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ORIGINAL ARTICLE

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Expression of a glypican-related 62-kDa antigen is decreased in hepatocellular carcinoma in correspondence to the grade of tumor differentiation

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Abstract A monoclonal mouse antibody, Be-F4, was generated by means of immunization with a synthetic oligopeptide. In Western blots, this antibody recognizes an antigen with an apparent molecular mass of 62 kDa, termed p62. Immunohistochemical analysis of p62 in hepatocellular carcinoma (HCC) specimens (n=33) and in corresponding non-cancerous liver tissue was performed using monoclonal antibody Be-F4. All non-neoplastic hepatic cells showed, without exception, a moderate or strong staining intensity of the 62-kDa antigen, recognized by Be-F4. In contrast to the non-neoplastic hepatocytes, the cellular p62 content was unambiguously reduced in all malignant cells. The extent of decrease of p62 corresponded to the grade of histological differentiation of HCC cells (P<0.001). Using a semiquantitative scoring system, the median of p62 expression, which was 2.1 for normal hepatocytes, was significantly reduced to 1.2 for G1, 1.0 for G2, and 0.2 for G3 HCCs. These data suggest that neoplastic transformation is associated with a reduced p62 content.

Keywords Hepatocellular carcinoma · Glypicans · Tumor differentiation · Be-F4

Introduction

Proteoglycans are glycoproteins containing various sulfated glycosaminoglycan residues, e.g., heparan sulfate. These glycoproteins, designated as heparan sulfate proteoglycans (HSPGs), are widely distributed in many tissues, occurring in various forms of extracellular matrices, at cell surfaces, and in intracellular granules [1]. HS is a regulatory polysaccharide. It modulates specific growth factor–receptor interactions, accelerates the formation of specific proteinase inhibitor complexes, and mediates interactions of the cell surface with several enzymes and structural proteins. It occurs especially on the surfaces of embryonic cells.

Glypicans, also called glypican-related integral membrane proteoglycans (GRIPS), are one of two major families of transmembrane HSPGs. The glypicans are anchored to membranes by a glycosyl-phosphatidylinositol (GPI) anchor. This covalently attached GPI residue was the source of the term glypican, which is derived from glycosylphosphatidylinositol-anchored proteoglycan. Six members of the human glypican family are known. The core proteins of all glypicans are of similar sizes, in the range of 60 kDa, and contain Ser-Gly repeats in the C-terminal domains that compose sites for substitution with HS. Varying expression and turnover patterns of glypicans prevail in distinct cell types, membrane domains, and endocytotic machineries, and they are subject to strict developmental controls. This may suggest that each of the glypicans functions in a specific context and that these functions pertain to the transduction of signals emanating from the continuous interplay between matrix components, growth factors, and proteinases.

One member of the glypican family is glypican-3 (GPC3), also designated as MXR7 [10] or OCI-5 [4]. Changes in GPC3 cause the Simpson–Golabi–Behmel syndrome (SGBS) [17], also called Simpson Dysmorphia Syndrome (SDYS). SGBS is an X-linked condition characterized by pre- and postnatal overgrowth, resulting in visceral and skeletal anomalies. Patients are also at high risk for the development of embryonic tumors, including Wilms' tumor (WT) and neuroblastoma, during early childhood [9]. Consistent with this is the supposition that GPC3 is involved in morphogenesis and growth control during development.

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It was reported that GPC3-encoding messenger (m)RNA expression was silenced through hypermethylation of the GPC3 promoter region in a subset of ovarian cancer cell lines [14]. Reactivation of GPC3 mRNA expression resulted in inhibition of colony-forming efficiency in these ovarian carcinoma cells, indicating that GPC3 exhibits tumor-suppressing activity. Recently, this conclusion was supported by the observation that the expression of the GPC3 mRNA is markedly decreased in malignant mesothelioma cells [16]. Likewise, colony forming was inhibited in mesothelioma cells by ectopic expression of GPC3. It was also reported that expression of the GPC3-encoding mRNA was detected in 75% of primary and recurrent HCC taken from 154 patients but only in 3% non-tumor livers [8]. These findings suggested that GPC3 has oncogenic potential.

Despite the impact of GPC3 in development and cancer biology, hitherto no antibody directed against human GPC3 is available. In this study, we immunized mice using a synthetic oligopeptide representing a putative hydrophilic domain of the GPC3 core protein. By this approach, we obtained monoclonal antibody (mAb) Be-F4. The antigen recognized by mAb Be-F4 was analyzed. Additionally, the expression of the antigen recognized by Be-F4 was characterized in a subset of human HCCs and corresponding normal hepatocytes using immunohistochemistry and Western blotting with Be-F4.

