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Glypican-3 is Expressed in Chromophobe Renal Cell Carcinomas

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Glypicans are core proteins of heparan sulfate proteoglycans that have numerous functions. Glypican-3 (GPC3) expression has been detected to be altered in several tumors, including hepatocellular carcinoma, breast carcinoma, and embryonic malignancies. To date, no information on GPC3 status in renal cell carcinoma is available. The material for the study consisted of 625 cases of renal tumors diagnosed at our institution. Tissue microarrays were constructed using all acceptable quality tissue blocks. Immunohistochemistry for GPC3 was performed with 1G12 antibody (BioMosaics). We found strong positive staining in 15 cases, moderate in 4 and weak in 68. The reactivity was particularly evident in chromophobe renal cell carcinomas (32/40). Our findings are of note in cases when GPC3 may be used in differential diagnosis of tumors of uncertain primary location.

Introduction

Glypicans (GPC) form a family of heparan sulfate proteoglycans, which consist in mammals of 6 different core proteins [8, 10]. The function of these molecules is extensively studied; GPCs have been shown to participate in ontogenesis, cellular signaling and cancer. GPCs participate in renal development and their expression may be altered in renal diseases [36]. Recently, GPC3 has become a focus of interest, as it was discovered to be rather specifically expressed in hepatocellular carcinoma [29] and useful in germ cell tumor differential diagnosis [46]. It was also studied in embryonic tumors, including nephroblastoma [34] and several carcinomas [23, 32], but - to my surprise - not in renal cell carcinoma (RCC). Thus we decided to explore the topic.

Material and Methods

The material consisted of all consecutive cases of renal cell tumors diagnosed in our institution from 1992 to 2005. The material was formalin-fixed and paraffin-embedded by routine protocols. From the tissue blocks 3μm sections were prepared and stained with hematoxillin-eosin. Cases with extensive necrosis, cystic tumors with only tiny foci of neoplastic epithelium, angiomyolipomas, tumors of uncertain classification and secondary tumors were excluded from consideration. All the cases were reclassified according to the WHO system [9]. Reclassification was based on HE slides, with the use of Hale's colloid iron, PTAH, paS staining and immunohistochemistry for epithelial membrane antigen, pan-cytokeratin, cytokeratins 7, CD10, vimentin when appropriate. Tumors were also restaged by the AJCC system [1].

Hematoxylin-eosin sections were reviewed and in each case a section containing a representative and well-preserved tumor was selected. Selected paraffin blocks served for preparing tissue microarrays using a Tissue MicroArrayer MTA-1 (Beecher Instruments Inc., WI, USA). From each donor block, three 0.6mm cylinders were cut off. The acceptor paraffin blocks were prepared noting the location of each cylinder, and 3 μm-thick sections were cut.

For immunohistochemistry the standard staining protocol was used. Briefly, the slides were dewaxed, rehydrated and incubated in 3% peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (0.2% citric acid titrated to pH 6.0 with 2N NaOH) for 3x5 minutes at 750 W. Primary antibodies used in the study are listed in Table 1.

The GPC3 antibody was incubated overnight and other antibodies for 30 minutes. The ENVISION (DAKO,

TABLE 1
Primary antibodies used

antigen	clone	manufacturer	dilution
EMA	E29	DAKO, Denmark	1:100
panCK	MNF116	DAKO	1:50
CK7	OV-TL12/30	DAKO	1:50
CD10	56C	Novocastra Ltd, UK	1:50
vimentin	V9	DAKO	1:50
GPC3	1G12	BioMosaics Inc, USA	stock

Denmark) detection system was used. 3-amino-9-ethylcarbasole (DAKO, Denmark) was used as the chromogen. The slides were counterstained with Mayer hematoxylin (DAKO, Denmark).

The results of GPC3 staining were scored semiquantitavely from 0 to 3+, averaging the results between TMA cores. The assessment of the staining was done with a Zeiss Axioscope microscope (Zeiss GmbH, Germany) and the results of scoring introduced into the Excel (Microsoft Corp. USA) spreadsheet. The t-Student statistics, χ^2, Mann-Whitney U, Kruskall-Wallis ANOVA, and ANOVA test were used, when appropriate. The statistical analysis was done with Statistica 7.1 (Statsoft, Tulsa, USA). The significance level was set to 0.05.

