Silencing of *DMBT1* in neuroblastoma cell lines is not due to methylation of CCWGG motifs on its promoter*

J. MUÑOZ, J.S. CASTRESANA**

Molecular Neuro-Oncology Laboratory, e-mail: jscastresana@unav.es, University of Navarra, 31008 Pamplona, Spain

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DMBT1 is one of the putative suppressor genes on 10q25-qter, which frequently lacks expression in many different kind of tumors, such as glioblastoma, and lung, esophageal and colorectal cancer. However, little is known about the reasons for this lack of expression in neoplasia. In a previous report, our group demonstrated how MC-IXC, a neuroblastoma cell line which lacked *DMBT1* expression, restored it after a 5-Aza-2'-deoxycitidine treatment. So, we wondered whether *DMBT1* aberrant promoter methylation could be responsible for *DMBT1* silencing in several tumor cell lines, in spite of the fact that there is no CpG island near the 5' end of the gene. We studied the possibility that methylation in CCWGG sequences of the *DMBT1* promoter (where "W" means "A" or "T") is able to silence the gene, as had previously been reported for *TP53* in leukemia. We digested genomic DNA by the methylation sensitive restriction enzyme EcoR II (C↓CWGG), and made two PCRs to amplify the three CCWGG domains placed in the 1kb upstream *DMBT1* 5' end. After the PCRs, we could not find correlation between methylation in CCWGG domains and *DMBT1* lack of expression. A positive regulator of *DMBT1* might be silenced by aberrant methylation.

Key words: DMBT1, neuroblastoma, methylation, CCWGG, promoter

The *DMBT1* gene, located at 10q25.3-26-1, has been proposed as a putative tumor suppressor gene in many types of tumors, like glioblastoma, medulloblastoma [1], and lung [2], colon, gastric and esophageal cancer [3]. It has a repetitive structure of 54 exons, with an open reading frame of approximately 80 Kb. It codes for 13 highly homologous SRCR (Scavenger Receptor Cysteine Rich) domains, separated by 12 SID (Sequence Interspersed Domains) domains, and two CUB domains flanking the last 14th SRCR domain. A final ZP domain is located at the most C-terminus. Finally, there is a putative exon coding for a transmembrane domain located 1.4 Kb downstream the last exon [4]. The gene is believed to present alternative splicing, and at least two human isoforms, glycoprotein 340 [5], and salivary agglutinin, have been already described.

Although the physiological function of the gene is not clear yet, some data point it to have a role in mucosal protection and epithelial differentiation [7, 8]. *DMBT1* most frequent genomic alterations are allelic losses, homozygous deletions and structural rearrangements, while point mutations are rare in this gene [9, 10]. Lack or reduction of expression of *DMBT1* has also been found in glioblastoma, medulloblastoma [1], small and non small cell lung carcinoma [2, 11], esophageal, gastric and colon cancer [3], and skin cancer [12]. So on, lack of *DMBT1* function and abnormal localization of its protein product have been thought to aid in tumorigenesis [7, 13]. In a previous study [14], we found *DMBT1* homozygous deletions in 3 of 45 neuroblastoma primary tumors and 2 of 12 neuroblastoma cell lines, while its expression was highly reduced or absent in 2 of these cell lines, MHH-NB-11 and MC-IXC, respectively. However, we could not associate *DMBT1* lack of expression with *DMBT1* homozygous deletion, as only MC-IXC showed homozygous deletion, and this deletion was internal to the gene, without affecting most of the N-terminal domain. We wondered whether methylation of the *DMBT1* promoter might be the reason for lack of expression. Therefore, we treated these

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**Corresponding author

cells with 5-aza-2-deoxicitidine. *DMBT1* expression was restored in MC-IXC cells, while in MHH-NB-11 we could not see any change of expression.

In spite of these results, there is not a CpG island near the transcription starting point, which could make unlikely a disregulation of the *DMBT1* expression by promoter hypermethylation. However, there is increasing evidence of gene silencing by cytosine methylation in single CpG dinucleotides, or in CCWGG sequences, as have been reported by some authors for the B29 gene in lymphomas [15], and for TP53 in leukemia [16]. We could find three of these sequences in the 1 kb upstream the 5'-UTR of the gene (Fig. 1). We tried to find a correlation between methylation at these sites and lack of *DMBT1* expression. We designed an experiment with 2 restriction enzymes, EcoR II and BstN I, methylation sensitive and insensitive, respectively, to see whether there was methylation at these CCWGG sequences in several neuroblastoma cell lines.