Materials and methods

Patient tissue and histological grading

Normal liver tissue and tumor samples from 33 patients with histologically proven HCC (G1-G3) were investigated using immunohistochemistry. Patient samples contained cancerous and non-neoplastic-surrounding hepatocytes. The tissue from resection specimens were formalin-fixed, followed by paraffin embedding. Small samples and needle biopsies were not included. For Western-blot analyses material was obtained from sections of frozen tissue blocks used for intra-operative diagnosis. Tissue samples consisting of histologically proven HCC or corresponding non-neoplastic liver tissue from the same patient was stored at –80°C until protein isolation. The histological grading of tumor differentiation was performed independently by two pathologists according to Edmondson and Steiner [3]. Only grade-4 carcinomas were excluded, due to the difficulty in distinguishing those from other undifferentiated tumors or metastases of different origin.

Production of monoclonal mouse anti-peptide immunoglobulin G

A synthetic oligopeptide "c"-gnsqqatpkdneistfhnlg (amino acids residues 537–556), representing a putative hydrophilic domain of the GPC3 core polypeptide derived from the GPC3-encoding complimentary (c)DNA sequence [10] was designed. BALB/c mice were immunized with four i.p. injections with 0.1 mg oligopeptide, coupled to keyhole limpet hemocyanin (KLH) in complete Freud's adjuvant at 1-month intervals. A tail vein was injected i.v. with 0.1 mg peptide-KLH conjugate 7 days before fusion. BALB/c splenocytes were fused with NS0 mouse myeloma cells with polyethylene glycol 4000 using standard procedures [7]. The fused cells were plated into 96-well plates with peritoneal exudate cells from BALB/c mice as feeder layers. The cultures were fed with hypoxanthine, aminopterin, and thymidine-containing RPMI 1640 medium to select for hybrids. Hybridomas were screened using an enzyme-linked immunosorbent assay (ELISA). Plates (96 wells) were stacked with peptide-ovalbumin conjugate, blocked for 2 h with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and incubated with hybridoma culture supernatant. Binding of the first antibody was detected using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig)G with *O*-phenylenediamine and hydrogen peroxide as substrates. Color development was measured at 490 nm. Hybridoma-selected (Be-F4) was cloned fivefold. Cells (5×10^6) were suspended in PBS and injected i.p. into pristane-treated BALB/c mice. Approximately 2 weeks later, mice were killed, and the ascites was collected. The ascites preparation was cleared by means of centrifugation, and the antibodies were separated using protein-A column chromatography. Total IgG fraction was dissolved in tris-buffered saline (TBS) and 0.02% NaN_3 at a concentration of 5 mg/ml.

Determination of the molecular mass of mAb Be-F4 antigen

Western-blotting analysis was performed with Be-F4 for assessment of antigen molecular weight. In these experiments, total cell extracts obtained from the human gastric carcinoma cell line EPG85-257P and its atypical multidrug-resistant variant EPG85-257RNOV [2] were used. Both cell lines were established in our laboratory and cultivated as described previously [2]. The cell line EPG85-257RNOV was chosen because this line markedly overexpressed the GPC3-encoding mRNA when compared with the parental carcinoma cell line EPG85-257P [10]. Total cellular protein extracts were prepared using standard procedures, as described previously [11]. Cells were lysed by treating with detergent-containing RIPA buffer [150 mM NaCl, 50 mM tris-Cl, 5 mM ethyleneglycoltetraacetic acid (EGTA), 1% triton X-100, and 0.1% sodium dodecyl sulfate (SDS), pH 7.4, supplemented with the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µM pepstatin, 0.1% trasylol]. Samples of total cellular protein were loaded onto a SDS-polyacrylamide (PA) gel and run for 2 h at 30 mA. Separated proteins were transferred to a 0.2-µm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany) in a Bio-Rad trans blot cell, using a buffer consisting of 25 mM Tris-Cl, 86 mM glycine, and 20% methanol. To avoid unspecific binding, the filters were incubated in 5% non-fat dry milk, 0.1% tween-20 in PBS for 3 h. Thereafter, filters were incubated with mouse mAb Be-F4 diluted in the same solution (1:1000) for 2 h and, afterwards, with sheep anti-mouse IgG horseradish peroxidase (1:5000; Amersham). The protein–antibody complexes were visualized using chemoluminescence (ECL system, Amersham), according to the manufacturer's protocol.

