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DMBT1 Encodes a Protein Involved in the Immune Defense and in Epithelial Differentiation and Is Highly Unstable in Cancer[1]

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ABSTRACT

The gene *deleted in malignant brain tumors 1* (*DMBT1*) has been proposed as a candidate tumor suppressor for brain, gastrointestinal, and lung cancer. It codes for a protein of unknown function belonging to the superfamily of scavenger receptor cysteine-rich proteins. We aimed at getting insights into the functions of DMBT1 by expression analyses and studies with a monoclonal antibody against the protein. The *DMBT1* mRNA is expressed throughout the immune system, and Western blot studies demonstrated that isoforms of DMBT1 are identical to the collectin-binding protein gp-340, a glycoprotein that is involved in the respiratory immune defense. Immunohistochemical analyses revealed that DMBT1 is produced by both tumor-associated macrophages and tumor cells and that it is deregulated in glioblastoma multiforme in comparison to normal brain tissue. Our data further suggest that the proteins CRP-ductin and hensin, both of which have been implicated in epithelial differentiation, are the DMBT1 orthologs in mice and rabbits, respectively. These findings and the spatial and temporal distribution of DMBT1 in fetal and adult epithelia suggest that DMBT1 further plays a role in epithelial development. Rearrangements of *DMBT1* were found in 16 of 18 tumor cell lines, and hemizygous deletions were observed in a subset of normal individuals, indicating that the alterations in tumors may be a result of both pre-existing deletions uncovered by a loss of heterozygosity and secondary changes acquired during tumorigenesis. Thus, *DMBT1* is a gene that is highly unstable in cancer and encodes for a protein with at least two different functions, one in the immune defense and a second one in epithelial differentiation.

INTRODUCTION

The gene *DMBT1*[3] at chromosome 10q25.3-q26.1 is a member of the SRCR superfamily and has been proposed as a candidate tumor suppressor gene for medulloblastoma, GBM, lung, and gastrointestinal tumors based on homozygous deletions and on lack of expression (1–4).

The majority of the SRCR proteins has been implicated in functions within the immune system. The Mac2-bp, for example, interacts with the lectin Mac-2 (galectin-3) and plays a role in the mucosal immune defense and in the activation of natural killer cells as well as in tumor suppression (5–8). The Mac2-bp further has been demonstrated to mediate cell-cell and cell-extracellular matrix adhesion (9, 10). Like the Mac2-bp, the SRCR protein gp-340, a binding protein for the lung collectins SP-D and SP-A, has been proposed to play a role in the mucosal immune defense (11, 12).

Ebnerin, CRP-ductin, and hensin are SRCR proteins in rat, mouse, and rabbit, respectively, that share homology with DMBT1, but have different expression patterns (13–15). Whereas Ebnerin has been suggested to play a role in taste perception (13), it has been reasoned that CRP-ductin might be involved in epithelial differentiation because of its differential expression in the intestinal epithelium (14). Biochemical studies have established that hensin is able to switch the polarity and to induce processes of terminal differentiation in epithelial cells of the kidney collecting ducts (15, 16).

SRCR, ZP, and CUB domains are thought to mediate protein-protein interactions, and CUB domains have been recognized as a motif common to proteins involved in processes of embryogenesis and organogenesis (17–19). *DMBT1* codes for an extracellular protein with up to 14 SRCR domains, 2 CUB domains, and a ZP domain (1, 20). Alternative splicing takes place within the exons encoding the first 13 SRCR domains and the SIDs. These exons are part of locus-specific sequence repeats that potentially are prone to genomic instability (20). The function of DMBT1, however, and the nature of the molecular events that might lead to an inactivation during tumorigenesis have remained unknown. In this study, we aimed at getting insight into the functions of DMBT1 as well as into its expression and localization in brain tumors. We further wanted to test the hypothesis that the repetitive structure of the gene gives rise to an increased susceptibility to genomic instability.

MATERIALS AND METHODS

Cell Lines, Tissue Sections, and Normal DNA Samples. Tumor cell lines were obtained from the Tumorbank of the Deutsches Krebsforschungszentrum (Heidelberg, Germany). Sections for immunohistochemistry were obtained from tissues that had been removed for therapeutic or diagnostic purposes. No concerns were raised by the responsible ethics committees to use residual tissues for scientific purposes. Normal DNA samples were obtained from peripheral blood leukocytes of healthy volunteers without known previous history of cancer.

LOH and Deletion Analyses. High molecular weight genomic DNA was prepared from the tumor cell lines according to standard procedures. For the LOH analyses, the homozygosity/heterozygosity of the *DMBT1* flanking markers *D10S209* and *D10S587* and of a bp polymorphism (CCA/CCG) in codon 1707 of *DMBT1* (referred to accession no. AJ000342) was analyzed. We determined the heterozygosity of the bp polymorphism to be 46% in the normal population (32 of 70 individuals). The combined heterozygosity of the three markers allowed to predict LOH in the tumor cell lines with an error rate of 1/33. For analyzing the bp polymorphism, PCR reactions were carried out with 50 ng of genomic DNA as the template, 1× Taq polymerase buffer and 2 units of Taq polymerase (both Perkin-Elmer), 1.5 mM $MgCl_2$, 200 μM each dNTP, and 20 pmol each primer

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[3] The abbreviations used are: DMBT1, deleted in malignant brain tumors 1; SRCR, scavenger receptor cysteine-rich; Mac2-bp, Mac-2-binding protein; CUB, C1r/C1s Uegf Bmp-1; ZP, zona pellucida; SID, SRCR interspersed domain; GBM, glioblastoma multiforme; SP-A, surfactant protein A; SP-D, surfactant protein D; BAL, bronchoalveolar lavage; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; KLH, keyhole limpet hemocyanin.

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(dmbt1ex54dsf2: 5′-CTTGGTGAGAGCTAAGGGG-3′ and dmbt1ex54dsr3: 5′-CTCTGCACACCACCATTTTAC-3′) in a final volume of 50 μl. After an initial denaturation for 3 min at 94°C, 40 cycles consisting of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C a final extension for 10 min at 72°C followed. One μl of the PCR was added to 4 μl of dye marker (1× Tris-borate EDTA, 10% w/v Ficoll, and 0.01% w/v bromphenolblue) and 3 μl of H_2O, denatured for 3 min at 95°C, and stored on ice. The samples were loaded on a 12% polyacrylamide gel (11 cm × 13 cm; 29:1 acrylamide:bisacrylamide; 1× Tris-borate EDTA; prerun for 5 min at 100 V before loading), and electrophoresis was carried out for 24 h at 80 V at room temperature. Subsequently, the gels were silver-stained to determine the heterozygosity/homozygosity of the polymorphism. Tumor cell lines apparently homozygous for all three markers were classified to have an LOH at the *DMBT1* locus. Southern blot analyses of the *DMBT1* gene were carried out with the probes *DMBT1*/sr1sid2 and *DMBT1*/59m21 as described elsewhere (20).