Results

The material under study consisted of 625 cases. There were 254 females and 371 males. The mean age at the diagnosis was 61.0 years (range 26 to 92, SD 11.1); for females - 61.5 years (SD 11.5), for males - 60.6 years (SD 10.8, difference not significant). The age of the patients was significantly lower for chromophobe renal cell carcinoma (56.9). The histological diagnoses of cases under study are shown in Table 2. A sarcomatoid component was present in 32 cases. The stage of primary tumor was pT1 in 279 cases, pT2 in 66, pT3 in 268, and pT4 in 3. In 9 cases, the available data were insufficient for staging. The mean tumor diameter was 6.3cm (range 0.5 to 26.0, SD 3.5). The smallest tumors were papillary carcinomas (5.8cm) and the largest chromophobe renal cell carcinomas (7.2). These differences were not statistically significant. In 33 cases, the tumor was partially cystic. Lymph node metastases were present in 5 cases.

Staining for GPC3 (Fig. 1) was negative in 538 cases (86.1%), 1+ in 68 cases (10.9%), 2+ in 4 cases (0.6%) and 3+ in 15 (2.4%). In positive cases the reaction was cytoplasmic and homogenous throughout the tissue cores included in the TMA. The results of the staining according to histological types is shown in Table 3. It is noteworthy that the vast majority of strongly positive cases were chromophobe carcinomas. The differences between tumor types were statistically significant ($p<0.0001$). The GPC3 reactivity was not related to the size of tumor, stage, sarcomatoid transformation, presence of vascular invasion, positive surgical margins, multiplicity of the tumor, nor presence of cystic component. The patients with GPC-3 reactive lesions were younger as the age of the patients with GPC-3 negative tumors was 61.0, for 1+ tumors 62.4, for 2+ tumors 57.0 and for 3+ tumors 55.2 ($p<0.05$). This effect might depend on specific characteristics of chromophobe RCC. Indeed chromophobe RCC patients were younger (see above) and limiting analysis to chromophobe RCC caused disappear-

TABLE 2
Histological diagnoses in cases under study

diagnosis	N	(%)
clear cell carcinoma	502	80.3
papillary carcinoma	62	9.9
chromophobe carcinoma	40	6.4
oncocytoma	21	3.4

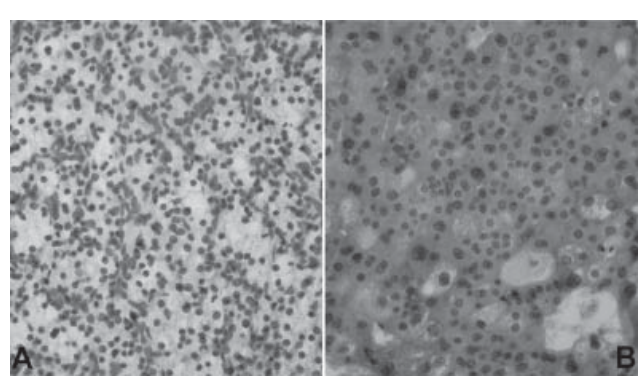

Fig 1. GPC3 staining results. A) Renal clear cell carcinoma. No staining is visible. B) Strong cytoplasmic reaction in a case of chromophobe renal cell carcinoma. Immunohistochemistry, lens magnification 40x.

TABLE 3
GPC3 scoring according to histological types

	GPC3 staining result				
	0	1+	2+	3+	total
clear cell carcinoma	475 (94.6%)	26 (5.2%)	1 (0.2%)	0 (0.0%)	502
papillary carcinoma	46 (74.2%)	13 (21.0%)	2 (3.2%)	1 (1.6%)	62
chromophobe carcinoma	8 (20.0%)	18 (45.0%)	0 (0.0%)	14 (35.0%)	40
oncocytoma	9 (42.9%)	11 (52.4%)	1 (4.8%)	0 (0.0%)	21
all cases	538 (86.1%)	68 (10.9%)	4 (0.6%)	15 (2.4%)	625

ance of age-GPC3 relationship statistical significance. Still, average age of patients with GPC-3 negative chromophobe RCC was 56.5, but 54.7 for 3+ tumors.

