DMBT1 is frequently downregulated in well-differentiated gastric carcinoma but more frequently upregulated across various gastric cancer types

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Abstract. Well-differentiated gastric carcinomas are considered to represent a distinct entity emerging via specific molecular changes different from those found in other gastric carcinoma types. The gene *deleted in malignant brain tumours 1 (DMBT1)* at 10q25.3-q26.1 codes for a protein presumably involved in cell differentiation and protection and has been proposed as a candidate tumour suppressor for brain and epithelial cancer. One study reported a loss of DMBT1 expression in 12.5% (5/40) of gastric cancer samples. Here, we examined in more detail DMBT1 protein and mRNA expression in 78 primary gastric tumour samples and corresponding normal gastric mucosa. DMBT1 was expressed in all non-tumour gastric mucosa tissues. Eleven out of 71 (15%) gastric tumours were negative for the DMBT1 protein in immunohistochemical analyses. Lack of DMBT1 expression was significantly more frequently found in well-differentiated gastric tumours (6/18 well-differentiated tumours vs. 5/53 other subtypes; P=0.025). Quantitative RT-PCR revealed a downregulation of the DMBT1 mRNA for 8/21 (38%) cases, while the remaining 13 cases (62%) displayed a substantial upregulation. Our data suggest that a loss of DMBT1 expression may preferentially take place in well-differentiated gastric carcinoma. However, an upregulation of DMBT1 expression is more frequently found across all gastric cancer types.

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Introduction

Human stomach carcinogenesis occurs after a multi-step process of genetic and epigenetic alterations. These sequential alterations differ between the two histological types, indicating that different genetic pathways exist for well-differentiated and poorly differentiated gastric cancers (1-6).

The gene *deleted in malignant brain tumours 1 (DMBT1)* at 10q25.3-q26.1 on the one hand has been proposed as a candidate tumour suppressor gene for brain, lung, esophageal, gastric, colorectal, skin and breast cancers, because genomic rearrangements and a lack of its mRNA expression have frequently been observed in these cancer types (7-17). On the other hand, however, it has been observed that epithelial cancer types such as lung, breast and pancreatic carcinoma may also show increased DMBT1 levels compared to normal tissues without pathophysiological alterations and it has been proposed that this may point to an activation of DMBT1 expression during early tumourigenesis (9,11,18,19).

DMBT1 encodes for a large secreted glycoprotein, which contains 14 SRCR (scavenger receptor cysteine-rich) domains that allow the protein to be classified as a member of the SRCR superfamily (20,21); the protein also presents 2 CUB (C1r/C1s-Uegf-Bmp1) and 1 ZP (Zona pellucida) domains. Thus, DMBT1 is composed of motives that are known to mediate protein-protein interactions (7,22). DMBT1 may represent a multifunctional protein related to the Mac2-binding protein (Mac-2bp) and the mucins. It has been proposed to exert functions in epithelial differentiation and mucosal protection (23-30). In particular, DMBT1 may play a role in defense against *Helicobacter pylori*, which is assumed to represent a causative agent in gastric carcinogenesis. In the gastric mucosa, DMBT1 is upregulated in response to *H. pylori* infection *in vivo* and it further binds and aggregates *H. pylori in vitro* (28,31). A potential role in the homeostasis of and/or defense against *H. pylori* defines DMBT1 as an interesting candidate for being involved in gastric carcinogenesis.

Although the prevalence of *Helicobacter pylori* significantly increases the risk of developing both subtypes of gastric

adenocarcinoma, the mechanisms underlying the development of well-differentiated cancer are better circumscribed than those for poorly differentiated gastric carcinoma. This dominant form seems to be related to the development of atrophic gastritis and intestinal metaplasia, and particulary to cag$^+$ strains of *Helicobacter pylori*. Cancer risk is believed to be related to *Helicobacter pylori* strain differences, inflammatory responses governed by host genetics and specific interactions between host and microbial determinants (32,33). *In vitro* studies further suggested that DMBT1 could play a role in the proliferation-differentiation transition of gastric epithelial cells (17). In a general approach, Mori and co-workers determined DMBT1 expression levels by RT-PCR in gastrointestinal tumours and reported on a loss of DMBT1 expression in 12.5% (5/40) of primary gastric cancer samples (13). Here, we aimed at a more precise delineation of DMBT1 expression in gastric cancer and studied the expression and localization of DMBT1 in 78 primary gastric tumour samples by immunohistochemistry and/or quantitative RT-PCR.