Immunohistochemistry and statistical analysis

Deparaffinized 3- to 5-µm sections were rehydrated, blocked with 20% normal goat serum and 1% BSA in TBS for 20 min, and incubated with 12.5 ng/µl Be-F4 diluted in TBS with 1% BSA for 1 h. The secondary biotinylated goat anti-mouse antibody and the horseradish peroxidase-conjugated streptavidin were applied according to the manufacturer's instructions (Super Sensitive System, BioGenex, San Ramon, Calif.). A chromogenic precipitate was obtained through incubation with AEC (3-amino-9-ethylcarbazole) substrate chromogen according to the manufacturer's instructions (Dako, Carpinteria, Calif.). After counterstaining with Mayer's hematoxylin, the sections were coverslipped with glycerol gelatin as mounting medium. Negative control slides were run in parallel, with an equivalent amount of mouse IgG fraction substituted for the primary antibody. The Be-F4 antigen expression level of each tumor cell and non-cancerous hepatic cell was counted by chance from 10 HPFs, i.e., magnification 400×. The proportion of negative, weakly, moderately, and strongly posi-

tive cells was rated. The score was calculated according to the formula: (% of negative cells ×0) + (% of weak positive cells ×1) + (% of moderate positive cells ×2) + (% of strong positive cells ×3). The results yield a range from negative ~0 to strongly positive ~3, a scale comparable with the histological grading of tumor differentiation. For the statistical analysis, the Kruskal–Wallis test was used. The procedures used included χ^2, degree of freedom, and significance (*P*-value).

SDS-PA gel electrophoresis and Western-blot analysis of HCC specimens

Total cellular protein extracts from HCC specimens were prepared as described for cell lines in the section "Determination of the molecular mass of Be-F4 antigen". The same is true for SDS-PA gel electrophoresis (GE) and Western blotting procedure.

Results

Generation of a mAb and determination of the molecular mass of the antigen

Using a synthetic oligopeptide representing a C-terminal domain of GPC3 in ELISA screening, five stable hybri-

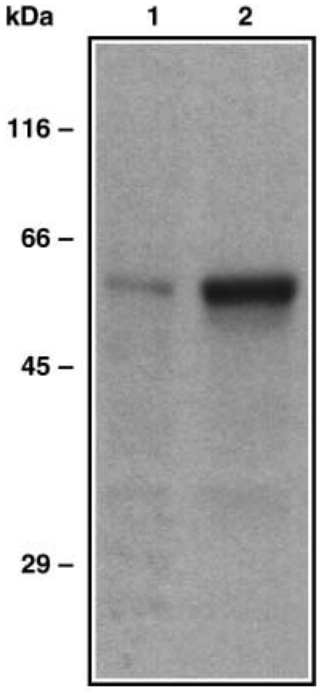


Fig. 1 Western-blot analysis of the drug-sensitive human gastric carcinoma cell line EPG85-257P (*lane 1*) and the atypical multidrug-resistant cell variant EPG85-257RNOV (*lane 2*). In both lines, 25 µg total cellular protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) and blotted to nitrocellulose. Incubation with monoclonal antibody (mAb) Be-F4 revealed a specific signal in the range of approximately 62 kDa. The non-resistant gastric carcinoma cell line EPG85-257P showed a moderate cellular content of p62, while the atypical multidrug-resistant variant EPG85-257RNOV showed a much more pronounced protein expression level

Table 1 p62 Expression in hepatocellular carcinoma (HCC) specimens. The score was calculated according to the formula (% of negative cells ×0) + (% of weak positive cells ×1) + (% of moderate positive cells ×2) + (% of strong positive cells ×3). The results yield a range from negative ~0 to strongly positive ~3