Discussion

Heparan sulfate (HS) proteoglycans are important components of extracellular matrix and cell surface, as it was first described by Kreamer in 1971 [20]. HS proteoglicans are divided into two families: syndecans, with short intracytoplasmic domain and extended extracellular domains positioning HS distantly from cellular membrane, and glypicans - globular proteins with HS insertion point located close to plasma membrane [2, 8, 12, 22]. Six different glypicans (GPC1 - GPC6) exist in mammals. Their aminoacid sequences are only moderately homologous, but quite similar in their three-dimensional structure. Of these, GPC3 is the best investigated, both *in vitro* and *in vivo* [10].

Glypican-3 was first identified as OCI-5 by Filmus et al, who screened cDNA libraries for genes expressed during intestinal development in rats [11]. The same group identified OCI-5 as a cell membrane associated heparan sulfate proteoglycan [13]. Human homologue of the OCI-5 gene was identified in cDNA derived from a mitoxantrone-resistant gastric carcinoma cell line by Lage and Dietel, and was labeled MXR7 [21]. Wichert et al. explored the subject in a more detailed way and found that GPC3 is not a mere mixantrone-resistance marker, but it participates in this resistance, as rybozyme–mediated removal abolishes the drug resistance [42].

As a rule, GPC3 is expressed in the embryo, especially in mesodermal tissues, but not in adult tissues. Some exception does exist, however; GPC3 expression may also differ in cancer and its tissue of origin [23, 32, 41]. In the normal adult kidney, the principal GPC is GPC1 [33]. In renal development, heterogeneity of proteoglycan core proteins and glycosaminoglycans act as a switching mechanism to regulate different stages of ureteric bud branching [36]. GPC3 in developing kidney modulates Bmp2-Smad signaling and inhibits branching of the ureteric bud [16]. This function and place of expression is interesting when we keep in mind the chromophobe renal cell carcinoma origin from the distant part of the nephron. Jain et al. found that GPC3 expression is decreased in renal dysplasia [19]. Loss of function of the GPC3 gene is implicated in Simpson-Golabi-Behmel syndrome (SGBS). SGBS consists of prenatal and postnatal overgrowth, renal malformations, especially renal medullary cystic dysplasia, pediatric embryonic tumors, including Wilms, inguinal or umbilical hernias, cardiac defects, skeletal abnormalities, such as polydactyly, vertebral and rib defects, cleft palate and facial deformities [10, 15]. Other glypicans may also participate in renal development and their mutation may cause renal lesions. Severe cases of SGBS were linked to Xq22 locus [5] and mutations in GPC4 [15]. GPC4 is expressed in developing kidney, especially in tubular epithelial cells [40]. GPC5 is located in 12q31-32, with deletions in this locus causing symptoms overlapping with SGBS [35]. SGBS demonstrates interesting similarities to Beckwith-Widemann syndrome [12]. It is worth remembering that Beckwith-Widemann syndrome is linked to biallelic expression of IGF-2, a factor related to GPC3 signaling.

The GPC3 gene is switched off in some cancers, such as ovarian carcinoma, thus it is a putative tumor suppressor gene [23]. The growth of mesothelioma cell lines is inhibited by GPC3 expression [28]. In breast cancer, glypican-3 prevents IGF-2-induced MMP-2 activation, thus GPC3-transfected cell lines form fewer metastases [32].

GPC3 was shown to induce apoptosis in breast carcinoma and mesothelioma cell lines [14]. The proapoptotic effect depends on GPC3 protein only and not on glycosaminoglycan chains, and is prevented by IGF-2. Further studies are needed to see if any relationship between GPC3 expression and prognosis exists also for renal tumors.

GPC3 immunohistochemistry may be useful for differential diagnosis of germ cell tumors. In non-neoplastic testis, no GPC3 reactivity is present. In germ cell tumors, it appears in a later stage of the disease, with intratubular neoplasia completely negative. Zyner et al. found GPC3 reactivity in all yolk sac tumors and choriocarcinoma areas present in their series, whereas only a minority of immature teratomas and even fewer embryonic carcinomas were positive; mature teratomas, seminomas and intratubular neoplasia were completely negative [46]. Ota et al. observed GPC3 immunoreactivity in all yolk sac tumors they examined, whereas embryonic carcinomas, seminomas and most of teratomas were negative. Focal reactivity was found in the choriocarcinoma component and weak reactivity in poorly differentiated areas of teratomas [31].