Expression Analyses by RT-PCR. Poly(A)+ RNAs from the different tissues of the immune system were purchased from Clontech. Total RNA from the cell lines U937, Raji, Jurkat, and HL-60 and from alveolar macrophages was prepared according to standard procedures. Alveolar macrophages were obtained by cytospin from human BAL. The reverse transcription was carried out with 1 μg of total RNA or 20 ng of mRNA, respectively, as described earlier (1). The subsequent PCR amplification was carried out in two steps. The first PCR contained 5 μl of the 1:5 diluted single-stranded cDNA from the reverse transcription, 1× PCR buffer (Perkin-Elmer), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.4 μl of a Taq:Pfu polymerase mix (9:1; Taq from Perkin-Elmer, 5 units/μl; Pfu from Stratagene, 2.5 units/μl), and 20 pmol each primer (dmbt1ex50scf3: 5′-TGGGACATTGAGGTGCAAAAC-3′ and dmbt1rtsr5: 5′-AGCTGACGTGAATACGGAGG-3′) in a final volume of 50 μl. Fifteen cycles with 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C were carried out followed by a final extension for 10 min at 72°C. Five μl of the first PCR were used as the template in a second PCR that was set up as described above except that 20 pmol each of the *DMBT1*-specific primers dmbt1ex51scf2 (5′-GTTC-CCCTCTCATTGCTCG-3′) and dmbt1rtsr5 (PCR product: 488 bp) were used in addition to 5 pmol each of the primers TFR5 (5′-GTCAATGTCCCA-AACGTCACCAGA-3′) and TFR3 (5′-ATTTCGGGAATGCTGAGAAAA-CAGACAGA-3′), which were specific for the transferrin receptor. Thirty cycles with 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C were carried out in this second PCR, followed by a final extension for 10 min at 72°C. Ten μl of the PCR products were subsequently separated on 3% Nusieve agarose gels.

Generation of a DMBT1-specific Monoclonal Antibody. A synthetic peptide, DMBT1/2640, corresponding to amino acids 26–40 (RTTD-YASLIPSEVPLC) of DMBT1 was coupled to activated KLH (Pierce). gp-340 was purified from BAL as described earlier (11). Four BALB/c mice were immunized by four i.p. injections at 3-week intervals (Eurogentec). As antigens, 50 μg of DMBT1/2640-KLH and 50 μg of purified gp-340, respectively, were administered with Freud's complete or incomplete adjuvant. Two mice were immunized with the KLH peptide only, whereas the other two mice obtained one injection with purified gp-340 and subsequently, three injections with the KLH peptide. One of the mice immunized according to the latter protocol produced the highest antibody titer against DMBT1, as determined by direct ELISA on DMBT1/2640-coated microtiter plates and was selected for the fusion of spleen cells. The fusion to the Sp2/*O*-Ag-14 cell line and the selection of hybridomas were carried out according to standard procedures at Eurogentec (Seraing, Belgium). IgG-producing hybridomas were screened against the target peptide antigen by direct ELISA. Hybridomas producing antibodies against the peptide epitope were cloned by limit dilution and tested for binding to a recombinantly expressed polypeptide containing the epitope by Western blotting (see below), yielding the monoclonal antibody anti-DMBT1h12. The subclass of the antibody was determined to be IgG1.

Expression of Recombinant DMBT1 Polypeptides. A 3.5-kb cDNA clone (*DMBT1*/c3.5) harboring the 5′ part of the 6-kb transcript was taken as the template for PCR. The primers dmbt1F3–3 (5′-CAAGTTGAATTCACAG-GTGGGTGGATCCCAAGGACT-3′) and dmbt1F3–4 (5′-TCGGATTCTA-GACCTGCTGTTGATGCATGTGAGGT-3′) were used for the amplification of the sequence that codes for a polypeptide comprising the leader peptide up to domain SID2 and containing the peptide sequence of DMBT1/2640. Primers dmbt1F3–5 (5′-GCATCGGAATTCGGATCCGAATCCAGTTTGG-CC-3′) and dmbt1F3–6 (5′-TCGGATTCTAGACCTACTGTCAATGCCGGTAAGT-3′) were used for the amplification of the part encoding SRCR6 to SID7 of the 6-kb transcript (accession no. AJ000342). PCRs were carried out with 25 ng of template, 1× Vent buffer, and 1 unit of Vent polymerase (both from NEB), 0.5 μl BSA (10 mg/ml), 200 μM each dNTP, and 20 pmol of each of the respective primers. After 21 cycles (1 min 95°C, 1 min 70°C, 1 min 70°C), the PCR product was digested with *Xba*I and *Eco*RI and purified by preparative agarose gel electrophoresis. The PCR products were then cloned into *Xba*I/*Eco*RI-digested pPICZalphaA vector (Invitrogen) that provides a combined myc and His(6) tag COOH-terminal to the DMBT1 polypeptides. The plasmids were transfected into *Escherichia coli* strain XL-1 Blue. The orientation and the integrity of the inserts were verified by sequencing. *Pichia pastoris* strain GS115 was transfected with the plasmids according to the suppliers instructions (*Pichia* Expression Kit, Invitrogen). For each transformation, 50 colonies were transferred to nitrocellulose membranes and analyzed for the expression of the myc epitope with a horse-radish peroxidase-conjugated anti-myc antibody (Invitrogen). One positive clone each, ppSRCR1/2 and ppSRCR6/7, respectively, was selected for further analysis. The expression and purification of the recombinant proteins were carried out as recommended by the supplier (Invitrogen). The dialyzed supernatants were applied on a column containing 1 ml of nickel chelate (Pharmacia) in buffer A [25 mM Tris (pH 8.0), 1 M Urea, 500 mM NaCl, 1 mM mercaptoethanol]. The recombinant proteins were eluted with a gradient of buffer A to buffer A with 1 M imidazole (60 min; flow rate, 1 ml/min), and the fractions were tested for the presence of the polypeptides by Western blotting and detection with an anti-myc antibody (Invitrogen). The fusion proteins eluted at an imidazole concentration of 100–150 mM, and the positive fractions were pooled. The predicted molecular weights of the two fusion proteins were M_r 39,000 for ppSRCR1/2 and M_r 31,000 for ppSRCR6/7. Both fusion proteins displayed a band doublet at about M_r 40,000 as well as several additional bands up to M_r 85,000. Western blot experiments with anti-DMBT1h12 revealed two closely spaced bands at about M_r 80,000 for ppSRCR1/2. PCR amplification with primers specific for the sequences flanking the integration sites (5′ AOX1 and 3′ AOX1; Invitrogen) delivered products of the predicted size, excluding that a concatamerization of the plasmid inserts had taken place during integration into the *P. pastoris* genome. The observed increase in the sizes of the fusion proteins therefore most likely results from posttranslational modification in *P. pastoris*.