Materials and methods

Patients. Seventy-eight patients with sporadic gastric carcinoma, who underwent surgery at Lisbon hospitals, Portugal, were included in this study. From each patient, tissue fragments from primary tumours and nonneoplasic mucosa were obtained under informed consent.

All the analyzed individuals were Caucasoid. The average age of the patients was 62±12 years (range: 36-85 years) and 61% were male.

Hematoxylin and eosin-stained sections were used to categorize tumours according to the World Health Organization (WHO) histological criteria (34). Data were obtained regarding grade of differentiation: 19 well-differentiated adenocarcinomas, 11 moderately differentiated adenocarcinomas, 30 poorly differentiated adenocarcinomas and 18 mucocellular carcinomas. The tumours were also classified according to Laurén's histological criteria (35): 58 intestinal and 20 diffuse.

Immunohistochemistry. Paraffin-embedded sections (thickness, 2 μm) of formalin-fixed tissue samples were used for immunohistochemistry. A streptavidin-biotin immunoperoxidase staining technique was performed. Briefly, the procedure was as follows: sections were mounted on ChemMate capillary gap microscope slides (Dako, Glostrup, Denmark), deparaffinized in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was performed, to unmask epitopes: tissue sections were heated in 0.01 M sodium citrate buffer pH 6.0 for 4 min. Immunostaining was automated using the ChemMate HRP/DAB detection kit (Dako) on the TechMate™ instrument (Dako). Endogenous peroxidase activity was blocked by incubation for 15 min with appropriated solution (HP-BK, Dako). For the subsequent steps, the TSA-Indirect kit was used to amplify the signal generated by horseradish peroxidase, following the instructions of the supplier (NEN Life Science Products, Boston, USA). Following 1-h incubation at room temperature with the primary antibody anti-DMBT1h12 (200 μg/ml), the sections were incubated for 45 min with a biotinylated goat anti-mouse antibody (AB2, Dako) and developed using the streptavidin-biotin-peroxidase complex technique (36). Peroxidase activity was detected with diaminobenzidine (DAB, Dako) as a substrate, for 5 min, which stains the structures containing the epitope brown. Sections were counterstained with hematoxylin for 1 min and mounted in Entellan (Merck, Darmstadt, Germany) for microscopic examination.

As standard negative control, the primary monoclonal antibody anti-DMBT1h12 was substituted by equal amounts of normal mouse IgG (Sigma-Aldrich, Madrid, Spain). Glioblastoma multiforme sections with DMBT1 accumulation were included in each experiment as internal positive controls. The relative amount of DMBT1-positive cells and their location in the normal stomach and gastric cancer were determined by independent visual inspection of two pathologists.

The monoclonal mouse antibody anti-DMBT1h12 was raised against a peptide comprising amino acids 26-40 of DMBT1, as described elsewhere (27). The antibody specifically recognizes this DMBT1 epitope in Western blotting experiments, and it has been demonstrated to be suitable for the detection of the protein in formalin-fixed and paraffin-embedded tissue specimens (27). The antibody is not commercially available yet, but may be provided by the authors upon request.

Real-time quantitative RT-PCR. Tissue samples were stored in RNAlater™ (Ambion, Austin, USA) at -20˚C immediately after surgery until RNA extraction. Total RNA was isolated from fresh tissues using the SV Total RNA Isolation System kit (Promega, Madison, USA), as recommended by the manufacturer. First-strand cDNA was synthesized using MultiScribe™ reverse transcriptase (Applied BioSystems, Foster City, USA), with random hexamers, according to the manufacturer's instructions in a total volume of 50 μl; samples were incubated for 10 min at 25˚C, 30 min at 48˚C and finally 5 min at 95˚C.