Material and methods

Cell cultures. Four neuroblastoma cell lines (BE(2)-C, SK-N-DZ, SK-N-SH and MC-IXC) were purchased from the American Type Cell Collection (ATCC, Manassas, VA). MHH-NB-11, IMR-32 and SK-N-MC neuroblastoma cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The glioblastoma cell line U87MG (European Collection of Cell Cultures) and the normal human astrocytes cell lineage (NHA, Cambrex, East Rutherford, NJ) were used as negative controls. SK-N-MC cells were grown in DMEM supplemented with 10% Fetal Bovine Serum, 2% L-Glutamine and MEM non essential aminoacids (5%), following manufacturer's instructions; IMR-32, and MHH-NB-11 cells were cultured with RPMI medium, supplemented with 10% FBS, L-Glutamine and MEM non essential aminoacids (5%); MC-IXC and Be(2)-C cells were grown in EMEM/Ham's F12 medium mixed in a 1:1 proportion, and SK-N-SH were cultured in EMEM medium with 2 mM L-Glutamine and Earle's BSS, both supplemented with 10% FBS and 5% MEM non essential aminoacids. NHA cells were grown in Astrocyte Growth Medium BulletKitc (AGM^c BulletKit^c, Clonetics). This medium contained: one 500 ml bottle of Astrocyte Basal Medium (ABM^c); 10 µ/ml hEGF (human recombinant Epidermal Growth Factor), 1 ml; 10 mg/ml Insulin, 1.25 ml; 25 µg/ml Progesterone, 0.5 ml; 50 mg/ml Transferrin, 0.5 ml; 50 mg/ml Gentamicin: 50 µg/ml Amphotericin-B, 0.5 ml; and 25 ml Fetal Bovine Serum. DNA was extracted with phenol-chloroform procedures.

Methylation analyses. 1 µg DNA from each sample was digested in a final volume of 15 µl, with 1.5 µl 10X restriction buffer and 1 µl (10 U/µl) restriction enzyme EcoR II (Sigma-Aldrich Inc., Chicago, IL), which does not work if the second cytosine of the target sequence (CCWGG) is methylated. The reaction was incubated overnight at 37 °C. A final 72 °C step for 20 min was used to inactivate the enzyme. For each reaction with EcoR II, two controls were made: one negative control, without enzyme, and a positive one, digested with BstN I, a methylation-insensitive enzyme. The reactions with BstN I were incubated at 60 °C overnight.

Three CCWGG sequences were screened in the 1000 bp upstream region of the *DMBT1* gene (Fig. 1). In the first region (M1), primers were designed to amplify a 195 bp fragment in which there were two of these sequences. 1.5 µl of digestion product were added to a reaction mix which contained 2.5 µl 10X reaction buffer, 0.8 mM dNTPs, 2.5mM Mg^{2+}, 5 pmol of each primer and 1 U of Taq Gold^{TM} DNA Polymerase (Perkin Elmer, Foster City, CA). The final volume for each reaction was 25 µl. Primers used were *5'-GCAGCATAGACTACTGATGA-3'* (sense), and *5'-GTTGCCTGACACTTATTTTT-3'* (antisense). Each reac-

a)

tggctgacacaggacatgggttcatggtacccttctagaat*gcagcatagactactgatgaacagtgcatggcaaaga*
*agccaagtgtcatttcatggcctcagcctctcagctgagaagcagggcacagctcac***cccagg***ctaggaaaacagaggc*
*aagtcctgg*aaagctgtctgcttttaaccaagagttactggccatcaagtgtcttggtta*aaaaataagtgtcaggca*
*ac*cttcttggtagagtgtgttgggggcattatcagagtctggtaatgacttctgagggtcccaaagagtgaagtgat
atttacatagcaaatccaaggagggggattgtgtgcaatatagtggaggtgggggcaggttttgtgggtttgccaag
ctccaagggtcatacaatgtgcatgtcaaggacaagaaatcaaagccatgtgaaatggttggaggtggttcagtttga
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AAGAACCCAGCAAA**ATG**

b)