Western Blotting. Human BAL was obtained from a patient with alveolar proteinosis whose lung was lavaged for therapeutic purposes, as described earlier (11). For SDS-PAGE, the NuPAGE Gel system (Novex) was used. Ten μl of the respective protein samples were mixed with 10 μl of loading buffer (NuPAGE Gel System, Novex) and heated for 10 min at 70°C. The proteins were separated on 4–12% polyacrylamide gradient gels with the 2-[*N*-morpholino]ethanesulfonic acid buffer system for 1 h and 15 min at 200 V and subsequently transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). The membranes were preincubated with PBS-T (0.1% Tween 20 in PBS) containing 5% w/v skim milk powder for 1 h at room temperature. As the primary antibody either anti-DMBT1h12 (8 μg/ml), anti-DMBT1h12 (8 μg/ml) preadsorbed with the synthetic peptide DMBT1/2640 (1 mg/ml), or monoclonal anti-gp-340 (50 ng/ml) in PBS-T with 5% w/v skim milk powder was used. After an incubation for 1 h at room temperature, three washes with PBS-T, an incubation for 1 h with alkaline phosphatase-conjugated antimouse IgG (Santa Cruz; diluted 1:1000 in PBS-T with 5% w/v skim milk powder), and three further washes in PBS-T followed. The membranes were then developed using nitro blue tetrazolium and potassium 5-bromo-4-chloro-3-indolylphosphate.

Immunohistochemistry. Paraffin-embedded sections (thickness, 3–4 μm) of formalin-fixed tissue samples were used for immunohistochemistry. Stainings with anti-gp-340 as the primary antibody were carried out as described earlier (11). For immunohistochemistry with anti-DMBT1h12, sections were deparaffinized and rehydrated by xylene and a series of graded ethanols. Endogenous peroxidase activity was blocked by incubation for 20 min with 1% H_2O_2 in PBS. For the subsequent steps, the TSA-Indirect Kit was used following the instructions of the supplier (NEN Life Science Products). Antibody anti-DMBT1h12 was used at concentrations of 40 or 80 μg/ml in a final volume of 200 μl/section and incubated overnight in a humidified chamber at 4°C. Peroxidase activity was detected with 3-amino-9-ethylcarbazole as a substrate (Sigma Chemicals). Sections were counterstained with Mayer's Hematoxylin and mounted in crystal mount (Biomeda) for microscopic examination. As a standard negative control, anti-DMBT1h12 was substituted by equal amounts of normal mouse IgG (Santa Cruz). All controls were negative. For image acquisition, digital analySIS 3.0 software, a 3CCD color video

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camera (Sony), and an Olympus BX-50 microscope (Olympus Optical) were used.

RESULTS

DMBT1 Plays a Role in the Immune Defense. The vast majority of the SRCR proteins has been implicated in different functions in the immune system, and database searches retrieved an expressed sequence tag (HSZZ14922) from the T-cell line Jurkat with match to the *DMBT1* sequence. We therefore examined the *DMBT1* expression in the immune system and related cell lines by RT-PCR. *DMBT1* was found to be expressed throughout the immune system, including alveolar macrophages, but not in the macrophage precursor cell line U937 and the promyelocytic cell line HL-60 (Fig. 1).

gp-340 is a SRCR protein that has been isolated from human BAL and shown to localize to alveolar macrophages (11). It has been demonstrated that gp-340 induces chemokinesis of alveolar macrophages and is a binding protein for the collectins SP-D and SP-A, which interact with carbohydrates on various bacteria, viruses, and allergens (11, 12, 21). Because oligopeptide sequences of gp-340 showed homology to the DMBT1 sequence, we hypothesized that gp-340 might represent an isoform of DMBT1. We generated a DMBT1-specific monoclonal antibody, anti-DMBT1h12, and found that it recognized the same high molecular weight bands in Western blots of BAL as a monoclonal antibody against gp-340, two closely spaced bands at about M_r 300,000 and a third band at M_r 200,000 (Fig. 2). The gp-340 cDNA has recently been isolated and shown to match the *DMBT1* sequence (22). Taken together, these data demonstrate that gp-340 is encoded by one (or more) of the alternative splice products of *DMBT1* and thus suggest that DMBT1 plays a role in the respiratory immune defense.

Expression and Localization of DMBT1 in the Normal Brain and Brain Tumors. Because *DMBT1* has been proposed to be involved in the tumorigenesis of medulloblastoma and GBM (1, 2), we investigated the localization of the protein in the normal brain and in brain tumors by immunohistochemistry. Anti-DMBT1h12 revealed a faint intracellular staining of only a few astrocytes, in the cerebellum as well as in the cerebral neocortex (not shown), whereas with anti-gp-340, a moderate intracellular staining of some astrocytes and neuronal cells was observed (Fig. 3*a*). Both antibodies stained subsets of immune cells. Anti-gp-340 reacted with perivascular macrophages in the cerebellum (Fig. 3*b*), and anti-DMBT1h12 reacted with leukocytes within an intact blood vessel in the vicinity of a tumor (Fig. 3*c*).

In 6 of 10 medulloblastomas analyzed with anti-gp-340, the tumor periphery was highly enriched with macrophages expressing DMBT1, whereas the tumor cells were DMBT1-deficient (Fig. 3*d*). Two of 10 GBMs analyzed with anti-DMBT1h12 were completely negative for DMBT1 expression. The remaining GBMs were composed of negative cell populations and cells that expressed the protein at high levels (Fig. 3*e*). DMBT1 localized to intracellular vesicles and/or to the cell surface (Fig. 3, *f* and *g*). On some occasions, the protein appeared to be confined to the sites of cell-cell interactions (Fig. 3*g*).