Expression levels of *DMBT1* in tumour samples relative to their normal adjacent tissues were investigated using real-time quantitative RT-PCR based on TaqMan fluorescence methodology. Gene-specific primers (5' CAA GTT CTA TCT ACA GGT GGG TGG ATC 3'; 5' GAA ATG GAG AAC CTT CTG CTA CAG TT 3') and a TaqMan probe (5' CAA GGA CTA CAG ACT ACG CTT CAC TGA TTC CC 3') labelled with 5'FAM and 3'TAMRA for *DMBT1* cDNA were designed using Primer Express software, version 1.0 (Applied BioSystems). To avoid detection of amplification of genomic DNA the probe was localized in an exon-exon boundary. Human *ß-actin* (TaqMan® pre-developed assay reagents for gene expression; Applied BioSystems) was used as the endogenous control gene. Reactions were performed with TaqMan Universal PCR Master mix (Applied BioSystems) in a 25-μl reaction volume. All reactions were performed in triplicate and included a negative control. PCR reactions were performed in the GeneAmp® 5700 sequence detection system (Applied BioSystems). Cycling conditions were 2 min at 50˚C, 10 min at 95˚C and 40 cycles of 15 sec at 95˚C and 1 min at 60˚C.

Validation experiments were performed to demonstrate that efficiencies of amplification of the target and reference genes are similar, and independent from the starting amount of cDNA (data not shown).