No.	Normal hepatic cells						HCC cells				
	No	Weak	Inter-mediate	Strong	Score	Histological grade	No	Weak	Inter-mediate	Strong	Score
1	–	–	–	–	–	1	0.2	0.2	0.4	0.2	1.6
2	0.0	0.0	0.8	0.2	2.2	1	0.3	0.5	0.2	0.0	0.9
3	0.0	0.0	0.6	0.4	2.4	1	0.0	0.0	0.9	0.1	2.1
4	0.0	0.0	1.0	0.0	2.0	1	0.3	0.2	0.5	0.0	1.2
5	0.0	0.0	1.0	0.0	2.0	1	0.5	0.3	0.2	0.0	0.7
6	0.0	0.0	1.0	0.0	2.0	1	0.3	0.4	0.3	0.0	1.0
7	–	–	–	–	–	1	0.0	0.2	0.8	0.0	1.8
8	0.0	0.5	0.5	0.0	1.5	2	0.4	0.6	0.0	0.0	0.6
9	0.0	0.0	0.8	0.2	2.2	2	0.5	0.5	0.0	0.0	0.5
10	0.0	0.2	0.8	0.1	1.9	2	0.0	0.2	0.8	0.0	1.8
11	0.0	0.0	1.0	0.0	2.0	2	1.0	0.0	0.0	0.0	0.0
12	0.0	0.0	0.9	0.1	2.1	2	0.7	0.3	0.0	0.0	0.3
13	0.0	0.0	1.0	0.0	2.0	2	0.0	0.0	1.0	0.0	2.0
14	0.0	0.0	1.0	0.0	2.0	2	0.2	0.3	0.4	0.1	1.4
15	0.0	0.0	0.7	0.3	2.3	2	0.2	0.3	0.4	0.1	1.4
16	0.0	0.0	0.5	0.5	2.5	2	1.0	0.0	0.0	0.0	0.0
17	0.0	0.0	0.3	0.7	2.7	2	0.0	0.0	0.5	0.5	2.5
18	0.0	0.0	1.0	0.0	2.0	2	0.0	0.5	0.5	0.0	1.5
19	0.0	0.0	0.5	0.5	2.5	2	0.3	0.1	0.3	0.3	1.6
20	–	–	–	–	–	2	0.4	0.4	0.2	0.0	0.8
21	0.0	0.0	0.2	0.8	2.8	2	0.3	0.5	0.2	0.0	0.9
22	0.0	0.0	0.8	0.2	2.2	2	0.4	0.3	0.2	0.1	1.0
23	0.0	0.0	1.0	0.0	2.0	2	0.5	0.5	0.0	0.0	0.5
24	0.0	0.0	0.8	0.2	2.2	2	0.5	0.5	0.0	0.0	0.5
25	0.0	0.0	0.6	0.4	2.4	2	0.2	0.6	0.2	0.0	1.0
26	0.0	0.0	1.0	0.1	2.1	3	1.0	0.1	0.0	0.0	0.1
27	0.0	0.0	1.0	0.0	2.0	3	0.9	0.0	0.1	0.1	0.3
28	0.0	0.0	0.5	0.5	2.5	3	0.6	0.3	0.2	0.0	0.6
29	0.0	0.2	0.4	0.4	2.2	3	0.4	0.2	0.2	0.2	1.2
30	0.0	0.0	1.0	0.0	2.0	3	1.0	0.0	0.0	0.0	0.0
31	0.0	0.7	0.3	0.0	1.3	3	1.0	0.0	0.0	0.0	0.0
32	0.0	0.0	1.0	0.0	2.0	3	0.3	0.4	0.3	0.0	1.0
33	–	–	–	–	–	3	1.0	0.0	0.0	0.0	0.0