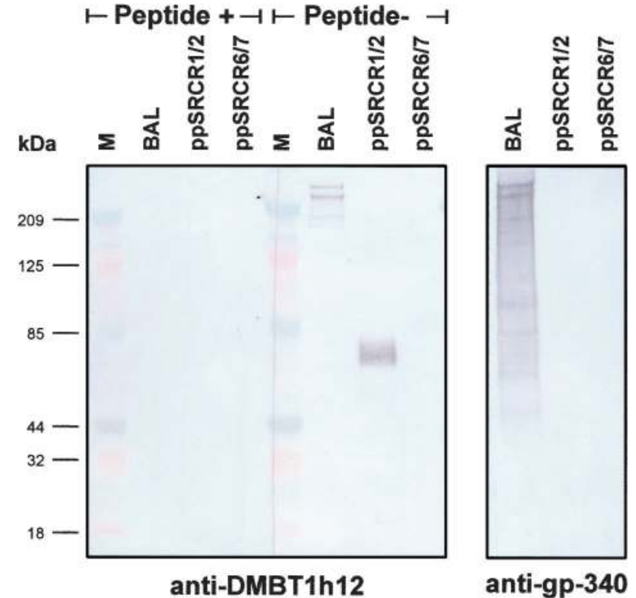

Fig. 2. gp-340 is an isoform of DMBT1. Ten μl of human BAL and of the two purified recombinant DMBT1/myc fusion proteins, ppSRCR1/2 and ppSRCR6/7, were separated by SDS-PAGE and transferred to membranes. ppSRCR1/2 comprises the amino-terminal sequence of DMBT1 up to SID2 and contains the peptide sequence used for immunization. ppSRCR6/7 comprises domains SRCR6 to SID7 of the isoform encoded by the 6-kb transcript of *DMBT1* (accession no. AJ000342). *Left panel,* the membrane was cut in two halves and incubated with the monoclonal antibody anti-DMBT1h12 with (*Peptide+*) or without (*Peptide−*) preadsorption with the synthetic peptide DMBT1/2640 used for the immunization. The antibody specifically recognizes ppSRCR1/2, but not ppSRCR6/7 that lacks the peptide epitope, and preadsorption abolishes binding, demonstrating the specificity of the antibody. Anti-DMBT1h12 recognizes three protein bands in BAL, a doublet at about 300 kDa and a band at 200 kDa. *Right panel,* duplicate Western blot incubated with monoclonal anti-gp-340. The three bands at the *top* are identical to the bands recognized by anti-DMBT1h12. Anti-gp-340 does not detect any of the two recombinant DMBT1-polypeptides, indicating that the two antibodies recognize different epitopes within the protein. Occasionally, several smaller bands were also detected by anti-gp-340, as shown in the *right panel*. Similar observations were also made with a rabbit polyclonal anti-gp-340 antiserum (Ref. 11),[4] suggesting that these bands represent degradation products and/or further variants. *M,* size marker.

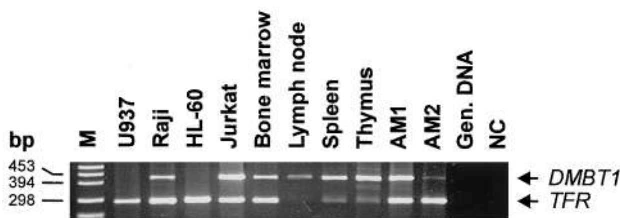

Fig. 1. RT-PCR analysis of the *DMBT1* expression in the immune system. A *DMBT1*-specific product was coamplified with an internal control product of the transferrin receptor (*TFR*) from the cDNA of different tissues of the immune system and related cell lines. A PCR product was obtained from all tissues tested as well as from alveolar macrophages isolated from two different individuals (*AM1* and *AM2*), the T-cell line Jurkat, and the B-cell line Raji, but not from U937 (macrophage precursor cell line) and HL-60 (promyelocytic cell line). *M,* DNA size marker; *Gen. DNA,* PCR with genomic DNA as the template; *NC,* negative control without template.

DMBT1 Plays a Role in Epithelial Differentiation. The three rodent proteins, Ebnerin, CRP-ductin, and hensin, share homology with DMBT1 and have a similar domain organization. The expression patterns of the four genes, however, substantially differ from each other (13–15). When we aimed at isolating the murine homologue of *DMBT1* by using cDNA probes specific for the 5′- and 3′-end of *DMBT1*, we retrieved a set of clones from different mouse cDNA and genomic libraries that all turned out to contain parts of the *CRP-ductin* coding sequence (not shown). This suggested that *CRP-ductin* represents the closest relative of *DMBT1* in mice and in turn that also *Ebnerin* and *hensin* might be *DMBT1* homologues in the other rodents. Hensin has initially been characterized as a protein localizing to the kidney collecting duct epithelium (15), and both CRP-ductin and hensin localize to the intestinal crypts and surface epithelium (14, 16). We therefore analyzed these tissues for the localization of DMBT1 by immunohistochemistry. We found that anti-DMBT1h12 likewise stained epithelial cells in the collecting ducts of the cortical kidney (Fig. 4*a*). In the duodenum, the crypt bases showed strongest staining, whereas in the colon, the crypt tops and the surface epithelium were found to be positive (Fig. 4, *b-d*). DMBT1 therefore closely resembles the distribution of CRP-ductin and hensin in these organs. Because