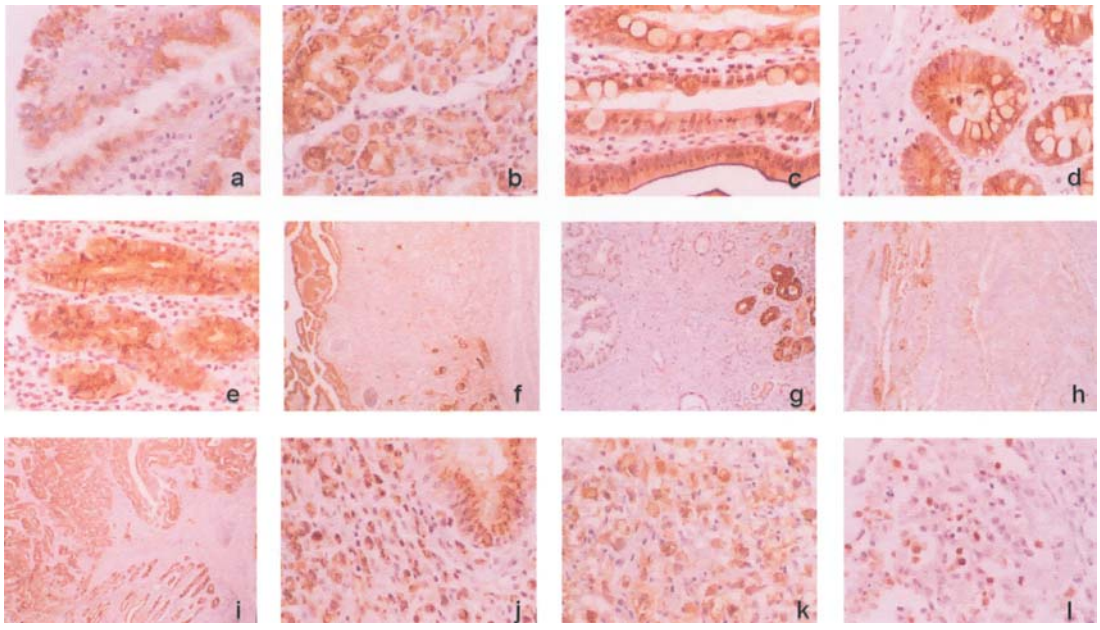

Figure 1. Immunohistochemical analysis of the DMBT1 expression and location in human normal stomach and gastric tumour samples. (a) Corpus gastricum mucosa with inflammation. Supranuclear granular staining of the surface epithelium (x400). (b) Corpus gastricum mucosa. Peripheral granular cytoplasmic staining of the parietal cells, adjacent to the cytoplasmic membrane (x400). (c) Gastric mucosa with intestinal metaplasia. Supranuclear and, in some cells, diffuse cytoplasmic expression. Linear staining of the apical border with irregular expression (x400). (d) Gastric mucosa with intestinal metaplasia. Supranuclear and diffuse cytoplasmic staining (x400). (e) Low-grade dysplasia. Diffuse cytoplasmic staining with supranuclear accentuation (x400). (f and g) Well-differentiated adenocarcinoma. Tumour cells negative vs mucosa without tumour positive (x40 and x100, respectively). (h) Well-differentiated adenocarcinoma. Focal expression in the tumour cells (x100). (i) Moderately differentiated adenocarcinoma. Expression in the tumour cells and in the tumour-flanking mucosa (x100). (j) Poorly differentiated adenocarcinoma. Diffuse cytoplasmic staining, granular type (x400). (k) Mucocellular carcinoma. Diffuse cytoplasmic staining, granular type (x400). (l) Nuclear DMBT1 signals in the cells containing picnotic nuclei vs absence of expression on the morphologically preserved (non-apoptotic) cells (x400).

Table I. Frequency of the presence and absence of DMBT1 protein in the gastric cancer samples.

Histological group	n	Presence	Absence
1 Well-differentiated adenocarcinomas	18	67%	33%
2 Moderately differentiated adenocarcinomas	8	87%	13%
3 Poorly differentiated adenocarcinomas	28	86%	14%
4 Mucocellular carcinomas	17	100%	0%

n, total number of samples under study. 1 vs 2+3+4 populations: P=0.0249 (two-tailed Fischer's exact test).

Relative quantification of the mRNA levels of *DMBT1* (quantity of *DMBT1* transcripts in tumour samples relative to normal tissues) was determined using the $\Delta\Delta C_T$ method (37). The threshold cycle (C_T) numbers were averaged and *DMBT1* levels in tumour samples were given by the formula $2^{-\Delta\Delta CT}$, in which $\Delta\Delta C_T = \Delta C_{T,s} - \Delta C_{T,cb}$ (the difference between any sample C_T and the calibrator C_T). Briefly, the amount of target (*DMBT1*) was normalized to an endogenous reference gene (*ß-actin*) and its expression in tumour samples was calculated relative to a calibrator (normal adjacent sample). Final results are expressed as N-fold difference in tumour expression relative to non-cancerous adjacent tissue.

Statistical analysis. The relation of protein expression and histological types of gastric tumours was evaluated by Fisher's test performed in SPSS 13.0 for Windows. Differences were considered significant when the P values were <0.05.

Results

Absence of DMBT1 protein expression is most frequently found in well-differentiated gastric carcinoma. To initially determine the DMBT1 expression and localization in primary gastric cancer samples, we studied 71 primary gastric tumours and the respective matched adjacent normal mucosa samples by immunohistochemistry. The specificity of immunoreactivity was confirmed by replacing the primary antibody with normal mouse IgG. As a technical positive control, we used a glioblastoma multiforme section with strong staining for the DMBT1 protein.

All normal (non-tumour) gastric mucosa samples displayed positivity for the DMBT1 protein varying from single cells to single or multiple cell foci. DMBT1 was rarely detected in a basal localization. Most frequently, a cytoplasmic granular staining with a more pronounced supranuclear location was observed (Fig. 1a and b). Intestinal metaplasias displayed a virtually identical pattern with additional pronounced staining