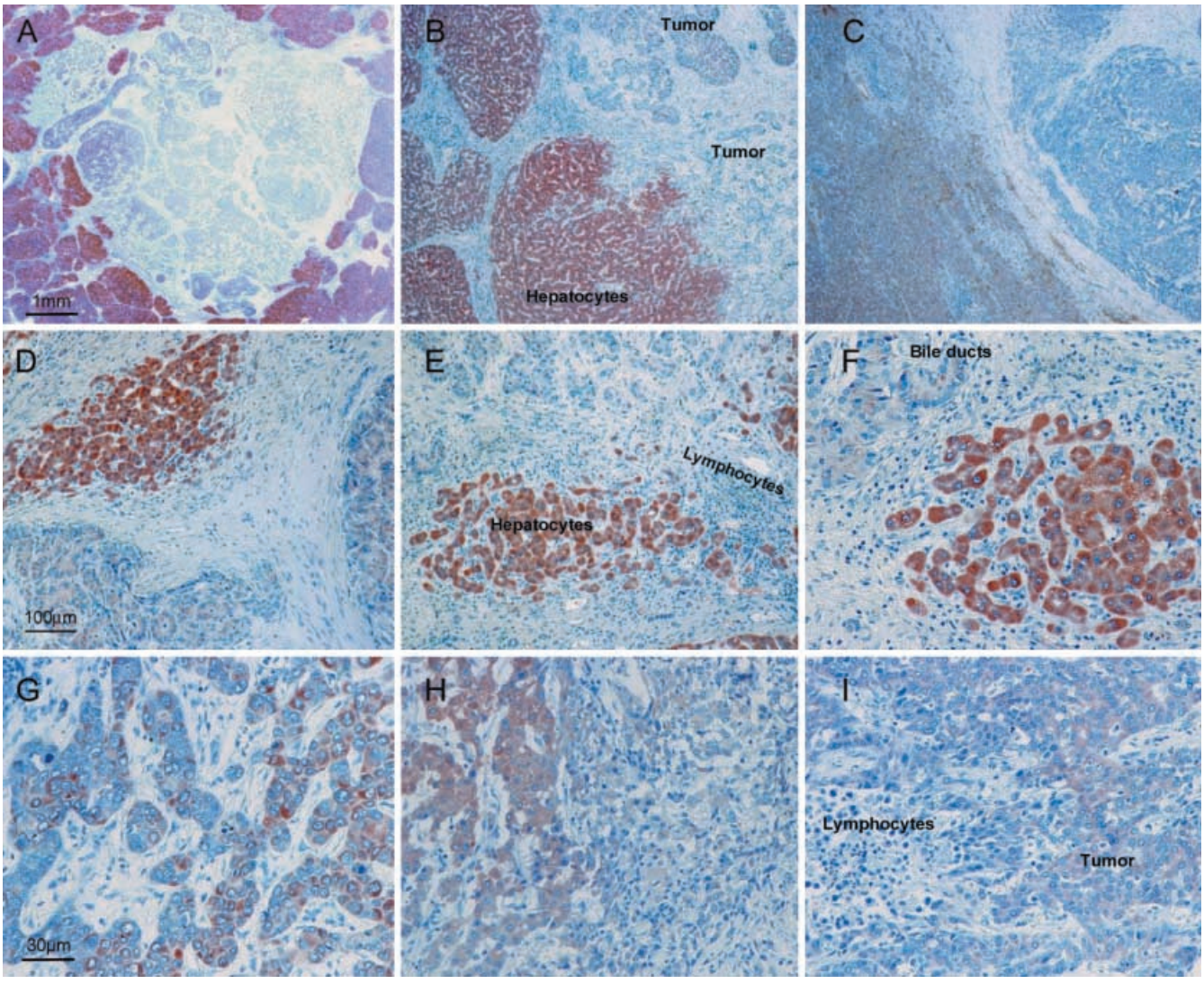
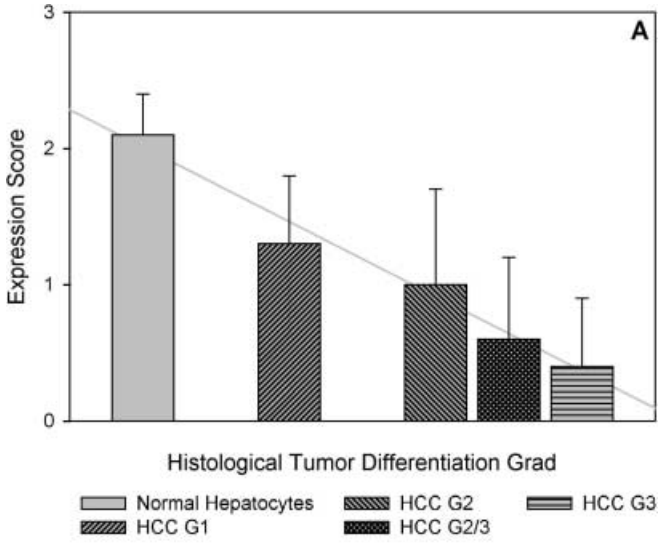


Fig. 2 Immunohistochemical staining of a representative hepatocellular carcinoma (HCC) specimen using monoclonal antibody (mAb) Be-F4. **A** Overview of a G3 HCC (unstained tumor nodule in the center), surrounded by stained normal hepatocytes. **B** Diffuse infiltrative growth of poorly differentiated and weakly stained HCC cells (*right side*) in contrast to well-stained non-cancerous hepatic cells (*left side*). **C** Figure **A** in more detail. **D**, **E**, **F** High magnification of non-cancerous hepatic cells that show distinct staining of intracellular cytoplasmic granules. Proliferating bile ducts are weakly positive or negative, whereas stromal cells and lymphocytes are completely negative. **G**, **H**, **I** Malignant HCC cells show a decreased staining intensity (**G** and **H**, both G2) in a diffuse (**H**) or patchy pattern (**G**) or show no staining signal (**I**, G3). The decreasing staining intensity of tumor cells corresponds to an increasing level of dedifferentiation. In each case, interlobular septum, lymphatic and blood vessels and Kupffer cells were completely free of any staining signal