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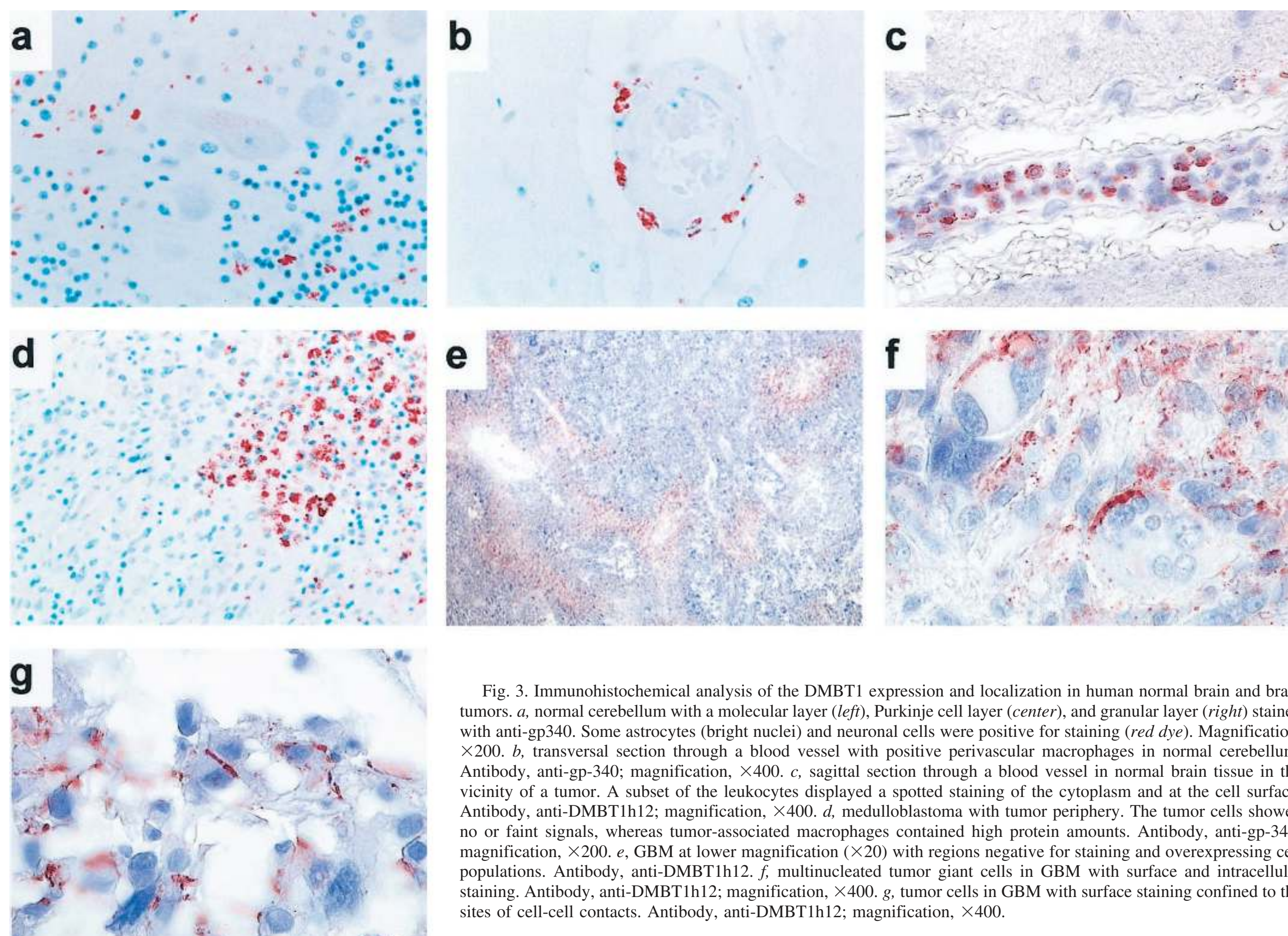

Fig. 3. Immunohistochemical analysis of the DMBT1 expression and localization in human normal brain and brain tumors. *a,* normal cerebellum with a molecular layer (*left*), Purkinje cell layer (*center*), and granular layer (*right*) stained with anti-gp340. Some astrocytes (bright nuclei) and neuronal cells were positive for staining (*red dye*). Magnification, ×200. *b,* transversal section through a blood vessel with positive perivascular macrophages in normal cerebellum. Antibody, anti-gp-340; magnification, ×400. *c,* sagittal section through a blood vessel in normal brain tissue in the vicinity of a tumor. A subset of the leukocytes displayed a spotted staining of the cytoplasm and at the cell surface. Antibody, anti-DMBT1h12; magnification, ×400. *d,* medulloblastoma with tumor periphery. The tumor cells showed no or faint signals, whereas tumor-associated macrophages contained high protein amounts. Antibody, anti-gp-340; magnification, ×200. *e*, GBM at lower magnification (×20) with regions negative for staining and overexpressing cell populations. Antibody, anti-DMBT1h12. *f,* multinucleated tumor giant cells in GBM with surface and intracellular staining. Antibody, anti-DMBT1h12; magnification, ×400. *g,* tumor cells in GBM with surface staining confined to the sites of cell-cell contacts. Antibody, anti-DMBT1h12; magnification, ×400.

both rodent proteins have been implicated in epithelial differentiation (14, 16) and a role for DMBT1 in the carcinogenesis of epithelial tumors has been proposed (3, 4), we compared the DMBT1 expression and localization in fetal and adult epithelia. Both the fetal intestinal epithelium (Fig. 4, *e* and *f*) and the fetal skin (Fig. 4, *g-i*) displayed higher protein amounts than the adult counterparts (Fig. 4, *b-d* and *j*, respectively), and the subcellular localization of DMBT1 was different in the fetal epithelia.

***DMBT1* Is Highly Unstable in Cancer and Polymorphic in Normal Individuals.** Deletions in or of *DMBT1* have been identified in brain, gastrointestinal, and lung tumors by PCR (1–4). We have recently reported on locus-specific repeating units in the region where the exons encoding the SRCR domains of *DMBT1* are located (Fig. 5*a*) and proposed that these may confer a susceptibility to genomic instability (20). To test this hypothesis, we analyzed a set of lung and brain tumor cell lines for LOH and genomic alterations at the *DMBT1* locus. LOH was observed in 3 of 12 lung and 6 of 9 brain tumors (included in Fig. 5*b*). Remarkably, 16 of 18 tumors had aberrations in *DMBT1*, as revealed by Southern blot analyses using the restriction enzyme *Rsa*I (Fig. 5*b*). Five tumors (H69, H128, H4, U118-MG, and U343-MG) showed internal homozygous deletions. For a direct comparison, we analyzed the genomic DNA of 36 normal individuals for the configuration of the gene using the same restriction enzyme. We found that 72% (26 of 36) of the normal individuals had two full-length copies of *DMBT1*, whereas 10 individuals (28%) showed different hemizygous deletions (Fig. 5*c*). One of these configurations (G3 in Fig. 5*c*) is compatible with a hemizygous loss of the region coding for SRCR4 to SRCR7, similar to the deletion observed in cell line H4. This may indicate that one of the mechanisms by which homozygous deletions arise is the presence of one deleted copy in the constitutional DNA that is uncovered by an LOH that eliminates the intact copy.

