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A splicing variant leads to complete loss of function of betaine–homocysteine methyltransferase (BHMT) gene in hepatocellular carcinoma☆

Hélène Pellanda, Farès Namour, Ma'atem Fofou-Caillierez, Aude Bressenot, Jean-Marc Alberto, Céline Chéry, Ahmet Ayav, Jean-Pierre Bronowicki, Jean-Louis Guéant*, Thierry Forges

INSERM U954 «Nutrition, Genetics, and Environmental Risk Exposure», Faculté de Médecine, Nancy Université, 9 Avenue de la Forêt de Haye, 54500 Vandoeuvre les Nancy, France

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ABSTRACT

The remethylation of homocyteine into methionine is catalyzed either by methionine synthase (MTR) or by betaine–homocysteine methyltransferase (BHMT), in the liver. Choline/betaine deficiency and impaired BHMT pathway have been associated with hepatocellular carcinogenesis, in animal models. The molecular mechanisms that impair the BHMT pathway are unknown. We aimed to investigate *BHMT*, *BHMT2*, and *MTR* expression in HepG2 cells and human hepatocarcinoma tissues. Transcripts were quantified by RT-qPCR and splicing was assessed by analysis of exon junctions and sequencing of variants. Protein expression was studied by Western Blot, immunohistochemistry and enzyme activity. Tumor tissue was compared with surrounding healthy tissue. RT-qPCR of HepG2 cells and of tumor samples showed a strong decrease of transcripts of *BHMT* and *BHMT2*, compared to normal. *MTR* transcript levels were not different. The decreased *BHMT* expression resulted from the transcription of a splicing variant that produced a frameshift in exon 4, with a premature termination codon in exon 5 and a loss of function of the gene. This splicing variant did not fit with any mechanism resulting from known splicing consensus sequences and was not detected in normal adult and fetal liver. Consistently, BHMT activity was abolished in HepG2 and protein expression was not detectable in HepG2 and in 5 of the 6 tumor samples, compared to normal tissues. In conclusion, a transcription variant of exon 4 produces a loss of function of *BHMT* in human hepatocarcinoma. Whether this abnormal transcription of *BHMT* is part or consequence of liver carcinogenesis should deserve further investigations.

1. Introduction

The sulfur-containing amino-acid methionine is either incorporated into various peptides and proteins or converted into S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT) which transfers an adenosyl group from ATP to methionine. SAM is the main methyl donor for DNA, RNA, protein and phospholipid methylation (Pajares and Perez-Sala, 2006). As an essential amino acid, methionine has to be provided by the diet, but it can also be regenerated from homocysteine (HCY) through two different remethylation pathways, which account each for half of the remethylation capacity in the liver (Finkelstein and Martin, 1984). In most tissues, HCY is remethylated into methionine by methionine synthase, encoded by *MTR*, with methyltetrahydrofolate acting as the methyl donor and cobalamine (vitamin B12) as a cofactor. The second remethylation pathway depends on betaine–homocysteine methyltransferase (BHMT) (Fig. 1A). In contrast to the methionine synthase pathway, human BHMT, encoded by *BHMT*, is expressed only in kidney and liver (Delgado-Reyes et al., 2001) where it accounts for 0.6–1.6% of total protein content (Garrow, 1996). Betaine, the substrate of BHMT is originating either from the diet with wheat, spinach, sugar beets, and crustaceans as the major sources (Craig, 2004), or from choline oxidation in the mitochondria. A second gene called *BHMT2* has been located upstream of *BHMT*, encoding a 73% homologous protein (Chadwick et al., 2000). The functional significance of BHMT2 has not yet been clearly identified (Li et al., 2008; Szegedi et al., 2008).The diet deficient in choline, folate, B12 and the methionine-deficient diet were shown to cause hepatocarcinogenesis (Copeland and Salmon, 1946; Ghoshal et al., 1983; Ghoshal and Farber, 1984). This was related with DNA hypomethylation, overexpression of proto-oncogenes such as *c-myc*, *c-fos* and *c-Ha-ras* (Pogribny and James, 2002; Pogribny et al., 2004; Wainfan et al., 1989; Wainfan

Abbreviations: MTR, methionine synthase; BHMT, betaine homocysteine methyltransferase; SAM, S-adenosylmethionine; MAT, methionine adenosyltransferase; HCY, homocysteine; HCC, hepatocellular carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POLR2A, RNA polymerase II; SAH, adenosylhomocysteine.