doma clones were isolated secreting mAbs specific for the peptide. Screening with whole cellular denatured protein extracts immobilized on nitrocellulose ensured that the mAbs selected would be useful in the detection of antigens in Western blots. One stable hybridoma clone was ultimately isolated, yielding a mAb (Be-F4), which appeared to be specific for the synthetic oligopeptide. The application of mAb Be-F4 in Western-blot analysis of the drug-sensitive human gastric carcinoma cell line, EPG85-257P, and an atypical multidrug-resistant cell variant, EPG85-257RNOV, derived from that cell line, revealed a specific signal in the range of approximately 62 kDa (Fig. 1). According to the accessed molecular mass of 62 kDa, the antigen recognized by mAb Be-F4 was designated p62. Figure 1 demonstrates that the p62 expression level is more pronounced in the atypical multidrug-resistant gastric carcinoma cell line EPG85-257RNOV than in the non-resistant parental line EPG85-257P. This observation is in accordance with the results of Northern blot analyses, detecting GPC3 mRNA expression level in those cell lines. It was demonstrated previously, that the non-resistant gastric carcinoma cell line, EPG85-257P, showed a moderate GPC3 mRNA expression level, while the atypical multidrug-resistant variant, EPG85-257RNOV, showed a much more increased GPC3 mRNA expression [10].



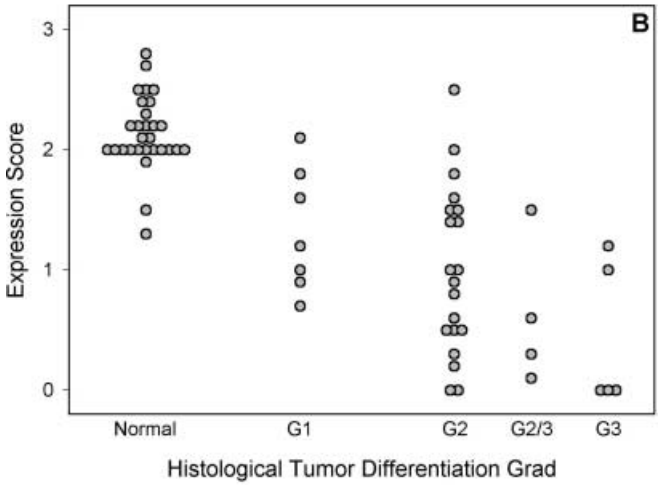


Fig. 3 Be-F4 antigen expression levels in non-cancerous hepatic cells and various hepatocellular carcinomas of different tumor differentiation grades (**A**, **B**). The expression score was determined as described in Materials and methods. **A** The median (*line*) and standard deviation (*error bars*). **B** The score of grouped single tumors

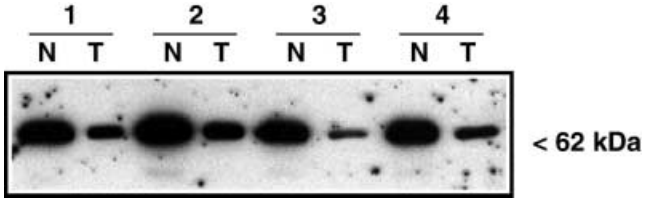


Fig. 4 Western-blot analysis of four specimens of hepatocellular carcinoma (HCC) and corresponding non-neoplastic liver tissue from the same patient. In each track, 50 µg total cellular protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%), blotted to nitrocellulose, and incubated with monoclonal antibody (mAb) Be-F4. In all cases, the expression level of Be-F4 antigen was much lower in HCC than in tumor surrounding non-cancerous tissue. Histopathological data of the tumors examined: case 1, well-differentiated HCC (*G1*), stage IIIA (*T*), and non-neoplastic liver cirrhosis (*N*); case 2, well-differentiated HCC (*G1*), stage I (*T*), and non-cancerous liver cirrhosis (*N*); case 3, moderately differentiated HCC (*G2*), stage IIIA (*T*), and non-tumorous liver without cirrhosis (*N*); and case 4, moderately differentiated HCC (*G2*), stage IIIA (*T*), and non-neoplastic liver cirrhosis (*N*)