DISCUSSION

DMBT1 is localized at chromosome 10q25.3-q26.1, within a region that shows frequent LOH in a variety of tumors. Because homozygous deletions and lack of expression have been reported for brain, gastrointestinal, and lung cancer, *DMBT1* is considered a candidate tumor suppressor gene (1–4). Its functions, however, and therefore possible mechanisms by which DMBT1 might participate in tumorigenesis have remained unknown. We aimed at gaining insight into the functions of the protein and into molecular events that potentially lead to an inactivation of the gene.

Unambiguously, DMBT1 has at least two distinct functions, one in the immune system and a second one during epithelial differentiation, as suggested by several lines of evidence. The cloning of the gp-340 cDNA that has been shown to be identical to one of the 8-kb transcripts of *DMBT1* (22) and the Western blot studies in the present report demonstrate that the collectin-binding protein gp-340 corresponds to one or more of the isoforms encoded by *DMBT1*. gp-340 and therefore DMBT1 is a putative receptor for bacteria, viruses, and allergens that are opsonized by the collectins SP-D and SP-A and has

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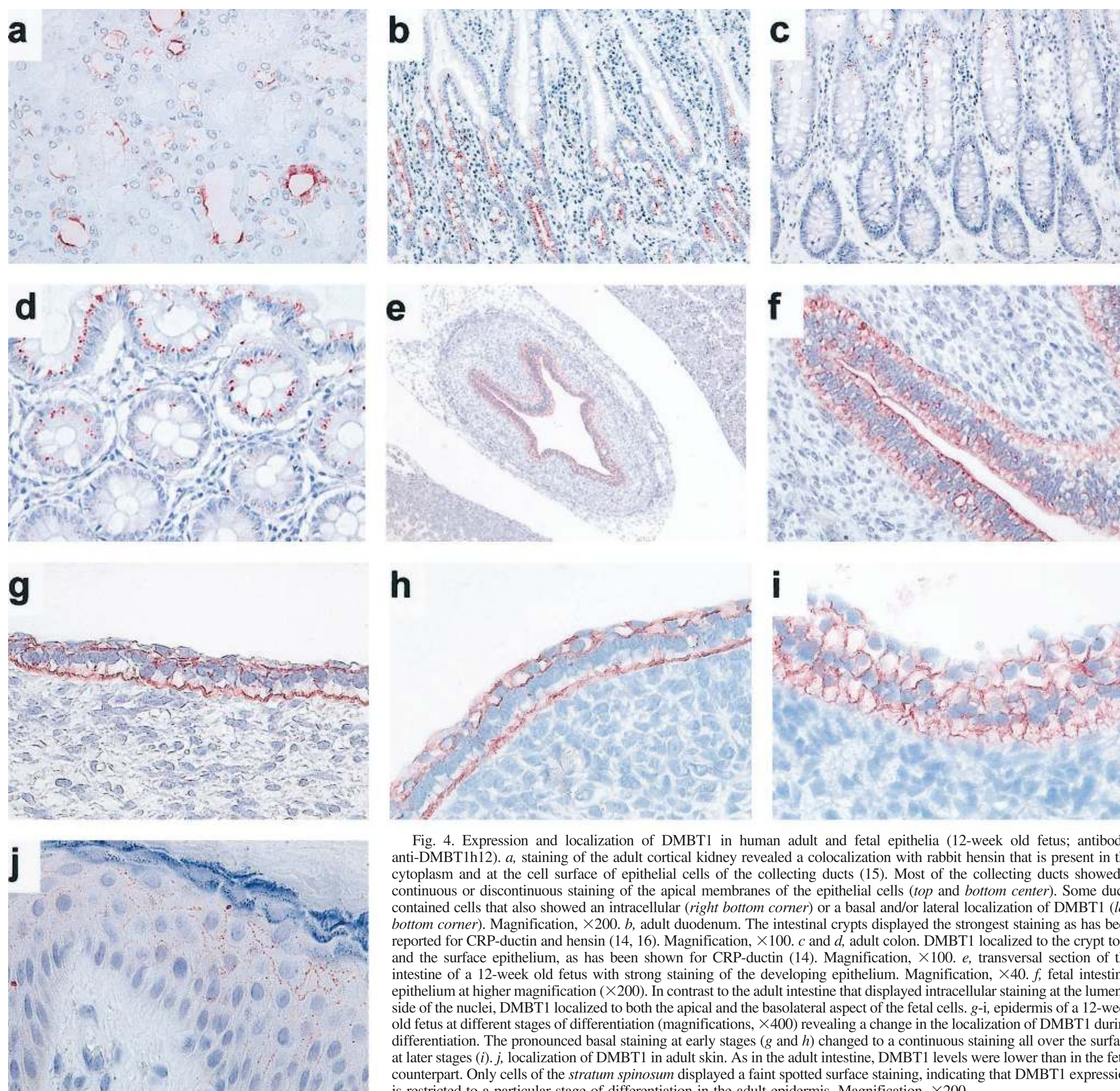

Fig. 4. Expression and localization of DMBT1 in human adult and fetal epithelia (12-week old fetus; antibody, anti-DMBT1h12). *a,* staining of the adult cortical kidney revealed a colocalization with rabbit hensin that is present in the cytoplasm and at the cell surface of epithelial cells of the collecting ducts (15). Most of the collecting ducts showed a continuous or discontinuous staining of the apical membranes of the epithelial cells (*top* and *bottom center*). Some ducts contained cells that also showed an intracellular (*right bottom corner*) or a basal and/or lateral localization of DMBT1 (*left bottom corner*). Magnification, ×200. *b,* adult duodenum. The intestinal crypts displayed the strongest staining as has been reported for CRP-ductin and hensin (14, 16). Magnification, ×100. *c* and *d,* adult colon. DMBT1 localized to the crypt tops and the surface epithelium, as has been shown for CRP-ductin (14). Magnification, ×100. *e,* transversal section of the intestine of a 12-week old fetus with strong staining of the developing epithelium. Magnification, ×40. *f,* fetal intestinal epithelium at higher magnification (×200). In contrast to the adult intestine that displayed intracellular staining at the lumenal side of the nuclei, DMBT1 localized to both the apical and the basolateral aspect of the fetal cells. *g-i,* epidermis of a 12-week old fetus at different stages of differentiation (magnifications, ×400) revealing a change in the localization of DMBT1 during differentiation. The pronounced basal staining at early stages (*g* and *h*) changed to a continuous staining all over the surface at later stages (*i*). *j,* localization of DMBT1 in adult skin. As in the adult intestine, DMBT1 levels were lower than in the fetal counterpart. Only cells of the *stratum spinosum* displayed a faint spotted surface staining, indicating that DMBT1 expression is restricted to a particular stage of differentiation in the adult epidermis. Magnification, ×200.