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* Corresponding author at: INSERM U 954, Faculté de Médecine – BP 184, 54511 Vandoeuvre les Nancy, France. Tel.: +33 3 83 68 32 92; fax: +33 3 83 68 32 79.
E-mail address: Jean-Louis.Gueant@medecine.uhp-nancy.fr (J.-L. Guéant).

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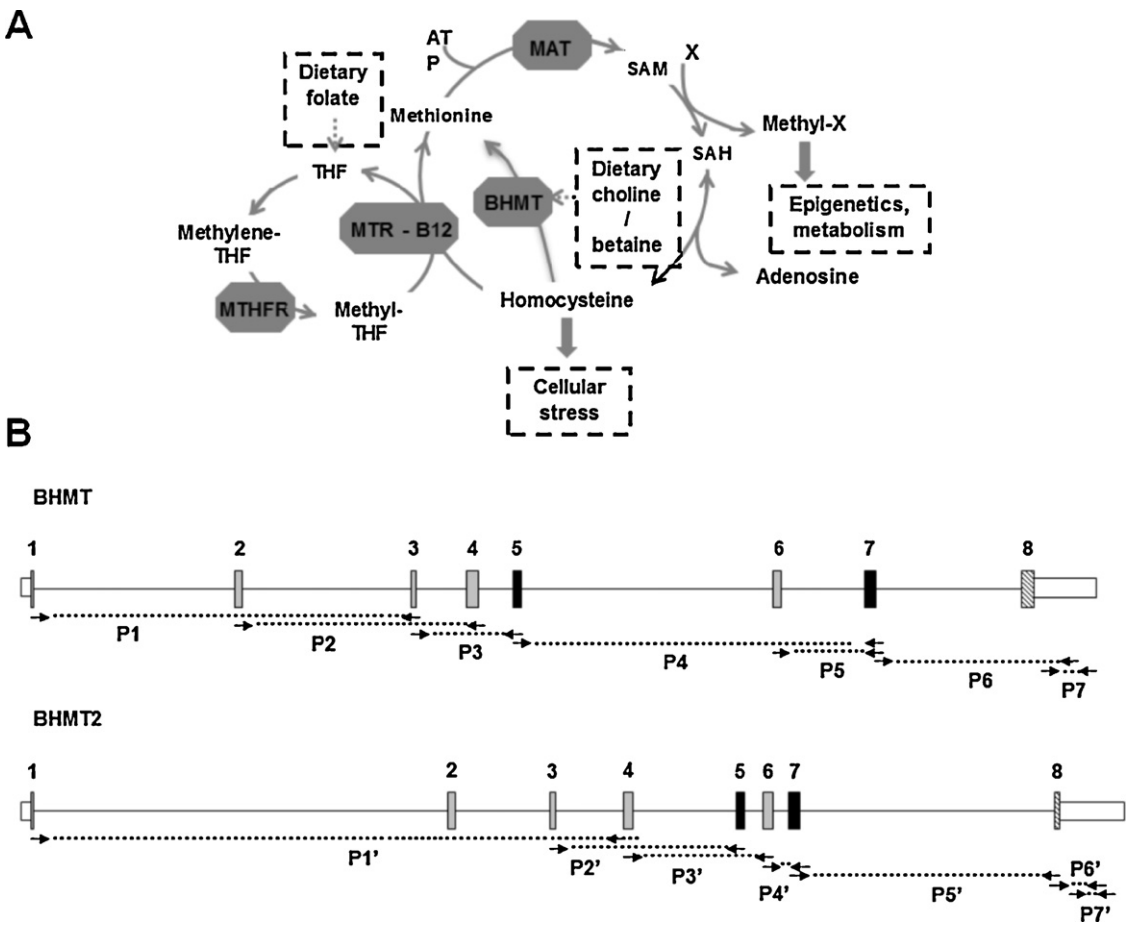