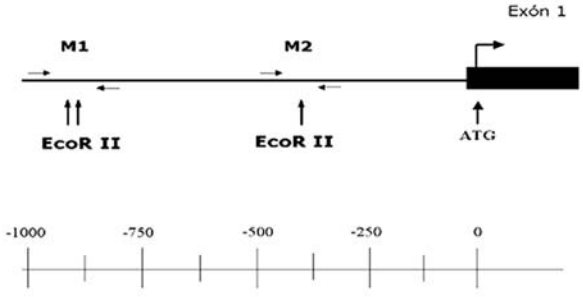

Figure 1. a) The 1000 pb. DMBT1 sequence upstream the initial ATG codon. Non-exonic DNA is in lower case, 5'-UTR is in upper case. Nucleotide sequences for regions M1 and M2 are depicted in italic, with the primers used for the amplification of both regions underlined. Target sequences for EcoR II are shadowed, in bold, both in M1 and M2 regions; these sequences are at -930, -905 and -398 nucleotides, respectively. Initial ATG of DMBT1 is depicted in bold.
b) Scheme illustrating the base-pair position of both three target sequences for EcoR II in the 1 Kb fragment upstream the initial ATG, and the position of primers which amplify both M1 and M2 regions.

tion was heated at 95 °C 10 min in a first denaturation step. Then, we used 28 cycles of amplification, at 94 °C, 1 min; 52 °C, 45 s; and 72 °C, 45 s. A final extension step at 72 °C, 10 min, was used.

The third sequence was screened by a different PCR reaction, in which we amplified a 326 bp fragment (M2). Briefly, 1.5 µl of digestion product were mixed in a final volume of 25 µl, with 2.5 µl 10X reaction buffer, 0.8 mM dNTPs, 2.5 mM, Mg^{2+}, 5 pmol of each primer, and 1 U Taq Gold^{TM} DNA polymerase (Perkin Elmer, Foster City, CA). Primers used were *5'-TCCCTCATGACTTCTGCTCT-3'* (sense), and *5'-AACGCACTGACATAAGTGAAAA-3'* (antisense). Thermocycler conditions used were: 95 °C, 1 min; 28 cycles at 94 °C, 1 min, 58 °C, 45 s and 72 °C, 45s; 72 °C, 10 min.

We also amplified exon 6 of the *TP53* gene as an internal control, to ensure that all our PCRs had approximately the same amount of DNA. This region had been previously assessed not to have any target sequence for EcoR II [16]. 1.5 µl of digestion product was added to a 50 µl reaction mix, which contained 5 µl 10X reaction buffer, 0.8 mM dNTPs, 1.5 mM Mg^{2+}, 5 pmol of each primer, and 2.5 U Taq Gold^{TM} DNA polymerase (Perkin Elmer, Foster City, CA). Primers for TP53 exon 6 amplification were *5'-GGGCTGGGACCCAGGCCTCTGATTCCTCAC-3'* (sense), and *5'-AGACCCCAGTTGCAAACCAG-3'* (antisense). Temperature conditions for this last PCR were: 95 °C, 10 min; 30 cycles of 94 °C, 1 min, 61 °C, 1 min, and 72 °C, 1 min; 72 °C, 10 min. PCR products were visualized in 2% agarose gels stained with ethidium bromide (0.1 µg/ml).

DMBT1 gene expression before and after 5'Aza-2'-deoxycitidine treatment was assessed by RT-PCR in a previous report [14].

Results and discussion

While MC-IXC cells lacked methylation both in M1 and M2 regions, nearly all the rest of cell lines examined showed methylation in at least one region (Fig. 2). U87MG cells had methylation in both regions, and IMR-32, Be(2)-C, SK-N-DZ, MHH-NB-11 and SK-N-SH showed the M2 region methylated. SK-N-MC and NHA cells, which showed *DMBT1* expression, had no methylation in any region. Results are summarized in Table 1.