Immunohistochemistry of HCC specimens

The antigen detected by mAb Be-F4 shows a homogeneous intracellular distribution of cytoplasmic granules in non-cancerous hepatic cells and in hepatocellular cancer cells if HCC cells are stained. In each case, cells of interlobular septum, lymphatic and blood vessels, and Kupffer cells were completely unstained (Fig. 2). Furthermore, in most cases, bile ducts were free of any specific staining. However, few cases showed a weak staining signal in bile ducts. Without exception, all non-cancerous hepatic cells showed a moderate or strong staining intensity with mAb Be-F4 (Fig. 2). The median score for p62 expression in these non-cancerous liver cells was 2.0; the mean value was 2.1. HCC cells constantly showed a decreased staining intensity when compared with the staining signal obtained in non-cancerous liver cells from the same section (Fig. 2). Be-F4-specific staining signal was completely absent in 28% of poorly differentiated G3 tumors and 5.6% of moderately differentiated HCC cases (Table 1). As shown in Fig. 3 and Table 1, the decrease in p62 expression corresponded to an increasing level of dedifferentiation of the hepatocellular carcinoma cells. This is in accordance with the observation that no specimen of well-differentiated G1 tumors showed completely reduced Be-F4 antigen expression. Well-differentiated G1 HCC specimens showed a mean p62 expression value of 1.3 (±0.5; median: 1.3), G2 HCC carcinoma cells showed a mean p62 expression value of 1.0 (±0.7; median: 1.0), and poorly differentiated G3 tumors reduced Be-F4 antigen expression with a mean value of 0.4 (±0.4; median 0.2). The correlation between grade of differentiation and p62 expression level was statistically significant ($P<0.001$, $\chi^2=38.4$, and degree of freedom 3).

Western-blot analysis of HCC specimens

As shown in Fig. 4, Western-blot analysis using mAb Be-F4 revealed, in a subset of specimens of non-cancerous liver tissues, a strong signal in the range of 62 kDa. In all cases, corresponding HCC from the same patient showed an unambiguous reduction of total cellular p62 content when compared with the surrounding non-neoplastic hepatic tissue.

Discussion

A formalin- and paraffin-compatible mAb, Be-F4, was generated. Western-blot analysis revealed that the specif-

ic protein band recognized by mAb Be-F4 corresponded to a molecular mass of approximately 62 kDa. Hence, the antigen was designated p62. The assessed value of 62 kDa for the antigen recognized by Be-F4 is in accordance with the data reported for the rat variant of GPC3, which was designated OCI-5 [4]. The application of mAb 12CA5-I directed against a nine amino acid epitope of hemagglutinin A (HA) of the influenza virus revealed in Western-blot experiments the detection of three specific HA epitope-tagged OCI-5 fusion proteins of 41, 65, and 69 kDa [5]. The 69-kDa band might correspond to the non-glycanated HA-tagged protein core of the gypican, whereas the 41-kDa band is the result of proteolytic cleavage. Another study [12] using polyclonal antibodies directed against rat OCI-5 demonstrated that three specific OCI-5 bands of 45, 65, and 70 kDa were detectable in rat cancer cell lines. The 62-kDa Be-F4-specific protein signal of the human glypican-related antigen could correspond to a non-glycanated GPC3 protein core after cleavage of a putative 25 amino acid signal peptide, since the open reading frame of the GPC3 cDNA encodes a protein of similar size [10]. As it could be demonstrated for the GPC3-encoding mRNA [10], the Be-F4 antigen was expressed on a much higher level in the atypical multidrug-resistant human gastric carcinoma cell line, EPG85-257RNOV, than the corresponding non-resistant, parental variant EPG85-257P. However, the congruity of the human Be-F4 antigen with the human GPC3 core protein has not yet been proven, since in Western-blot experiments cell lines transfected with the GPC3-encoding cDNA did not show any unambiguously Be-F4-specific signal (data not shown). If the antigen recognized by mAb Be-F4 is not identical with the GPC3 polypeptide, it might be a glypican-related protein, since Be-F4 detects a peptide domain derived from a member of the glypican family. Further experimental studies, which are beyond the scope of this paper, are required to clarify the identity of p62.

Since hitherto, despite many efforts, no mAb specifically recognizing the GPC3 polypeptide could be generated, it appears obvious that GPC3 is only weakly or not immunogenic in mice or that this protein is not accessible by using standard procedures of protein detection.

Using mAb Be-F4 in immunohistochemical staining of paraffin sections containing cancerous and non-neoplastic hepatocytes, it was observed that non-cancerous hepatic cells showed a distinct staining of intracellular cytoplasmic granules, which probably belong to the huge intracytoplasmic membrane system of hepatocytes, since glypicans and related polypeptides are membrane-attached. It could be demonstrated that p62 is expressed at high level in all normal hepatocytes, while, in HCC cells, the Be-F4 antigen content is downregulated in correspondence with the grade of dedifferentiation. Poorly differentiated grade-G3 tumor cells showed, statistically significant (P>0.001), the lowest Be-F4 antigen content. In other words, tumor dedifferentiation in HCC is associated with a decreased expression of the Be-F4 antigen. In accordance with these immunohistochemical data, a decreased cellular p62 content was observed in HCC when compared with corresponding non-neoplastic hepatic tissue from the same patient using Western-blot analysis.