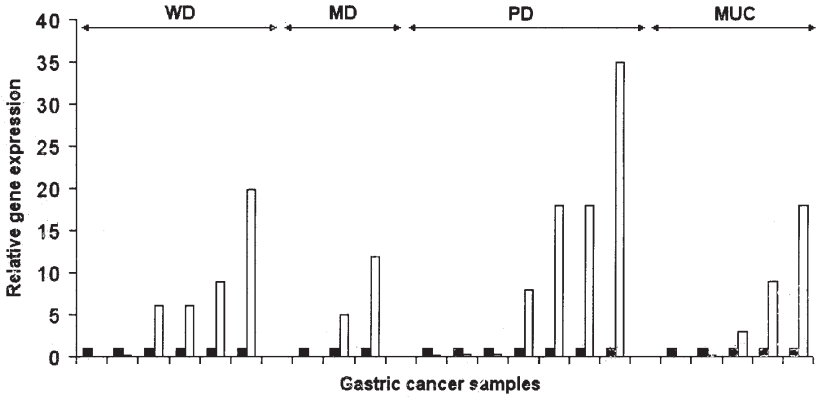

Figure 2. Relative expression of DMBT1 in histological subsets of gastric cancers (GC) (open bars) and normal adjacent mucosas (black bars) by TaqMan quantitative RT-PCR. WD, well-differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; MUC, mucocellular carcinoma.

of the brush border (Fig. 1c and d). In addition, 5/5 low-grade dysplasias contained within the sections were found to be positive for DMBT1 (Fig. 1e). In contrast, the DMBT1 staining in the well-differentiated adenocarcinomas was very weak and irregular and sometimes even negative (Fig. 1f, g and h). The moderately/poorly differentiated adenocarcinomas and the mucocellular type had more intense, diffuse or granular cytoplasmic staining (Fig. 1i, j and k). In general, gastric tumour cells always displayed a granular or diffuse cytoplasmic staining except for apoptotic cells, which showed signals in picnotic nuclei (Fig. 1l).

In total, 11 of the 71 gastric cancer samples (15%) lacked detectable signals in the tumour cells, while DMBT1 was well detectable in the tumour-flanking gastric mucosa. Remarkably, the majority of the DMBT1-negative cases were well-differentiated gastric carcinoma (6/18 well-differentiated gastric carcinoma versus 5/53 other gastric cancer types; Table I), which defined a statistically significant overrepresentation within this group (P=0.025; two-tailed Fisher's exact test).

DMBT1 mRNA expression is more frequently upregulated than downregulated in gastric carcinoma. In order to independently confirm these data and to monitor for cases with quantitative alterations of the DMBT1 expression levels, we performed quantitative RT-PCR analyses with 21 gastric carcinomas and matched non-cancerous mucosa samples. Fourteen of these also had been included in the previous immunohistochemical analyses.

Ten out of these 14 samples delivered concordant results, i.e., mRNA expression was detectable for an IHC-positive tumour. Three IHC-negative tumours displayed well-detectable mRNA expression, while one IHC-positive mucocellular carcinoma lacked a corresponding RT-PCR product (included in Fig. 2). Taken together, compared to the matched normal gastric mucosa samples, the DMBT1 mRNA expression levels were decreased in 8/21 (38%) of the tumours, but increased between 4- and ~35-fold in 13/21 (62%) of the gastric carcinomas (Fig. 2). The normal samples were normalized to one and it was considered overexpressed when the tumour samples had values >2 and underexpressed when the expression value was <0.5.

There was no association between the *DMBT1* mRNA level and the grade of differentiation of the tumours, since in all groups more than 57% of the tumours showed overexpression (Fig. 2); similar results were obtained according to the Laurén criteria: 64% (7/11) of the intestinal type and 60% (6/10) of the diffuse type carcinomas that were analyzed displayed *DMBT1* overexpression.

Discussion

DMBT1 located at chromosome 10q25.3-q26.1 has been proposed as a candidate tumour suppressor for brain, lung, skin, breast and digestive tract cancers (7-17).

For the digestive tract, RT-PCR amplification has shown an apparent reduction in *DMBT1* mRNA in 23/43 (53%) esophageal, 4/24 (17%) colorectal, and 5/40 (12%) gastric cancers, compared with paired normal tissues (13). Here, we conducted a more comprehensive study of the DMBT1 expression in the normal stomach and gastric cancer by immunohistochemistry and quantitative RT-PCR.