Fig. 1. (A) Folate and homocysteine metabolism. In the liver, homocysteine (HCY) is remethylated into methionine by methionine synthase (MTR) which uses methyltetrahydrofolate and cobalamin as a co-factors or by BHMT which uses betaine as methyl donor. *Abbreviations*: MAT, methionine adenosyltransferase; X, substrate to be methylated. (B) Schematic representation of *BHMT* and *BHMT2* genes with primer pairs position. *BHMT* and *BHMT2* exons are shown as numbered boxes, introns are represented by solid lines. Exons with 80–100% homology are colored in black, exons with 60–80% homology are colored in grey and exon 8 displaying 30% homology is shaded. White boxes indicate 3′- and 5′-UTR. Arrows depict primer pairs position used in RT-PCR and numbered P1–P7 for *BHMT* and P1′–P7′ for *BHMT2*.

and Poirier, 1992), and decreased expression of tumor-suppressive genes such as *p53* and *p16*^{INK4A} (Pogribny and James, 2002; Pogribny et al., 2004). The methyl-deficient diet enhances the chemical induction of hepatocellular carcinoma (HCC) in rats exposed to diethylnitrosamine (DENA) (Mikol et al., 1983; Yokoyama et al., 1985). In contrast, choline supplementation appeared to reduce the incidence of HCC in these models, underlining the importance of the BHMT-dependent methionine regeneration pathway in the prevention of hepatocarcinogenesis (Ghoshal and Farber, 1984; Wainfan and Dizik, 1987). In humans, a decrease of *BHMT* expression has been reported in tumor tissues, compared with normal liver (Avila et al., 2000; Liang et al., 2005; Sun et al., 2007). However, the underlying molecular mechanisms are not known. The aim of the present study was therefore to investigate the changes in MTR, BHMT and BHMT2 expression and to elucidate the underlying molecular mechanisms in HepG2 cells and liver carcinoma.

2. Materials and methods

2.1. Cell culture

HepG2 cells were purchased from LGC Standards (Molsheim, France). These cells were cultured in 75 cm^2 flasks at 37 °C in a humidified CO_2 (5%) incubator in Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies) with 10% fetal calf serum (v/v) (PAA Laboratories) and 1% (100 U) penicillin–streptomycin (v/v) (Gibco). The medium was changed every two days until the cells reached 50%, 80% or complete confluence. Cells were then trypsinised after washing with 1× Dulbecco's phosphate buffered saline (Gibco), collected and centrifuged for 5 min at 200 × *g*, then frozen at −80 °C, until extraction of RNA and proteins.

2.2. Liver tissues

Adult liver tumor samples were obtained from 6 patients undergoing surgery for hepatocellular carcinoma (patients T3–T6) and cholangiocarcinoma (patients T1 and T2). After surgical removal, tumor tissue was carefully dissected and paired with a sample of surrounding healthy tissue (C3–C6 and C1, C2, respectively). Paired specimens were frozen in liquid nitrogen then stored at −80 °C until processing. The dissection was always performed by the same operator within 15 min after surgical removal. Representative sections were cut and subjected to histological examination, which showed that tumor samples (T1–T6) contained ≥80% tumor cells whereas their healthy counterparts (C1–C6) contained only normal cells. Age, sex, tumor type and etiology are given in Supplementary Table 1. Fetal liver tissue samples (F1 and F2) were obtained from two fetuses after spontaneous abortion at 27 and 38 weeks gestational age, respectively. All samples were obtained after patient consent. All study procedures were conducted in accordance with the institutional ethics committee approval.