If the 62-kDa antigen detected by mAb Be-F4 is indeed identical with the GPC3 core protein, the observation that poorly differentiated high-grade tumors showed the lowest level of p62 expression is in line with a phenomenon seen in IEC-18 cells, which were derived from normal rat small intestine. These cells reduced the GPC3 mRNA expression level when transformed by activated H-ras or v-src oncogenes [4]. Most interestingly, the degree of downregulation correlated with the extent of morphological changes produced by cell transformation by these oncogenes. In other words, depending on the grade of oncogene-mediated dedifferentiation of IEC-18 cells, the glypican mRNA expression level was reduced. Further in vitro studies using epithelial IEC-18 cells demonstrated that the GPC3 transcription level is regulated by changes in cell shape [13]. GPC3 mRNA synthesis is increased when cells attain a more rounded morphology, such as occurring in non-cancerous hepatic cells. In contrast, cells exhibiting an acquired more spread out, flattened, elongated or spindle shape morphology show a decreased glypican mRNA expression level. Therefore, it appears conclusive that a down regulation of GPC3 or related glypicans occurring during dedifferentiation of HCC cells could be associated with a modulated gene regulation mediated by changes in cell morphology.

The gene encoding GPC3, which is believed to be involved in morphogenesis and growth control [5], has been described as mutated in patients with SGBS [17], a disorder characterized by morphological changes, such as pre- and postnatal overgrowth and various visceral and skeletal dysmorphisms. Some of these dysmorphisms could be the result of a deficiency in growth inhibition or programmed cell death in certain cell types during development. However, it has been demonstrated that GPC3 is able to induce programmed cell death [6]. In simpler terms, a reduction of the glypican content in tumor cells mediates a growth advantage by a decrease of the cellular disposition to trigger a signal pathway leading to apoptosis. This argument is in line with the observation that the more malignant poorly differentiated G3 tumors showed a higher degree of reduction of p62 expression than the tumors of less malignant entities. More indications that GPC3 may function as a tumor suppressor have been reported from alternative cancerous cell systems. Similar to the observation that p62 expression is inactivated in HCC, expression of GPC3 was frequently (31%) lost in a subset of ovarian cancer cell lines [12]. Furthermore, it was reported from 16 of 18 primary malignant mesotheliomas and 17 of 22 human mesothelioma cell lines that the GPC3-specific transcript levels were markedly decreased [16]. Both studies support the hypothesis that glypicans could act as tumor-suppressing factors.

If p62 is identical with GPC3, the data presented in our study would be contrary to the observations by Hsu

et al. [8], who described the expression of GPC3-encoding mRNA in 143 (75%) of 191 primary HCC using Northern blotting analyses. However, these discrepancies might be explained by the possibility that mAb Be-F4 did not recognize GPC3 but recognized a glypican-related polypeptide or by the use of different methods for the detection of glypican expression. Northern blotting ana-lysis detects the GPC3-encoding mRNA in a tissue extract; the immunohistochemistry approach and the Western blotting procedure detect the protein directly.

Similar findings as those obtained in this paper were reported from an immunohistochemical study in which the expression of another glypican-related protein (other than p62), i.e., syndecan-1, a member of the second HSPG family, the syndecans, was investigated in HCC [15]. As it was demonstrated for p62, the expression of syndecan-1 was reduced in correspondence to the grade of tumor differentiation. In poorly differentiated HCC, the tumor cells showed loss of syndecan-1 protein expression, whereas well-differentiated HCC cells and normal hepatocytes exhibited a positive staining reaction. Although the syndecan-1 core protein has a molecular weight of approximately 65 kDa, it is unlikely that p62 is identical with syndecan-1, because the oligopeptide used for immunization of mice did not show any similar sequences to that of syndecan-1. However, it appears that loss of the expression of at least two different proteins that are related to the glypicans is closely related to the malignant character of HCC cells.

It has been demonstrated that p62 expression level is decreased in HCC cells in relation to the differentiation grade when compared with non-cancerous hepatic cells. Whether this phenomenon is involved in carcinogenesis of HCC or is a result from secondary events is not yet clear. However, the data suggest that neoplastic transformation of HCC is associated with a reduced cellular Be-F4 antigen content, since loss of Be-F4 antigen might result in neoplastic transformation. The correlation between a decreased p62 protein expression level and the histological grade of tumor dedifferentiation indicates that there may be a potential for the use of mAb Be-F4 in the morphological diagnostics of HCC. It may at least help to discriminate highly and moderately differentiated HCC. Further studies correlating Be-F4 antigen expression with the clinical follow-up of patients suffering from HCC are required to determine whether mAb Be-F4 is confirmed to be useful as an important prognostic indicator for HCC.

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