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been demonstrated to have a stimulatory effect on alveolar macrophages. Hence, DMBT1 appears to play a role in the mucosal immune defense, which is in line with its predominant expression in tissues with large epithelial surfaces, such as the lung and the small intestine. However, DMBT1 must have further immune-related functions, as is indicated by its widespread expression in the immune system and by the finding that perivascular macrophages and peripheral blood leukocytes express the protein. Moreover, in 6 of 10 medulloblastomas, the tumor periphery was highly enriched with *DMBT1* expressing macrophages. The collectin mannan-binding protein has been demonstrated to bind to glioma and colorectal carcinoma cells *in vitro* and to mediate a cellular antitumor response *in vivo* (23, 24). DMBT1/gp-340 secreted by tumor-associated macrophages could likewise participate in a cellular antitumor response, *e.g.*, by interacting with the collectins SP-D and SP-A and/or other molecules possibly bound to altered carbohydrate structures on the surface of the tumor cells.

Tumor cells within medulloblastomas were negative for staining with anti-gp-340, whereas a fraction of astrocytes and neuronal cells within the cerebellum was found to be positive. It would be premature to conclude that a loss of *DMBT1* expression is a general hallmark of medulloblastoma formation because the precursor cells of medulloblastomas are thought to be pluripotent stem cells that give rise to the different cell types of the cerebellum. Furthermore, there are some hints that the two monoclonal antibodies recognize epitopes that can be present in combination in some isoforms, as indicated by our Western blot experiments, but differentially used in other isoforms, as indicated by the finding that anti-DMBT1h12, in contrast to anti-gp-340, did not reveal staining of neuronal cells and only faint signals in astrocytes. The integrity of the signals detected by the two antibodies is confirmed by preliminary results obtained from the characterization of a third monoclonal antibody that does not appear to have a preference for specific variants and therefore recognizes the composite pattern of anti-DMBT1h12 and