The qRT-PCR analyses delivered results that were concordant with those obtained from the immunohistochemical studies for 10 out of 14 cases that could be analyzed by both approaches. Discordant results may be due to various factors such as, for example, heterogeneity of the tumour with regard to DMBT1 expression and the ratio of normal to tumour cells. DMBT1 mRNA expression did not result in immunodetectable protein expression in three cases, which could be based on the above-mentioned factors. Alternatively, the DMBT1 protein levels did not reach the sensivity limit of the antibody and/or the detected *DMBT1* mRNA is not translated into protein. There is evidence that some genes related to cancer development are regulated at the translational level (38). While we could detect the DMBT1 protein in all normal gastric mucosa samples, 11/71 (15%) tumours displayed absence of the protein, which is in good agreement with the data reported by Mori and co-workers (13).

Rather than representing lower-grade tumours, well-differentiated gastric carcinoma are considered to represent a distinct entity, as would be supported by the existence of at least two distinct genetic pathways of gastric carcinogenesis (1-6). The moderately and poorly differentiated gastric adenocarcinomas, as well as the mucocellular carcinomas, therefore most probably do not represent more progressive stages of well-differentiated gastric adenocarcinomas, but rather entities

that may have emerged via distinct molecular pathways. Six out of 18 (33%) well-differentiated gastric carcinomas did not display detectable DMBT1 protein levels, pointing to a loss or considerable reduction of DMBT1 expression, compared to only 5/53 (9%) of the other gastric cancer types. This defined a statistically significant overrepresentation of DMBT1-negative tumours among the well-differentiated gastric carcinoma and would suggest that a loss of DMBT1 expression preferentially takes place in this entity. In turn, this would be in agreement with the assumption that alterations in distinct molecular pathways play a role specifically in the genesis of well-differentiated gastric carcinoma.

A finding that was not suggested by previous studies is that the majority of the gastric carcinoma (13/21 or 62%) rather displays an upregulation than a downregulation of DMBT1 expression with no obvious preference for a particular subtype. Similar observations were made in the recent past for lung, breast, hepatocellular, and pancreatic carcinoma (9,11,18,19). Due to the observation that carcinogenic triggers such as the chemical carcinogens DMBA and 2-AAF result in induction of DMBT1 expression in the mammary gland and hepatic duct epithelium, it is assumed that DMBT1 may represent part of an early protective response (11,39), which may be followed by a loss of DMBT1 expression at later stages of tumourigenesis. For the stomach, it has been demonstrated that DMBT1 expression is upregulated after *H. pylori* infection *in vivo* (31) so that bacterial infection and/or consecutive inflammatory changes could account for the upregulation of DMBT1 expression in gastric carcinoma tissues.

Based on detailed studies *in vitro*, a localization of DMBT1 in the extracellular matrix (ECM) and a polymerization by galectin-3 are considered to be necessary for induction of terminal epithelial differentiation by DMBT1 (25,40). By contrast, lumenally secreted variants are considered to play a role in pathogen defense because of their ability to interact with and aggregate various bacteria, in particular *H. pylori* (28). With regard to a secretion to the ECM, our data were not informative. A basal staining of the cells was only occasionally observed. The pronounced staining at the apical side of the nuclei observed in our study is suggestive of a predominant lumenal secretion by the gastric mucosa.

Apoptotic cells represented an exception, because they displayed a nuclear staining. The presence of DMBT1 protein in the nucleus can be explained by the cellular events taking place during apoptosis. An apoptotic event includes dismantling of nuclear pores, disruption of nucleocytoplasmic barrier, and nuclear entry of some proteins (41). The proteins that undergo nuclear translocation can be directly involved in apoptosis, as is known for cytochrome c or caspases (42). To date, however, there are no studies for DMBT1 available that analyzed its relation to apoptotic processes.

In conclusion, these results suggest that a loss or reduction of DMBT1 expression most commonly is found in well-differentiated gastric carcinoma. Simultaneously, however, an upregulation of DMBT1 expression is even more frequently found across all gastric cancer types. Follow-up studies are required to determine as to whether this reflects a non-causative role of DMBT1 expression changes or a more complex involvement in the etiology of gastric carcinoma.

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