GPC3 is also present in embryonic tumors. Saikali found no GPC3 in normal tissue accompanying Wilms tumor [34]. Toretsky et al. [39] found an increased GPC3 expression in Wilms tumor and hepatoblastoma. In normal tissue surrounding the lesion, a weak GPC3 expression was found. The authors suggested a tumor promoting action for GPC3, rather than tumor-suppressive described in carcinomas. The mechanisms of increased GPC3 expression in embryonic tumors are unclear. White et al. found that the GPC3 gene is mutated in some 5% of Wilms tumor cases [41]. According to Boily et al. [3], promotor methylation is altered, with loss of methylation in neuroblastoma, but gain of methylation in nephroblastoma. However, no correlation with GPC3 expression was observed and it was suggested that other mechanisms are involved. Also in normal tissue promotor methylation seems to be similar in GPC3 expressing and non-expressing tissues, suggesting that promotor methylation is not involved in GPC3 expression regulation [18]. On the other hand, in rat mesothelioma, aberrant methylation of the GPC3 promotor region was detected [28].

Recently, the GPC3 research concentrates on liver cancer diagnosis. Although rather rare in developed countries, hepatocellular carcinoma (HCC) is the 5th cancer worldwide, chiefly because of the high hepatitis C virus (HCV) prevalence. A further increase in frequency may also be expected [4, 37]. Thus, there is need for new serum markers for screening purposes; new histochemical markers to assist the diagnosis are needed as well. GPC3 in HCC was first detected by gene profiling, combining microarray and representational difference analysis [29, 37, 43]. In HCC, GPC3 mRNA increases 21.7-fold comparing to normal liver, and 7 to 10-fold as compared to benign hepatic lesions. Interestingly, GPC3 mRNA expression is higher in invasive than in noninvasive tumors [45]. Zhou et al. found GPC3 mRNA in 76.6% cases of HCC, whereas it was low or absent in normal liver, focal nodular hyperplasia and cirrhosis [44, 45]. In HCC precancers it was present in 13% [44]. GPC3 protein is upregulated in 42% cases of HCC [27]. Llovet et al. found that GPC3 together with survivin and LYVE1 may be useful in differentiating dysplastic nodules from early foci of HCC [24]. On the other hand, Luo et al. showed the gene expression pattern to be similar in carcinoma and surrounding, non-neoplastic cirrhotic liver tissue [25]. Capurro et al. found positive GPC3 in 53% of HCC patient sera. Importantly, this marker was independent of αFP [6]. In Nakatsura series, GPC3 was positive in the serum of 40% HCC patients [29], also in case where all other markers were negative. GPC3 was also proposed as a target for antibody-mediated treatment strategies. GPC3 was increased in both HCC and hepatoblastoma [43], but decreased in cholangiocarcinoma [26]. Sutcliffe proposed using immunohistochemistry for GPC3 for detecting bone marrow micrometastases in patients expecting liver transplantation [38]. The function of GPC3 in HCC remains poorly understood: it stimulates cell proliferation through WNT signaling [7], may be involved in WNT - TGF-β signaling pathway [12] and stimulates proliferation by inhibiting FGF2 and BMP-7 [27].

While analyzing the present findings, it must be remembered that in chromophobe renal cell carcinoma, although now superseded by immunohistochemistry, Hale's colloid iron positivity is the most classical feature. This method detects cytoplasmic polysaccharidic compounds [30]; it is thus in concert with GPC3 positive immunohistochemistry. However, so far no specific data on GPCs expression in RCC were available. An extensive search for the genetic profile of renal cell carcinoma was performed by Higgins et al. [17]. The authors assessed expression of over 22 000 sequences and were able to basically reproduce the morphologic classification of renal tumors. GPC3 was not listed among genes strongly differentiating tumor types in that study; however, the authors made the results available on the Internet. Thus it is possible to check any sequence used in the study; looking at the GPC3 gene, it is evident that its expression is stronger in chromophobe carcinomas that in the conventional group; this confirms our results.

In summary, it was shown that some of RCC, especially chromophobe RCC, may express GPC3. From the practical point of view, as chromophobe renal cell carcinoma is a rather rare malignancy and patients to be screened for HCC are mainly HCV carriers, our findings should not hamper the use of GPC3 for HCC detection. However, some caution may be needed while using GPC3 immuno-

histochemistry for tumors with uncertain primary location, tumors in an otherwise healthy liver or cytologic material of an intraabdominal malignancy. It would also be interesting to check if GPC3 appears in the serum of RCC patients.

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