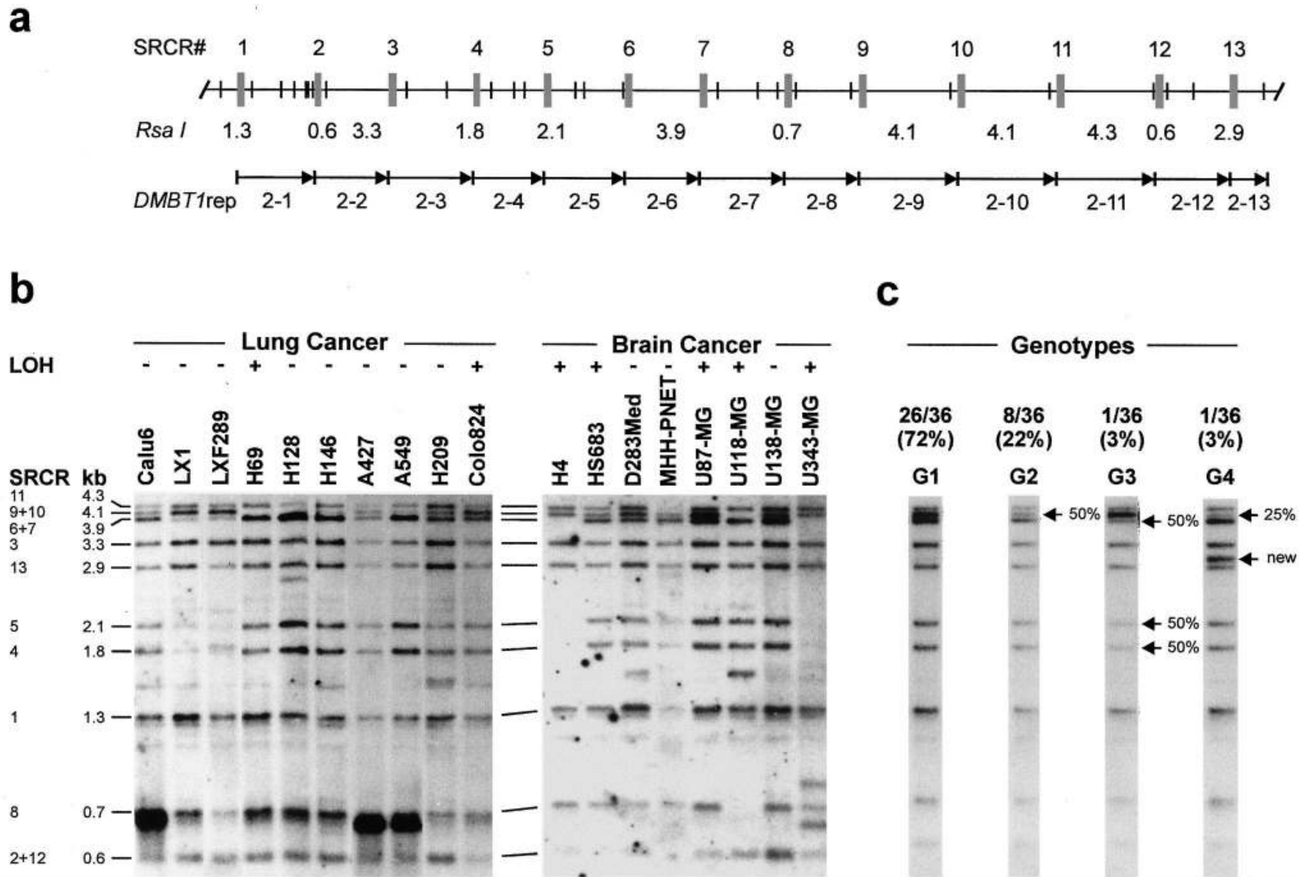

Fig. 5. Analysis of the genomic configuration of *DMBT1* in tumors and normal individuals. *a,* schematic presentation of the region containing the exons that encode the SRCR domains and SIDs (20). Only the SRCR exons are shown (*gray boxes*). *Black vertical lines,* symbolize the recognition sites for *Rsa*I. The size of the restriction fragments hybridizing with probe *DMBT1*/sr1sid2 is depicted below the line. *Arrows,* the position of the highly homologous repeating units at the *DMBT1* locus (*DMBT1*reps). Numbering of the SRCR domains and the *DMBT1*reps is as has recently been proposed (20). *b,* genomic aberrations in lung and brain cancer cell lines. The *Rsa*I digested DNA was transferred to membranes and hybridized with probe *DMBT1*/sr1sid2, which cross-hybridizes with the exons coding for the 13 amino-terminal SRCR domains. The presence (+) or absence (−) of LOH in the *DMBT1* region is indicated at the *top* of the panels. Only cell lines U87-MG and Colo-824 were devoid of rearrangements. The remaining 16 tumors showed aberrations in the gene, as indicated by the absence or increases or decreases in the signal intensity of restriction fragments. In several cases, *e.g.*, in H128 and U343-MG, additional bands, presumably fusion fragments, were observed. *c,* analysis of the *DMBT1* configuration in normal individuals. *Rsa*I-digested genomic DNA from peripheral blood leukocytes of 36 normal individuals was probed with *DMBT1*/sr1sid2. G1 to G4 are representatives of the four genotypes that were identified. The frequency of the respective genotype is depicted on the *top* of the panels. G1 is representative of the band pattern that would be expected in the presence of two full-length copies of *DMBT1*. *Arrowheads* in genotypes G2 to G4 mark the *Rsa*I fragments with an altered signal intensity. The remaining signal intensity in percent, as estimated by visual inspection, is given at the *right* of the *arrowheads*. All Southern blot results were confirmed by the use of a second probe, *DMBT1*/59m21, recognizing an overlapping band pattern (20).

anti-gp-340.[4] In summary, this raises the possibility that distinct DMBT1 isoforms might exist in the brain that are subjected to a differential regulation.

Our data demonstrate that the isoform(s) recognized by anti-DMBT1h12 are deregulated in GBM. Whereas a subset of the GBM is DMBT1-deficient, the major fraction contains cell populations that express these variants at levels higher than those observed in normal astrocytes. Furthermore, a surface-linked form appears to be ectopically expressed in a subset of the tumor cells. One possible explanation could be that the expression of aberrant forms or the ectopic expression of naturally occurring variants may even offer growth advantages to the tumor cells. However, DMBT1 apparently shares a set of features with the group A SRCR protein Mac2-bp that likewise is either secreted or surface-linked, has a function in the mucosal immune defense, interacts with a lectin, is expressed by both tumor and immune cells, and activates immune cells (5–7, 9, 25). Mac2-bp is present at elevated levels in the sera of cancer patients (25, 26) and has a local and systemic tumor suppressive effect *in vivo* probably due to the activation of natural killer cells (7, 8). It is therefore thought that Mac2-bp secreted by tumor cells acts as an alarm signal stimulating an antitumor response. Based on the parallels between DMBT1 and Mac2-bp, it is possible that DMBT1 secreted by tumor cells is likewise involved in mechanisms of tumor surveillance, *e.g.*, by stimulating macrophages in the local environment. Therefore, a loss of expression or the overexpression of aberrant variants could alternatively interfere with such processes.

There are several lines of evidence that CRP-ductin and hensin represent the rodent orthologs of DMBT1. CRP-ductin and hensin (and also Ebnerin) share a high homology with DMBT1 and have an overall similar domain organization. Our initial efforts identified *CRP-ductin* as the genomic homologue of *DMBT1*. Likewise, Takito *et al.* (27) have shown in a recent report that *DMBT1*, *CRP-ductin*, *hensin*, and *Ebnerin* are homologues in men and rodents. Our analyses demonstrate that DMBT1 colocalizes with CRP-ductin and hensin in the gastrointestinal tract and the kidney, supporting that the three proteins share a common function. Detailed biochemical studies have revealed that hensin is able to switch the polarity of collecting duct epithelial cells and to induce processes of terminal differentiation (15, 16). Most likely, hensin exerts its effect by multimerization and interaction with the extracellular matrix (28). Because differences in the protein levels and the spatial distribution of DMBT1 were found when comparing fetal and adult epithelia, we must anticipate that

[4] U. Holmskov, unpublished data.

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DMBT1 also participates in epithelial differentiation, probably by similar mechanisms.

However, whereas an involvement in processes of differentiation supports a potential role of *DMBT1* as a tumor suppressor, it is not yet clear if and how the gene might be inactivated during tumorigenesis. As proposed by its repetitive genomic structure (20), *DMBT1* was found to be highly unstable in tumors. Most likely, the *DMBT1* repeats give rise to an increased rate of recombination that eliminates part of the exons coding for the SRCR domains and SIDs or leads to other rearrangements within the gene. In most of the cell lines analyzed, the rearrangements are complex and a detailed determination of the genomic configuration of the gene is in progress, but is hampered by the repetitive genomic structure. Moreover, deletion polymorphisms that are observed in normal individuals further complicate these analyses and indicate that some of the homozygous deletions arise by the uncovering of a hemizygously deleted allele by an LOH at chromosome 10q. In fact, these results in part are challenging because they raise the question as to whether alterations in *DMBT1* are a cause or a consequence of tumorigenesis. Alternatively, however, this could raise the possibility that a subset of the population is a carrier of alleles that increase the cancer risk or facilitate tumorigenic growth once the formation of a tumor has taken place.

SRCR, CUB, and ZP domains have been implicated in the mediation of protein-protein interactions (17–19). DMBT1/gp-340 is secreted in the oligomeric form in the lung (11), and hensin exerts its effects in dependency of its state of oligo- or multimerization (28). The SRCR domains of DMBT1 therefore are likely involved in either the oligomerization of the protein or the mediation of interactions with ligands. Thus, major questions to be solved in the future are what the function of the SRCR domains and SIDs is and whether these repetitive domains have distinct or redundant functions.

In conclusion, our data indicate that *DMBT1* is subjected to alterations in a high number of tumors and plays a role in epithelial differentiation, in the immune defense, and eventually, in the immune surveillance of tumors. *In vitro* studies are in progress to test these models and to determine whether the different DMBT1 variants differ in their functional properties. Moreover, knock-out mice that will help to further elucidate the functions of DMBT1 and its possible role in tumorigenesis are under construction.

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