest ID score was achieved by the monoclonal one; therefore, the present approach to Lf distribution seems to indicate the monoclonal antibody is more specific and sensitive, able to bind all reactive epitopes either masked or unmasked. However, the exposure and/or conformation of the epitopes for the monoclonal antibodies appeared not to be affected by the conformational changes subject to incorporation of iron in Lf, allowing thus a greater affinity between antigenic determinants and monoclonal antibodies (29).

Many biological functions of Lf have been addressed including regulation of iron homeostasis, antioxidant and immunoregulatory activities, bacteriostatic and bactericidal effects as well as the capability to curb the proliferation of other microbes such as fungi and viruses (30-37). In our study, Lf immunoexpression was found in areas of renal parenchyma adjacent to neoplasms, mainly in the proximal tubular structures, while glomeruli were unstained. It could be argued that the presence of Lf in tubular non-neoplastic cells may be related to its endogenous production in order to activate the host defence system by Lf specific functions,

such as antioxidant role, interleukin activation and T-lymphocyte proliferation. Interestingly, although the histogenesis of RCC has been widely attributed to tubular cells (23), a quote of our cohort of CCC variant was unstained for Lf. However, this iron-binding multi-function protein is coded by a gene present in the short arm of chromosome 3 (3p) (38-40) and 3p-regions may be deleted in some RCC (23,41). Therefore, the evidence of a reduced Lf immunoexpression in CCC should refer to the down-regulation of Lf gene due to the frequent deletion of 3p regions in this RCC variant. Consequently, from a practical point of view, the immunohistochemical analysis of Lf distribution could help to identify renal tumours with a loss of this chromosomal region. Conversely, the presence of Lf in renal neoplastic elements might be related to the production of this iron-binding protein in the tumour itself as suggested by Lf immunostaining, although this interpretation should be controlled by methods other than morphological analysis. Alternatively, the cytoplasmic immunolocalization of Lf in neoplastic cells could not reflect an intracellular synthesis, being instead the consequence of defective or functionally impaired Lf receptors; specific Lf-binding sites have been shown in a human neoplastic cell line (42) and a receptor-mediated endocytosis could be hypothesized, similarly to that demonstrated for transferrin in growing cells (43); in this manner, the intracellular presence of Lf immunoreactivity may reveal the degree of transmembranous iron transfer.

It has been suggested that Lf inhibits cell proliferation and suppresses tumour growth *in vivo* (44-46), although the molecular mechanisms underlying these effects remain unknown. Nevertheless, it has been reported that treatment of breast carcinoma cells MDA-MB-231 with human Lf induces growth arrest at the G1 to S transition of the cell cycle (45); in addition, in adult rats, it has been shown that Lf treatment significantly inhibited the VEGF(165)-mediated response in terms of microvessel spatial extension, overall vascularity and incidence of crossover (46). Finally, these effects of Lf on target cells appear to depend on the cell phenotype; in fact, Lf does not modify the susceptibility to lysis of haematopoietic cells such as Jurkat and K-562 cells, but does significantly increase that of the breast and colon epithelial cells (44). On the other hand, it is well known that Lf has a high affinity for iron, which has been considered an essential nutrient for cells that are dividing rapidly such as tumour cells, taking part in various metabolic cellular processes such as oxydative phosphorylation and RNA and DNA synthesis (47,48); therefore, neoplastic renal cells should be able to utilize Lf in order to have a greater availability of iron for their turnover, similarly to that suggested in other neoplasms (12,19,20). However, Lf has been shown to mediate the hyposideremia of acute inflammation (49) and a role for Lf as a mediator of the anemia associated with renal adenocarcinoma has been hypothesized since 10/24 RCC with low iron levels were positive with both FITC and peroxidase-conjugated anti-lactoferrin (14). We were unable to confirm the suggested relationship between anemia encountered in different neoplasms and a corresponding Lf immunoexpression (15,17); moreover, in the present study, Lf immunostaining was not found in relation to the grade of RCC, site and stage of the disease neither with the degree of terminal anemia.

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