Then, several cell lines which have cytosine methylation in at least a CCWGG domain, do express the gene, while MC-IXC, which had no methylation in any CCWGG, lacked *DMBT1* expression. As a consequence, it seems proved that lack of expression of *DMBT1* in this cell line seems not to be related to aberrant methylation in CCWGG sequences.

However, we could demonstrate how expression of *DMBT1* could be restored in this cell line when it was treated with 5-Aza-2'-deoxycytidine [14]. This discrepancy might be explained by the methylation-related silencing of a different positive regulator of *DMBT1*; after a treatment with a

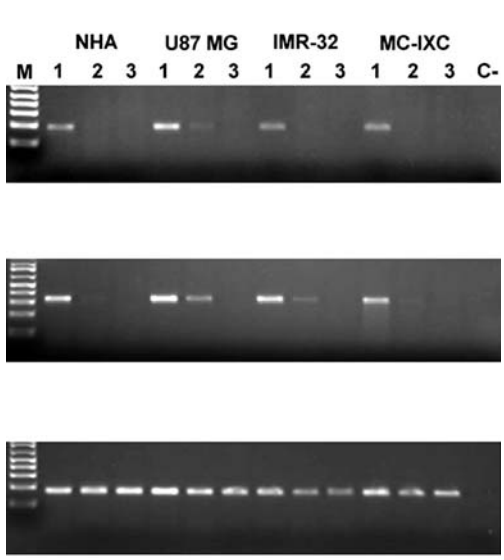

Figure 2. Methylation at CCWGG sites. a) PCR of the M1 region, which contains two CCWGG domains. b) PCR of the M2 region, which contains one CCWGG site. c) PCR of the exon 6 of the TP53 gene, which has not any CCWGG on its sequence, and was used as negative control. NHA cells did not show methylation at any region, while U87MG cells presented cytosine methylation both at M1 and M2 regions. MC-IXC did not show methylation, neither at M1, nor at M2 region. M: 1Kb plus DNA marker; 1: genomic DNA, not digested; 2: genomic DNA, digested with EcoR II, a methylation-sensitive restriction enzyme; 3: genomic DNA digested with BstN I, a restriction enzyme, methylation-insensitive. NHA: Normal Human Astrocytes; U87MG: glioblastoma multiforme cell line; IMR-32 and MC-IXC, neuroblastoma cell lines. MC-IXC lacked DMBT1 expression, but it could be restored after a treatment with 5-Aza-2'-deoxycytidine. However, methylation-related silencing of DMBT1 in this cell line seems not to be a consequence of methylation at CCWGG sites on its promoter.

Table 1. Promoter hypermethylation and expression of DMBT1 in neuroblastoma cell lines

Cell line	CCWGG methylation		Gene expression	Gene expression after 5/Aza treatment
	M1	M2		
NHA	–	–	+	n.a.
SK-N-MC	–	–	+	n.a.
MC-IXC	–	–	–	+
IMR-32	–	+	+	n.a.
SK-N-DZ	–	+	+	n.a.
SK-N.SH	–	+	+	n.a.
Be(2)-c	–	+	+	n.a.
MHH-NB-11	–	+	+/–	+/–
U87 MG	+	+	+	n.a.

M1 and M2: regions of DMBT1 promoter which contain at least one CCWGG motif; +: presence of CCWGG methylation/gene expression; –: absence of CCWGG methylation/gene expression; +/–: slight gene expression; n.a.: data non available

demethylating agent, this gene might be re-expressed and activate *DMBT1* expression.

LUALDI et al [17] identified regulatory regions in a 3.7 Kb fragment upstream the initial ATG. In this fragment, they reported both putative sequences for positive regulation (a TATA binding domain at 75 nt upstream the initial ATG) and for negative regulation (a CACCT binding site for the repressor of gene expression Delta EF1). An Alu sequence at -2657 nt upstream the initial ATG might be a negative regulator of *DMBT1* expression.

More studies must be done not only in order to understand the regulation of *DMBT1*, but also to increase our knowledge of the role of *DMBT1*, both in cellular physiology and in cancer promotion. It will also be of great interest to discover which gene or genes regulate *DMBT1* expression, and to see whether aberrant methylation of any of them might participate in *DMBT1* silencing.

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